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ORIGINAL PAPER

Impact of caloric restriction on the *Wnt*/β-catenin pathway in the hippocampus and cortex of a Kindled rat model

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ABSTRACT

Introduction and aim. Epilepsy is a common neurological disorder, and despite numerous treatment options, approximately 30% of patients have drug-resistant epilepsy. This situation prompts the exploration of alternative treatments such as caloric restriction (CR), whose mechanisms of antiepileptic action need to be fully elucidated. One of the key overactivated pathways in epilepsy is the *Wnt*/β-catenin pathway.

Material and methods. To explore the potential regulatory effects of CR on this pathway, we conducted a study using twenty-eight male Wistar rats divided into four groups (7 animals each): Control, Sham (20% CR), kindling ad libitum (KAL), and kindling with CR (KCR). Caloric restriction rats received 80% of their daily food intake based on body weight, compared to those fed ad libitum. The kindling model was achieved by the introduction of an electrode in the basolateral nucleus of the amygdala. Immunofluorescence and Western blot techniques were used for the analysis of protein levels (*Wnt*, β-catenin, *GSK3*β, and cyclin D) in the frontal cortex and hippocampus.

Results. Electroencephalographically and behaviorally, the KCR group exhibited a shorter duration of seizures and an increased behavioral threshold compared to the KAL group. Protein analysis revealed an increase in *Wnt* pathway proteins (*Wnt*, β-catenin, and cyclin D) in the KAL group compared to the control group. In contrast, CR reduced protein levels in animals that were induced to kindling.

Conclusion. These findings suggest that CR may exert its antiepileptic effects through the regulation of the *Wnt* pathway by inhibiting its activity in the hippocampus and cortex of kindled rats.

Keywords. caloric restriction, drug-resistant epilepsy, epilepsy, *Wnt*/β-catenin

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Introduction

Epilepsy is defined as a neurological disorder marked by recurrent, unprovoked seizures due to abnormal electrical activity in the brain.[1] Epilepsy is associated with significant neurobiological, cognitive, psychological and social consequence.[1] Its etiology is multifactorial, involving structural, genetic, metabolic, immune, infectious, or sometimes unknown causes.[2] The cornerstone of epilepsy treatment is drug therapy with antiseizure medications (ASM) and lifestyle changes. However, at least one-third of people with epilepsy do not achieve seizure control despite the use of appropriate ASM or surgical interventions.[3]

In recent decades, dietary modifications, such as caloric restriction (CR), have been used in the treatment of drug-resistant epilepsy (DRE), demonstrating neuroprotective effects.[4] However, the mechanisms underlying these effects remain unknown. CR usually consists of a diet in which 20% of total calories are restricted, which simulates a fasting state and induces significant metabolic adaptations. These adaptations facilitate a shift from glucose to free fatty acids as the main cellular energy source.[5] CR-induced chronic ketosis activates several molecular mechanisms. It enhances neuronal hyperpolarization through ATP-sensitive potassium channels (K_{ATP}), increases the seizure threshold, and modulates gut microbiota and inflammation, notably reducing interleukin-1B and other cytokines in murine models.[5-8] In the CNS, CR is associated with increased mitochondrial biogenesis; enhanced antioxidant enzyme activity such as superoxide dismutase in the cerebral cortex and increased neuronal activity in the hippocampus of aged rats.[9-11]

The *Wnt* signaling pathway, integral to cell survival and apoptosis, includes a canonical pathway dependent on β-catenin and noncanonical pathways such as the Ca^{2+}-dependent and planar cell polarity pathways (Fig. 1).[12] In the canonical pathway, β-catenin is essential for the transmission of *Wnt* signals to the nucleus, allowing subsequent activation of *Wnt* target genes via TCF/β-catenin complexes. In the absence of *Wnt*, β-catenin is targeted for degradation. It is phosphorylated by GSK3β with the assistance of CK1α, AXIN, and APC, forming a destruction complex.[12-14] However, when *Wnt* ligands bind to Frizzled receptors (Fzd), it leads to the oligomerization of Dishevelled (Dvl) at the plasma membrane. This process repositions GSK3β and Axin, promoting the phosphorylation of the co-receptor LRP5/6. This sequence of events allows CK1γ to phosphorylate Dvl, allowing it to block *GSK3*β by binding to its binding protein (GBP) and to promote the degradation of Axin within the *Wnt*/Fz-LRP5/6 complex.[14-16] When these components are inhibited, the β-catenin degradation complex -catenin does not form, and β-catenin is free to travel to the nucleus. There, it binds to the TCF/LEF family of transcription factors, resulting in the release of Groucho, a transcriptional inhibitor, and facilitating gene transcription. In the nucleus, β-catenin also recruits other basal components of the transcriptional machinery, such as B-cell lymphoma 9 (Bcl-9), p300, and cyclic adenosine monophosphate (cAMP), which collectively activate the transcription of genes crucial for cellular functions, including those coding for cyclin D, the Myc family, and axin2.[12,14,15]

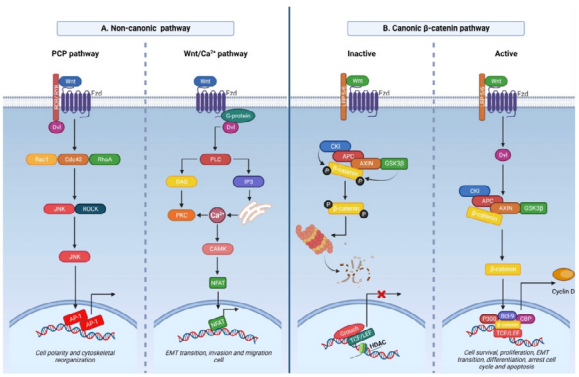

Fig. 1. A: Two non-canonical *Wnt* pathways have been described, a polar cell pathway that mainly regulates the reorganization of the cytoskeleton and cell polarity, and another intracellular Ca^{2+}-dependent pathway that regulates cell migration and invasion, as well as epithelial-mesenchymal transition. B: The canonical pathway will depend on whether it is activated or inactivated by Wnt family ligands. When the pathway is inactive, the β-catenin protein is phosphorylated by the destruction complex and destroyed by the ubiquitin proteasome system; this will result in blocking gene transcription in the nucleus. On the other hand, when the pathway is activated, the destruction complex is inhibited so that β-catenin remains free in the cytosol and translocates to the nucleus where it will promote the transcription of genes such as those of the Myc family, cyclin D, among others. The canonical pathway regulates cell survival, proliferation and differentiation, cell arrest, and apoptosis, created with https://www.biorender.com

Previous research suggests that CR can reduce seizures by stabilizing neuronal membranes through K_{ATP} channel-mediated hyperpolarization and inhibiting the mTOR signaling cascade via GSK3β. Understanding how CR affects the *Wnt*/β-catenin pathway, which is critical for central nervous system homeostasis and is involved in epilepsy and other neural diseases, is essential.[12-17] This could provide the foundation for new therapies for refractory epilepsy, offering new strategies for patients who are resistant to existing treatments.

Aim

Therefore, the aim of this study was to evaluate the impact of caloric restriction on the *Wnt*/β-catenin pathway in the hippocampus and cortex of a kindled rat model.

Material and methods

Experimental subjects

Twenty-eight male Wistar rats (120-130 g) were obtained from the Neurological Diseases Medical Research Unit of the Specialty Hospital CMN S. XXI. Our handling and treatment of the animals adhered to institutional protocols, complying with national regulations (NOM-062-ZOO-1999) and international ethical standards (Council for International Organizations of Medical Sciences, CIOMS) study approval number 100/17. The rats were housed individually in transparent cages with corn-cob bedding, maintained at 23±1°C with a 12-hour light-dark cycle (lights on at 07:00 h). The cages were cleaned regularly and kept dry. The sample size was determined using a G-Power analysis[18], with the aim of minimal animal use while achieving statistical strength; this analysis specified a sample of n=28 with an error value of 0.75.

Experimental procedure

Rats were randomly assigned to one of four groups: a control group (n=7) that received standard dietary pellets and ad libitum; a sham group (n=7) under caloric restriction without stimulation; and two groups that underwent a standardized amygdala kindling stimulation protocol, one with caloric restriction (KCR, n=7) and one with an ad libitum diet (KAL, n=7). CR was implemented by providing rats with 80% of the daily amount of a standard diet (Harlan Standard Commercial Diet No. 2018S Teklad Global 18% Protein, Harlan Laboratories), adjusted daily based on recorded body weight. This regimen was compared to a control group of rats allowed to feed ad libitum. The diet restriction protocol was established based on previous studies and accumulated experience.[19,20] Sixteen rats, four from each group, were used for Western blot analysis and twelve, three from each group, for immunohistochemistry. Caloric restriction was established at 20% below the individual weight to prevent hypoglycemia, which could confound the results by triggering seizures. This restriction began upon receiving the rats and continued until they reached 250-300 g. For those subjected to kindling, the restriction was maintained during the stimulation period.

Amygdala Kindling model

The Kindling model involves administering a subthreshold electrical stimulus that triggers electroencephalographic and behavioral changes, culminating in generalized seizures.[21] For this study, stereotaxic surgery was performed in rats to implant an electrode in the basolateral nucleus of the amygdala. They were anesthetized with sodium pentobarbital (50 mg/kg i.p., Pfizer Laboratories, Mexico City, Mexico), and once anesthesia was administered, their heads were fixed in a stereotaxic frame to introduce the electrode into the amygdala (Ap=-6.2 mm, L=5 mm relative to Bregma suture; H=1.5 mm interaural, according to the Paxinos and Watson Atlas). The electrode was then secured with dental acrylic. Post-surgery, rats were monitored and administered Gentamicin (Aurofarma) at a dose of 4-5 mg/kg b.w. intramuscularly every 24 hours for 10 days after surgery. Stimulation was carried out using a Grass S88 Stimulator at 50 microvolts of amplification. Rats received daily stimulation at a frequency of 60 Hz, with a pulse duration of 1.0 ms and an intensity of 400 μA. Behavioral changes were assessed using Racine's stages, and the duration and intensity of the ictal and postictal states were recorded. The Kindling stimulation protocol was deemed complete when a rat experienced 10 generalized tonic-clonic seizures, reaching Racine's stage 5. All rats were sacrificed on postnatal day 85, the day following their final stimulation, after undergoing a total of 64 days of caloric restriction (CR).

Immunofluorescence

At the end of the experimental phase, rats were anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by 4% paraformaldehyde. The brains were then removed and embedded in paraffin and sagittal sections (7 μm) were prepared. These sections were deparaffinized, hydrated with graded alcohols and blocked with bovine serum albumin (Sigma, St. Louis, MO, USA). They were incubated overnight with primary antibodies including Wnt-3, β-catenin, GSK3β, actin, and Cyclin-D (all mouse monoclonal, diluted 1:100, Santa Cruz). Following three washes with PBS, secondary antibodies were applied: Rhodamine Red for *GSK3*β and Cyclin-D and Fluorescein IsoThioCyanate for Wnt-3 and β-catenin, producing red and green fluorescence, respectively. The sections were mounted with Vectashield containing DAPI (ab104139) for nuclear staining. The imaging was performed with an Olympus IX81-F3 microscope equipped with a Q-Imaging digital camera, using a 40X objective in a field of 520 μm^2. Areas imaged included the CA3 zone and the apical area of the frontal cortex because they constitute the brain structures most susceptible to epileptogenesis. Protein densities were quantified using ImageJ software (Rasband, 1997-2016, version 1.45).

Western blot

For Western blot analysis, tissue samples from the hippocampus, dentate gyrus, subiculum, and frontal cortex of the rats were homogenized. Protein concentrations were quantified using the Lowry method.[19] Fifty micrograms (μg) of protein were separated by SDS-PAGE and subsequently transferred to PVDF membranes (BioRad, Hercules, CA, USA). The mem-

branes were blocked with 5% milk in PBS containing 0.01% Tween-20 for two hours. Santa Cruz Biotechnology primary antibodies, including those against β-actin (1:3000), Wnt-3 (1:2000), β-catenin (1:3000), cyclin D (1:500) and *GSK3*β (1:3000), were incubated overnight. The following day, the membranes were washed and incubated with horseradish peroxidase conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) at a dilution of 1:15,000. Protein bands were visualized using the UVP ChemiDoc Imaging System (Upland, CA), and band density was quantified with ImageJ software (Rasband, 1997-2016, version 1.45).

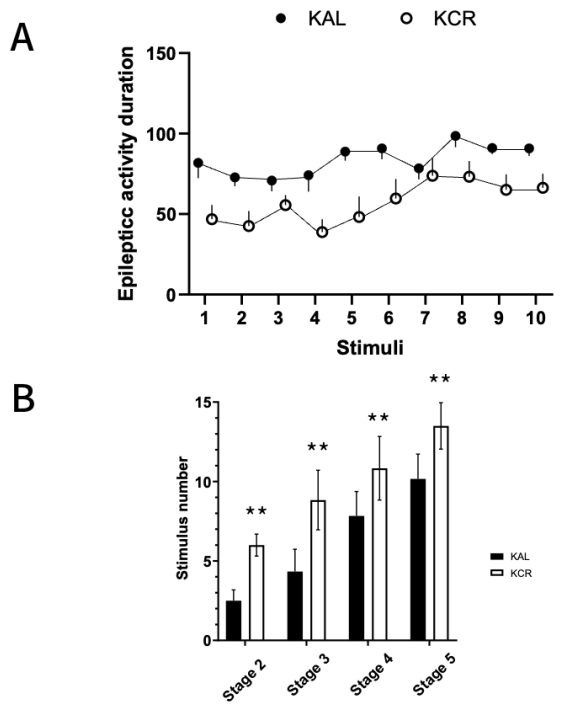

Fig. 2. Kindling model measurements in the KAL and KCR groups. Two bar graphs showing the result of what was observed and recorded during the application of the Kindling model. A: Graph showing the duration as a function of the stimuli given to the rat (x-axis). The solid line represents the KAL group and the dotted line the KCR group; note the differences in duration along the 10 stimuli. (F=45.75 p<0.0001). B: Graph showing the number of stimuli required to reach each of the Racine stages. The white bar represents the KAL group, and the black bar represents the KCR group. The KAL group needed a smaller number of stimuli to reach each stage compared to KCR (F=11.79 p<0.0014). *p<0.05, **p<0.01 and ***p<0.001 compared to KAL

Statistical analysis

Data were presented as mean values with standard deviations (mean±SD). The normality of the data distribution was evaluated using IBM SPSS Statistics v29.02.0 (Armonk, NY, USA). For datasets following a normal distribution, a two-way analysis of variance (ANOVA) was employed to investigate the effects of caloric restriction on the *Wnt* pathway. Alternatively, for nonnormally distributed data, a one-way of variance ANOVA was performed. Tukey's post hoc test was applied to analyze the kindling data, whereas Duncan's post hoc test was used for the other datasets. Statistical significance was established at $p<0.05$.

Results

Statistical analysis using a two-way ANOVA revealed significant differences in the durations after discharge between the KCR and the KAL ($F=45.75$, $p<0.0001$). Specifically, the KCR group exhibited shorter after discharge durations, not exceeding 75 seconds, even after ten stimuli, while durations in the KAL group surpassed 90 seconds at the final stimulus (Fig. 2). Similarly, significant differences were observed in the number of stimuli required to progress through Racine's stages ($F=11.79$, $p<0.0014$). The KCR group required a higher number of stimuli to reach Racine stage 5 compared to the KAL group, indicating a mitigated progression of seizure severity under dietary restriction.

Immunofluorescence

Using immunofluorescence, thus providing dual validation of the findings through two methodologies. In the hippocampus (Fig. 3A), all proteins except *GSK3*β exhibited higher concentrations in the KAL group compared to other groups, notably β-catenin ($p<0.001$ $F=4.57$), *Wnt* ($p<0.015$ $F=6.52$), and cyclin D ($p<0.030$, $F=5.020$). Further analysis revealed that the Sham group had the lowest protein concentrations relative to all other groups, with values that closely matched those of the control group. *GSK3β* was significantly increased in the KCR group ($p<0.002$, $F=12.79$) in comparison to the KAL group. A subsequent Duncan post hoc test identified significant differences across all groups, particularly from Sham to all other groups without exception, and between KAL and KCR for all proteins analyzed.

In the cerebellar cortex (Fig. 4A), the concentration of β-catenin protein ($p<0.01$ $F=9.87$) was found to be higher in the KAL group than in the other groups. Duncan's post hoc test showed a significant difference between the Sham group and the control group, as well as differences between KCR and KAL. *The Wnt* protein ($p<0.001$ $F=7.897$) and cyclin D ($p<0.037$ $F=4.615$) showed higher values in the KAL group compared to the rest of the groups, Duncan's post hoc test shows significant differences in *Wnt* in the KAL group compared to Sham and KCR. Similarly for cyclin D we found the same differences between the groups, as well as a difference between KAL and the control.

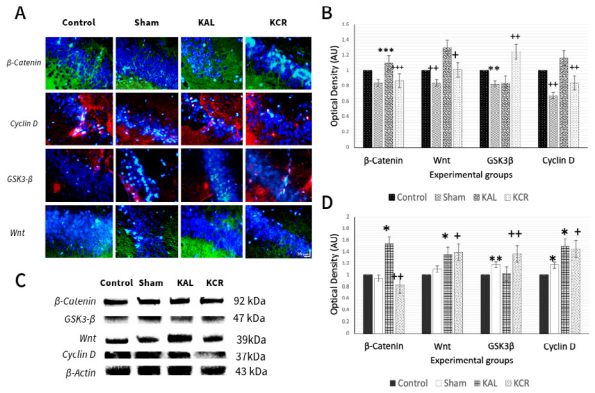

Fig. 3. Effect of caloric restriction and the amygdala Kindling model on *Wnt* pathway proteins. A: *Wnt* pathway proteins by immunofluorescence technique in the hippocampus, apical area with a 40X objective in a 520 mm2 field (Scale Bar 20 µm). Cell nuclei (DAPI) in blue and proteins analyzed in red for cyclin D and *GSK3*β, and green for *Wnt* and β-catenin and B: analysis of proteins of the *Wnt* pathway in each of the experimental groups. C: Representative images in the hippocampus and D: Densitometric analysis of β-catenin, *Wnt,* GSK3β and cyclin D expression levels. Mean±SEM graphed, using one-way ANOVA followed by a post hoc Duncan's test. *p<0.05, **p<0.01 and ***p<0.001 compared to the control group, $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 compared with the KAL group

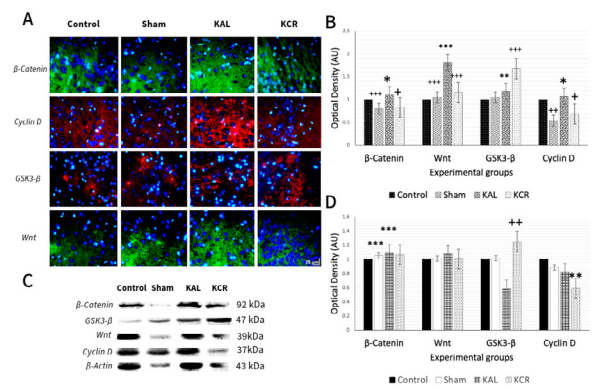

Fig. 4. A: *Wnt* pathway proteins by immunofluorescence technique in the cerebral frontal cortex, apical area with a 40X objective in a 520 mm2 field (Scale Bar 20 µm). Cell nuclei (DAPI) in blue and proteins analyzed in red for cyclin D and *GSK3*β, and green for *Wn*t and β-catenin and B: analysis of proteins of the *Wnt* pathway in each of the experimental groups. C: Representative images in the cerebral cortex and D: Densitometric analysis of β-catenin, *Wnt, GSK3*β and cyclin D expression levels. Mean±SEM graphed, using one-way ANOVA followed by a post hoc Duncan's test. *p<0.05, **p<0.01 and ***p<0.001 compared to the control group, $^{+}$p<0.05, $^{++}$p<0.01 and $^{+++}$p<0.001 compared with the KAL group

Western blot

In the Western transfer technique, the analysis of the same proteins confirmed the results obtained by immunofluorescence, statistically significant results were observed. The group subjected to the amygdala kindling model was expected to result in overexpression of *Wnt* pathway proteins, as demonstrated in previous studies.[22] Therefore, a one-way ANOVA was performed to investigate the decrease in *Wnt* pathway proteins in the hippocampus and cortex in groups subjected to caloric restriction compared to the other groups. At the hippocampal (Fig. 3B) level, β-catenin concentrations were higher in the KAL group (p<0.005 F=9.476), and lower in the KCR group. This indicates an increase in β-catenin expression where the kindling amygdala model was applied and a decrease in groups undergoing caloric restriction. Given β-catenin's role in signal transmission to the nucleus and transcription activation, an elevated concentration of cyclin D was anticipated. Our findings reveal an increase (p<0.035 F=3.98) in both groups subjected to the kindling model with a more pronounced expression in the KAL group One of the most significant results pertains to GSK3β, a component of the β-catenin destruction complex. Its expression was expected to decrease in groups without Wnt stimulation. Indeed, *GSK3*β levels were reduced in the KAL group (p<0.023 F=4.62) and increased in the KCR group due to reflex inhibition by *GSK3*β. The control group exhibited the lowest protein concentrations across all groups. Compared to the Sham group, the differences were not statistically significant, except for cyclin D concentrations, which did not vary significantly between the Sham and Kindling caloric restriction groups. A post hoc test revealed significant differences in β-catenin concentrations in the hippocampus between the KAL group and the remaining groups, mainly with the control group and KCR. In contrast, *GSK3β* protein had significant differences between the Sham group and the control group and KCR with respect to KAL. *Wnt* had differences in the KAL group compared to the Control group, as did KCR compared to KAL. In the cyclin D protein we found differences in all groups compared to the control, and differences between KCR compared to KAL.

In the cerebral cortex (Fig. 4B), the findings followed trends similar to those observed in the hippocampus, although the differences between groups were less pronounced for each protein, particularly for β-catenin and *Wnt*. β-catenin displayed higher concentrations in the KAL group (p<0.001 F=14.72), as did *Wnt*, although *Wnt* concentrations were lower in the KCR group. In the cortex, there was a significant decrease in *GSK3*β levels in the KAL group compared to all other groups (p<0.030 F=4.196), with a notable increase in the KCR group. To validate these findings, a Duncan's test was performed, which confirmed significant differenc-

es between the groups. At the level of the cerebral cortex, we did not find significant differences for the *Wnt* protein.

Discussion

The primary objective of this study was to explore the efficacy of CR as an alternative therapy for DRE and to elucidate the molecular mechanisms involving the *Wnt*/β-catenin pathway and its potential modulation.

It has been suggested that molecular changes may be associated with *Wnt*/β-catenin signaling as many components of this pathway are acutely elevated after seizures.[23] Two decades ago, it was confirmed that seizures induced by electroconvulsive events lead to positive up-regulation of β-catenin and *Wnt*2 ligand in neurons of the rat dentate gyrus.[24] Similarly, elevated expression of *Wnt*3a, β-catenin, and Cyclin D1 was observed in the rat hippocampus, peaking 14 days post-status epilepticus.[25] Additionally, in astrocytes, seizures induced by pentylenetetrazole were linked to β-catenin overexpression and increased susceptibility to seizures.[26]

Our findings are consistent with these observations, confirming a close relationship between the overexpression of these proteins in an established epilepsy model. Differential protein concentrations were observed in both the hippocampus and the cerebral cortex between the experimental groups. In the KAL group, increased concentrations of *Wnt* proteins, β-catenin, and Cyclin D were found, along with decreased *GSK3*β levels of GSK3 compared to the KCR group and the other groups. This suggests that there is indeed an overexpression of this pathway and its components in this epilepsy model. On the other hand, all *Wnt*/β-catenin pathway proteins, except for *GSK3*β, exhibited lower optical densities in the KCR group compared to the KAL group. It should be noted that despite the reductions caused by caloric restriction in the KRC group, it was not possible to reach the concentration levels of the control group, as this group had the lowest values compared to the other groups in the hippocampus in the Western blot. On the contrary, the cerebral cortex showed similar findings, with lower concentrations in the simulated group compared to the other groups. Among the pathway components, β-catenin and *GSK*3β showed the most significant statistical differences in both regions of the brain. Although the *Wnt* protein showed significant statistical values in the hippocampus, it did not show significant differences in the cerebral cortex. Furthermore, the Sham group, where caloric restriction was applied without the kindling epilepsy model, showed lower protein concentrations compared to the other groups in both brain structures in immunohistochemistry. The rest of the immunofluorescence findings were similar for the other groups, mainly for KAL and KCR.

As noted previously, the *GSK3*β protein is a crucial component of the β-catenin destruction complex, which facilitates transcription within the nucleus.[27] Given its role in an inhibitory complex, it is reasonable to expect higher concentrations of *GSK3β* in the KCR group compared to those in KAL, as a mechanistic approach to counteracting pathway deregulation. However, it appears that elevated levels of *GSK3*β do not reach overexpression but can instead contribute to other molecular changes. It is plausible that the observed *GSK*3β levels in the KCR group facilitate proper ubiquitination of β-catenin, thus preventing the complete deregulation of the *Wnt* pathway. Several studies have established that inhibition of *GSK3*β exhibits anticonvulsant effects, similar to protective effects against hippocampal neuronal damage observed with lithium treatment after seizures.[28,29] However, previous research also suggests that excessive inhibition of this protein could adversely affect neuronal plasticity and cognitive dysfunctions.[30-32] In light of these complexities, our study identifies the need for future research to determine optimal levels of *GSK3*β for proper regulation of the *Wnt*/β-catenin pathway. Such research could significantly enhance our understanding and improve treatments that target this pathway.

Our results indicate significant modulation of the *Wnt*/β-catenin pathway by CR, as evidenced by the differential expression of key proteins. We propose a bidirectional relationship, starting from the assumption that an increase in the expression of the components of the *Wnt* pathway, which ultimately allow for the translation of signals to the cellular nucleus (*Wnt* and β-catenin), is directly related to seizure activity (postictal activity). On the other hand, the decrease in protein concentrations allows for regulation of seizure activity, minimizing postictal activity, as reflected by the effects of caloric restriction, as observed in the KCR group findings. Increase in GSK3β through caloric restriction leads to inhibition of the pathway via β-catenin ubiquitination, allowing a decrease in signal translation and an increase in the expression of proteins such as Cyclin D, and consequently seizure activity. These molecular changes, along with increased seizure thresholds and decreased duration observed in the KCR group, are likely to persist and be reinforced over time, highlighting the potential of CR to influence neuronal signaling and alter epileptic activity.

Based on the data presented, our objective is to propose a model to elucidate the relationship between CR, the *Wnt* pathway, and epilepsy, as shown in Figure 5. This model suggests that the inhibitory effects of CR on epilepsy could be mediated through several mechanisms within the canonical *Wnt* pathway. These include mTOR inhibition, reduced glucose absorption due to decreased affinity for GLUT3, electrochemical stabilization of the membrane through the opening of K_{ATP} channels, and enhanced mitochondrial biogenesis and stabilization.

Collectively, these mechanisms contribute to the overall inhibitory influence of CR on epileptogenic activity.

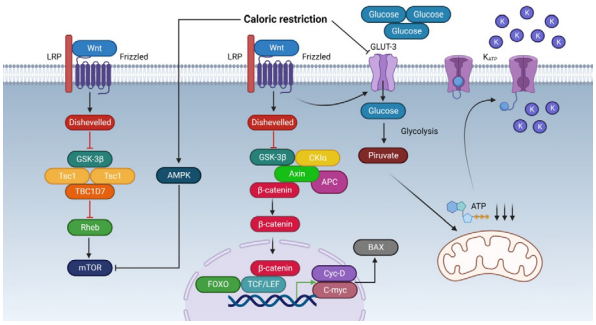

Fig. 5. Molecular mechanisms of the antiepileptic effect of caloric restriction through the regulation of the *Wnt*/β-catenin pathway, created with https://www.biorender.com

Mutations that lead to mTOR hyperactivation are significantly implicated in the pathophysiology of epilepsy, contributing to the production of reactive oxygen species and subsequent neuronal damage.[33] The *Wnt*/β-catenin pathway intersects with mTOR signaling, which is a likely target of the inhibitory actions of CR. Specifically, activation of the canonical *Wnt* pathway results in inhibition of *GSK3β*, a component of the β-catenin destruction complex. This inhibition triggers the activation of the tuberin protein complex, encoded by the tuberous sclerosis genes TSC1 and TSC2.[34] Activation of TSC1 and TSC2 subsequently inhibits Ras homologous enriched brain protein (Rheb), leading to an indirect inhibition of mTORC1, since Rheb is a direct activator of mTORC1.[35]

In the context of CR, reducing carbohydrate intake alters the dynamics of cellular energy and confers a state of cellular stabilization. Activation of the Wnt pathway has been specifically linked to increased affinity of GLUT3 channels for glucose in neurons, rather than to changes in their expression or functional activity. This increase in glucose uptake, particularly observed in the cortex, is stimulated by ligands such as Wnt3a, which enhance glucose uptake through this enhanced affinity mechanism.[36] Associating these dynamics with our findings, where a decrease in *Wnt* pathway signaling was observed in the KCR group, suggests that CR can exert homeostatic control over glucose metabolism through inhibition of the *Wnt* pathway. This inhibition likely results in a reduced affinity of GLUT3 channels for glucose and impacts the enzymes responsible for stimulating glycolysis. Consequently, this reduction in enzyme activity would decrease the energy supply and metabolic capacity of neurons necessary to propagate synaptic signals, conferring cellular stability.

Although this study provides important information on the effects of CR on the *Wnt*/β-catenin pathway and its potential implications for the treatment of DRE, some limitations must be acknowledged. First, reliance on animal models, although necessary, may not fully replicate the complexities of human epilepsy, which could limit the generalization of our findings. Therefore, it is crucial to advance clinical research with controlled clinical trials and specifically apply caloric restriction as a dietary therapy to patients with refractory epilepsy. Additionally, the study's focus on specific proteins within the *Wnt*/β-catenin pathway, while providing significant data, does not encompass all possible molecular interactions and pathways affected by CR. Similarly, only Cyclin D was measured as a from signal transduction result of this pathway, excluding C-*myc* and other proteins that are part of the final outcome of this pathway, which will be considered in future work.

Another limitation is the exclusion of the cerebellum from our analyses. Taking into account the documented involvement of the cerebellum in epilepsy and its association with the *Wnt*/β-catenin pathway in epileptic animal models, including this region could have potentially provided more comprehensive information on the systemic effects of CR.[37-39] Furthermore, the biochemical assays used, though precise, do not capture dynamic interactions in vivo that may influence the behavior of these proteins under different physiological conditions. Future research should aim to address these limitations by incorporating more diverse biological models, including cerebellar evaluations, a broader range of molecular evaluations, and dynamic in vivo analyzes to better understand the therapeutic potential of CR in human epilepsy.

Although we observed promising results indicating potential regulation of pathway overexpression, these findings are limited to the duration of our study and do not encompass chronic periods. Recognizing this limitation, we strongly advocate for future research to extend these investigations. We suggest implementing caloric restriction in longer-term studies to fully assess its effects on the *Wnt*/β-catenin pathway and its implications for epilepsy treatment.

Conclusion

Epilepsy is one of the most prevalent neurological disorders, with up to 30% of patients showing resistance to conventional treatments. This study explored CR as adjuvant therapy, particularly its impact on the canonical *Wnt*/β-catenin pathway, known to be involved in the pathophysiology of epilepsy. The results revealed that CR reduces key proteins in the amygdala initiation model, with a compensatory increase in *GSK3*β. These results suggest that CR can exert anticonvulsant effects through mechanisms such as neuronal membrane stabilization, ROS reduction, mTOR inhibition, and modulation of inflammatory signaling. This highlights the

potential as a therapeutic modifier in epilepsy, which merits further investigation.

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Declarations

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Author contributions

Conceptualization, C.R. and M.R.O.; Methodology, E.U. and E.O.; Software, H.R.P.; Validation, C.R., M.R.O. and H.R.P.; Formal Analysis, A.L.L.; Investigation, A.L.L. an D.V.; Data Curation, H.R.P.; Writing – Original Draft Preparation, A.L.L.; Writing – Review & Editing, C.R.; Visualization, D.V.; Supervision, L.M.A.C. and G.G.G.; Project Administration, C.R.

Conflicts of interest

We declare that there are no conflicts of interest on the part of any of the authors in this study.

Data availability

All clinical and statistical data and materials are available for the benefit of science.

Ethics approval

Our handling and treatment of the animals adhered to institutional protocols, complying with national regulations (NOM-062-ZOO-1999) and international ethical standards (Council for International Organizations of Medical Sciences, CIOMS) study approval number 100/17.

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ORIGINAL PAPER

Interleukin-13 as a potential biomarker in the management of pediatric asthma – a longitudinal study

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ABSTRACT

Introduction and aim. Asthma is predominantly a Th2 type hypersensitive disorder, with interleukin (IL) 4 and IL-13 playing a pivotal roles. Interleukin 13 is one of several cytokines that cause persistent inflammation associated with asthma. The aim was to examine the relationship between the response to treatment in asthma and serum IL-13.

Material and methods. This study, conducted at the SRM Medical College Hospital and Research Center, in Tamil Nadu, involved 68 children aged 6 to 12 years of age diagnosed with asthma. The study included medical history, including age of onset of wheezing, history of allergic rhinitis/atopic dermatitis, food allergies, use of inhalational corticosteroids, hospital admissions, and family history. Spirometry was performed, and treatment with inhalational corticosteroids was started according to GINA guidelines. Blood was collected prior to and after 3 months of treatment.

Results. A substantial positive correlation was observed between gender and IL-13 levels. An improvement in forced expiratory volume in the first second (FEV_1) was observed after treatment [(74.72% vs 95.05%) ($p<0.0001$)]. A negative correlation was discovered between IL-13 and FEV_1. A statistical significance between IL-13 levels before and after treatment ($p=0.005$).

Conclusion. Inhalational corticosteroids reduced serum IL-13 levels, indicating its role as a prognostic marker in pediatric asthma.

Keywords. asthma, biomarkers, interleukin-13

Introduction

Chronic asthma is an inflammatory illness of the airways marked by recurrent episodes of airflow restriction brought on by bronchospasm, edema, and increased mucus production. The term "atopic triad" refers to common conditions that are associated with asthma, including eczema (atopic dermatitis) and seasonal allergies (allergic rhinitis).[1]

Asthma is predominantly a Th2 type hypersensitive disorder, with interleukin 4 (IL-4) and IL-13 playing pivotal roles. Among the several cytokines that cause persistent inflammation associated with asthma is IL-13. In response to antigen-specific stimulation, activated Th2 CD4 and CD8 cells generate IL-13.[2-3] In the absence of appropriate preventive therapy, patients with asthma who have elevated serum IL-13 values may experience irreversible airway modelling due to airway hyper-responsiveness, mucus hypersecretion, fibroblast activation, and airway smooth muscle hyperplasia and hypertrophy.[4]

The clinical heterogeneity observed in individuals with poorly managed asthma requires the development

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of biomarkers that may facilitate the identification of a specific subgroup of patients. It has been demonstrated that in type 2 asthma, the pleiotropic cytokine IL-13 plays a significant role in the etiology of asthma. Induction of goblet cell metaplasia, increased mucus production, increased airway hyperreactivity, and indirectly, chemotaxis-mediated eosinophil trafficking to the site of tissue damage are among the effects of IL-13 in these situations.[5] Mechanistic insights into the involvement of this cytokine in inducing eosinophilia have been unveiled by data from preclinical models and clinical studies including IL-13 inhibitors in humans.

In the era of personalized medicine, to deliver an effective approach to asthma, it is important to identify biomarkers that can predict the course of the disease and response to therapy. A biomarker is a measurable indicator that can evaluate normal or pathological biological processes or the pharmacological response to a therapeutic intervention. Among the few biomarkers studied in asthma, serum IgE lacks specificity, eosinophils show elevated trends in other conditions, such as protozoal infections, serum periostin is elevated in growing children and fractional exhaled nitric oxide requires the availability of specialized equipment.

There is a general lack of literature on the study of biomarkers in assessing therapeutic response in asthma, specifically in the pediatric population.

In the present study, we provide a perspective on the utility of IL-13 as a predictor for evaluating the response to therapy in pediatric asthma patients, as evidenced by an improvement in forced expiratory volume in the first second (FEV_1).

The maximum amount of air expelled during the first second of a forced expiration from a position of fully expressed inspiration is known as the FEV_1. The degree of airway obstruction in asthma is determined by the FEV_1 marker. Reversibility with bronchodilator administration is indicated by a 12% or 200 mL increase in FEV_1.[6-7]

A receiver operating characteristic (ROC) curve, a graphical illustration and fundamental tool used for the evolution and comparison of diagnostic systems that allow comparisons of sensitivity and specificity.[8]

Aim

Examine the relationship between children's response to treatment in asthma and serum IL-13 levels.

Material and methods

The current longitudinal study was conducted at the SRM Medical College Hospital and Research Center, a tertiary hospital in Tamil Nadu, with the approval of the Institutional Ethics Committee (IEC No: ST0922-797). The research project started in September 2022 and spanned six months. In total, 68 patients were recruited for the investigation (n=68).

After receiving informed consent from parents and a duly signed consent form from the research participant, a total of 68 children aged between 6 and 12 years, recently diagnosed with asthma and starting inhalational corticosteroids as defined in the guidelines of the Global Initiative for Asthma (GINA) were recruited into the study.[9] They were followed up after 3 months.

Children with acute exacerbations of asthma that required systemic steroids in the previous 3 months, those already on asthma treatment, those unwilling to give consent, children on steroid therapy for any other conditions such as dermatological and endocrine disorders, those on anti-allergic medications, children <6 years of age and those who developed acute exacerbations during the study period were excluded from the study.

A comprehensive medical history with information on the age of onset of wheezing, history of allergic rhinitis/atopic dermatitis, food allergies if any, prior use of inhalational corticosteroids, hospital admissions related to wheezing and familial history of wheezing or asthma was acquired from the parents.

Children were instructed not to engage in physical activity since the morning of the study. The procedure was carried out with the patient in an upright position, dressed in lightweight clothing, and with their legs uncrossed. Dentures that caused any disruption of the procedure were removed. Occluding the nares manually with nose clips limited air leakage via the nasal passages. The calibration of the spirometer was verified on the day of examination. The patient is required to introduce the mouthpiece into his mouth. After verifying the absence of any leaks and ensuring that the patient is not obstructing the mouthpiece. The process is executed in the following manner:

1. The patient should inhale deeply, taking in as much air as possible, and hold their breath for less than 1 second at the maximum amount of air their lungs can hold.
2. The mouthpiece is placed within the oral cavity, specifically between the teeth, immediately after deep inhalation. To prevent any air leakage, it is important to tightly seal the lips around the mouthpiece. Exhalation should have a minimum duration of 6 seconds, or a duration recommended by the instructor. To solely measure forced expiratory volume, the patient should place the mouthpiece after completing step 1 and should refrain from breathing through the tube.
3. If any of the techniques are executed improperly, the technician must stop the patient's activity to prevent exhaustion and provide the patient with a renewed explanation of the operation.
4. The process is repeated at regular intervals of 1 minute until two matching results are obtained and satisfactory results are obtained.

5. To assess reversibility, following the above procedure, the child was administered 400 micrograms of bronchodilator (salbutamol).
6. The same procedure was repeated after 15 minutes of administration of bronchodilators.

A change in baseline FEV_1 by >12% was suggestive of a positive response, indicating reversibility, thus establishing the diagnosis of asthma.

Based on the medical history and the pulmonary function test, the children were classified into intermittent, mild persistent, moderate persistent and severe persistent asthma and treatment with inhalational corticosteroids was initiated accordingly according to guidelines.

Blood samples were obtained for the estimation of serum levels of IL-13 from the children in EDTA collecting tubes, without endotoxin / pyrogen and centrifuged at 1000 rotations/min for 10 minutes. The supernatant serum (250–500 µL) was stored in deep freezers at -70°C in the Molecular Biology Laboratory, SRM Institute of Science and Technology.

The subjects of the study were evaluated in the outpatient department after three months of adherence to the treatment regimen. In addition, a history of the frequency of symptoms and the need for reliever therapy was obtained. A repeat spirometry was performed and the average of three readings was tabulated against the initial reading. Blood samples were collected to analyze serum levels of IL-13 after therapy and preserved in a deep freezer at -70°C.

Once the research samples (both before and after treatment) were acquired, they were defrosted at room temperature and subjected to analysis using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Diaclone, Besancon Cedex, France). The IL-13 kit utilizes a solid phase sandwich ELISA. The wells of the microtiter strips have been coated with a monoclonal antibody that specifically targets IL-13. The wells are filled with samples, including known IL-13, control specimens, and unknowns, using a pipette. In the initial incubation, a biotinylated monoclonal antibody specific for IL-13 and the IL-13 antigen are incubated together. Following the washing process, the enzyme (streptavidin-peroxidase) is introduced. After incubation and extensive washing to remove any residual enzyme, a substrate solution is added to induce a chromogenic product of reaction in the enzyme that is attached to the substrate. The amount of IL-13 contained in the samples is directly correlated with the color of this product.

A linear standard curve was generated by plotting the average absorbance of each standard on the vertical axis versus the corresponding human IL-13 standard concentration on the horizontal axis. IL-13 levels in each sample were calculated by extrapolating optical density measurements against standard IL-13 concentrations based using the standard curve.

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM, Armonk, NY, USA) to compare blood IL-13 concentrations before and after 3 months of therapy using a paired t-test. A p-value of 0.05 or less was deemed statistically significant. Spearman's correlation analysis was used to analyze the relationship between serum IL-13 levels and treatment response indicated by an improvement in FEV_1 on spirometry, and also between IL-13 and all other demographic and clinical indicators. The ROC curve was used to assess the ability of a diagnostic test, specifically interleukin 13 in our case, to reliably differentiate between two patient conditions: mild asthma and moderate to severe asthma. In addition, to determine an adequate threshold value that will be beneficial for accurately diagnosing the presence or absence of a condition.

Table 1. Baseline characteristics of subjects their correlation with serum IL-13 before and after treatment

Baseline characteristics	Study subjects	Correlation coefficient of IL-13 before treatment (r value)	Correlation coefficient of IL-13 after treatment (r value)
Age in months			
Mean (SD)	101.4±25.2	0.180	-0.027
Gender n (%)			
Male	54 (79%)	0.396	0.135
Female	14 (21%)		
Height (cm)	127.8±16.9		
Weight (kg)	27.8±10.9		
Body mass index (kg/m^2)	16.3±3.8	-0.040	-0.060
Family history n (%)			
Yes	59%	0.052	-0.086
No	41%		
History of atopic dermatitis n (%)			
Yes	29%	-0.205	0.012
No	71%		
History of allergic rhinitis n (%)			
Yes	44%	-0.211	-0.110
No	56%		
Previous hospital admission n (%)			
Yes	33%	-0.075	0.196
No	67%		
FEV_1 (%)	74.72%	-0.274	-0.085
Severity based on spirometry			
Mild asthma	85%		
Moderate asthma	6%	-0.274	-0.085
Severe asthma	9%		

Results

In total, 68 patients were recruited for the investigation (n=68).

A significant positive correlation was observed between gender and circulating levels of IL-13 (r=0.396; p<0.05) was noted; that is, men had higher concentrations of IL-13 in their bloodstreams than females.[10] Serum IL-13 did not correlate significantly with the other clinical and demographic characteristics in our investi-

gation. Table 1 provides an overview of individual baseline characteristics and their correlation with serum IL-13 before and after treatment.

The pulmonary function test (FEV_1) classified the researchers into three groups: mild asthmatics (85%), moderate asthmatics (6%), and severe asthmatics (9%).[11] Children were initiated on inhaled corticosteroids according to starting therapy, blood samples were collected to determine serum IL-13 levels.

The average FEV_1 at the beginning of therapy was 74.72%, and after three months of treatment, the value was 95.05% (Fig. 1).

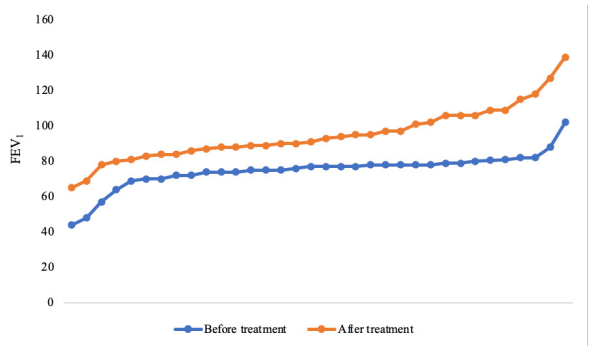

Fig. 1. Graphical representation of the trend of FEV_1 before and after treatment in children with asthma

Table 2. Descriptive statistics of asthma patients – FEV1, treated with ICS

	Mean (%)	Std. deviation	Min–max.	Median	p
Before treatment	74.72	10.18	44–102	77	<0.0001
After treatment	95.05	15.36	65–139	92	

Following therapy, serum IL-13 levels showed a downward trend.

The average serum IL-13 concentration prior to the start of treatment was 2.7±1.2 pg/mL. Following three months of appropriate treatment, the mean value decreased to 1.43±0.9 pg/mL. A difference of statistical significance was identified, as indicated by a p=0.005 (Fig. 2).

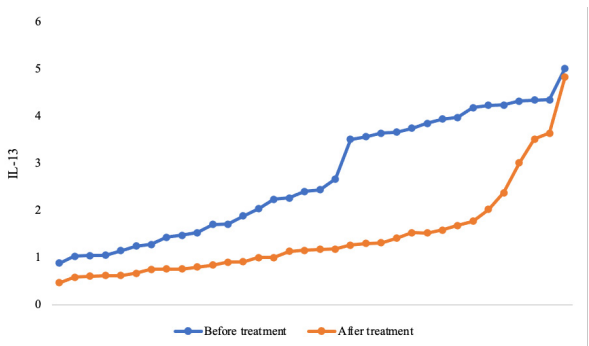

Fig. 2. Graphical representation of the trend of IL-13 before and after treatment in children with asthma

Table 3. Serum IL-13 concentrations in asthma patients treated with ICS

	Mean (pg/mL)	Std. deviation	Min–max.	Median	p
Before treatment	2.7	1.2	0.8–5.0	2.4	0.005
After treatment	1.43	0.9	0.4–4.8	1.1	

It is worth noting that a negative correlation was identified between IL-13 and FEV1. Specifically, after treatment, there was a reduction in serum IL-13 concentrations in conjunction with an improvement in FEV_1. However, the correlation between IL-13 and FEV1, as determined by the Spearman correlation table, did not have statistical significance (r=-0.274; p>0.05) (Fig. 3).

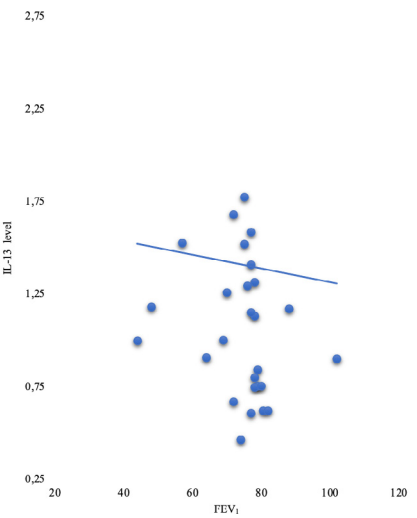

Fig. 3. Graphical representation of the correlation between serum IL-13 and FEV_1

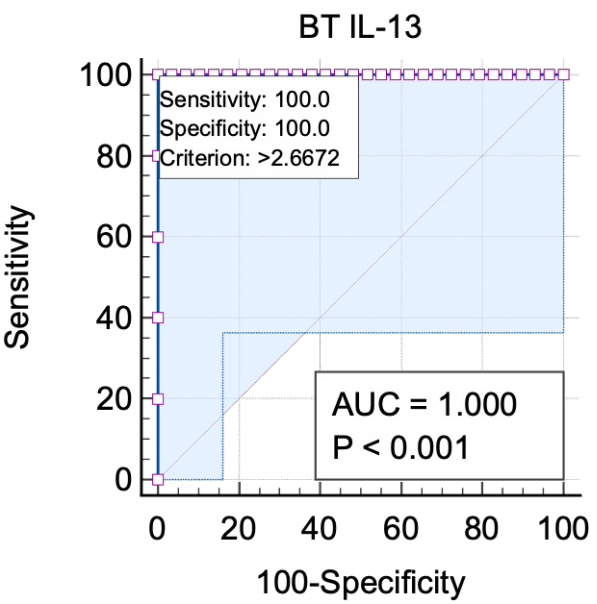

Fig. 4. ROC curve for IL-13 – determination of the cut-off point

Our study demonstrated that IL-13 can effectively differentiate between mild asthmatics and those with

moderate to severe asthma, with a sensitivity and specificity of 100%. The cut-off value to differentiate the groups was 2.6 pg / mL (p=0.001) as depicted in Figure 4.

Discussion

Asthma, one of the most prevalent noncommunicable significant diseases, substantially impacts the quality of life10. Bronchial asthma is a chronic inflammatory disease in which many produced cells and substances produced participate. Special significance is ascribed to eosinophils and lymphocytes of type 2 T helper, which induce IL-4, IL-5, IL-13. A deeper understanding of the mechanisms of the disease made possible by fundamental scientific investigations over the past twenty years has led to the development of highly specific treatments.[11]

A biomarker-driven methodology is being used to define a severe asthma endotype. There is no ideal biomarker, and unlike glycated hemoglobin, or HbA1c, in diabetes, or other certain conditions, the precise nature of asthma biomarkers is yet unknown.[12,13] Further evidence for the involvement in the pathophysiology of airway hyperresponsiveness comes from the overexpression of the protein in asthmatics' sputum, peripheral blood, bronchial submucosa, and mast cells in the airway smooth muscle bundle.[14]

Prevalent studies report elevated levels of IL-13 in asthmatics compared to healthy age matched controls, thereby implicating the role of IL-13 in the pathogenesis of asthma; however, there is paucity of literature among the pediatric population where severity of asthma and response to treatment have been followed up.[15]

In 2019, Saleh Jebur et al. studied 150 asthmatics and 50 healthy controls, aged 10 to 65, in order to estimate the levels of serum IL-13 and serum IgE levels in the blood of patients suffering from allergic asthma. Before starting inhalational corticosteroids, the total serum levels of IgE and IL-13 were measured in both the patients and the control group. Following completion of treatment, blood samples were collected to calculate the total level of serum IgE and IL-13. Consistent with our investigation, a statistically significant decrease in serum IL-13 concentrations was observed after treatment ($p<0.001$).[16]

Jovanovska Janeva et al. conducted a trial to identify the difference in serum IL-13 and FEV1, before and after treatment with ICS/LABA (or) Montelukast in addition to ICS/LABA, in 56 patients with uncontrolled severe persistent asthma in 2015. After therapy, FEV1 improved considerably ($p<0.001$). In conjunction with our study, after 6 months of medication, a substantial decrease in serum levels of IL-13 was found compared to the initial values in both groups ($p<0.0014$, $p<0.001$ respectively). However, the Montelukast group showed a more pronounced benefit.[17]

Gemou-Engesaeth and colleagues proposed that serum levels of IL-13 and surfactant protein D (SP-D) could reflect a disease status in children with asthma. A study was carried out on 20 asthmatic children and 15 controls of the same age. Serum levels of SP-D and IL-13 were initially determined. Children were administered inhaled glucocorticoids or sodium cromoglycate according to the criteria of the American Thoracic Society and European Consensus Guidelines. Serum levels of SD-P and IL-13 were monitored again 4-6 months after treatment. In this research, inhaled glucocorticoids had no significant impact on IL-13 levels, unlike our current study. Patients treated with sodium cromoglycate exhibited a tendency toward a lower level of IL-13, although this difference was not statistical significance.[18]

Recent research indicates that targeting the interleukin-13 pathway may be crucial in treating various asthma subtypes[19]. However, these studies are limited, especially in the pediatric population, and the role of IL-13 as a biomarker in asthma treatment has rarely been studied. A statistically significant difference in IL-13 levels was observed before and after treatment, suggesting the possibility of it being a potential biomarker in pediatric asthma management.

Study limitations

The study was carried out with a limited sample size, and the findings must be reconfirmed using a larger cohort. Also, the study must be analyzed in conjunction with a control population that is matched by age and sex. Furthermore, the incorporation of additional prospective biomarkers and their correlation would provide more insight.

Conclusion

In conclusion, after inhalational corticosteroids, a substantial reduction in serum IL-13 levels and an evident negative relationship between FEV_1 and IL-13 were observed, thereby supporting the role of IL-13 as a marker in predicting response to treatment in pediatric asthma.

Declarations

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Authors' contributions

Conceptualization, P.R.; Methodology, P.R. and P.S.; Software, V.A; Validation, S.S.; Formal Analysis, P.S. and L.K.V.; Investigation, P.S. and L.K.V.; Resources, P.R. and S.S.; Data Curation, P.R., P.S. and V.A.; Writing – Original Draft Preparation, P.S.; Writing – Review & Editing, P.R.; Visualization, V.A.; Project Administration, S.S.; Funding Acquisition, P.R.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The current longitudinal study was conducted at the SRM Medical College Hospital and Research Center, a tertiary hospital in Tamil Nadu, with the approval of the Institutional Ethics Committee (IEC No: ST0922-797).

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ORIGINAL PAPER

Mapping the landscape of gynecological cancer – analyzing presenting features by social network analysis

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ABSTRACT

Introduction and aim. The term 'gynecological cancers' refers to a wide range of malignancies that affect the female reproductive system. These types of cancer include ovarian, cervical, uterine, vaginal, and vulvar cancers. This study aims to employ SNA techniques to map the landscape of gynecological cancer by systematically analyzing the presenting features associated with different types of gynecological malignancies.

Material and methods. In this study, a total of 60 women diagnosed with gynecological cancer were included. An exploratory study design was used. A pre-tested questionnaire was used which includes basic demographic details, type of cancer and symptoms presented at the time of diagnosis.

Results. Nearly 44% of the women diagnosed with cancer were between the age of 51 to 60 years. Symptoms such as abdominal pain, lumps, mild and moderate symptoms that appeared to be highly connected and influential among the cancer patients. Abdominal pain, lumps, abdominal distension/bloating, and mild symptoms had a stronger connection with all other symptoms among the cancer patients.

Conclusion. Educating patients about the significance of symptoms such as abdominal pain, lumps, and abdominal distension/bloating in the context of ovarian cancer can empower them to seek timely medical attention. Increased awareness of the potential implications of these symptoms may prompt patients to undergo screening and diagnostic tests earlier, leading to improved detection rates and treatment outcomes.

Keywords. gynecological cancer, mapping, network visualization, social network analysis, symptoms

Introduction

Gynecological cancers encompass a diverse group of malignancies affecting the female reproductive system, including ovarian, cervical, uterine, vaginal, and vulvar cancers. These cancers collectively pose a significant burden on global health, contributing to substantial morbidity and mortality among women worldwide.[1] The estimation of cancer burden from the International Agency for Research on Cancer indicated that gynecological cancers accounted for 19% of worldwide cancer cases.[2] Gynecological cancers in India accounted for 30% of the total cancers among women.[3] A study reported in India, based on the cancer registries, indicated a decline in cervical cancer cases and a rise in breast, ovarian, and uterine corpus cancer cases across most of the registries over the full observation period. Four types of cancer, such as breast, cervix, corpus uteri, and ovary, constitute over 50% of all cancers in women.[4] Despite advances in early detection and treatment modalities, knowing the complexity and heterogeneity of gynecological cancers

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is important. Understanding the clinical characteristics associated with gynecological cancers is paramount for timely diagnosis, appropriate treatment allocation, and improved patient outcomes.[5] The characteristics encompass a spectrum of clinical manifestations, including symptoms, risk factors, demographic characteristics, and diagnostic markers, which can vary widely across different cancer types and stages of cancer.[6]

Conventional methods of analyzing presenting features focus primarily on examining individual variables separately, sometimes missing the complex interconnections and interdependencies between them. Researchers have started using innovative methodologies such as social network analysis to study the complex networks of relationships in medical data due to the limitations of standard techniques.

In recent years, the application of social network analysis (SNA) has emerged as a powerful tool in biomedical research for elucidating complex interactions among various elements within a system.[7] Social network analysis offers a powerful framework for investigating the interconnectedness of presenting features in gynecological cancer cases. By conceptualizing the presenting features as nodes and the relationships between them as edges within a network graph, SNA allows researchers to elucidate the underlying patterns, structures, and dynamics of these complex systems. Through quantitative metrics and visualization techniques, social network analysis enables the identification of key features, central nodes, and subgroups that may influence disease presentation, progression, or response to therapy.

Aim

This study aims to employ SNA techniques to map the landscape of gynecological cancer by systematically analyzing the presenting features associated with different types of gynecological malignancies. Through this approach, this study gains deeper insights into the complex etiology and phenotypic heterogeneity of gynecological cancers, ultimately facilitating more accurate diagnosis, risk stratification, and personalized treatment approaches.

Material and methods

In this study, an exploratory study design was used. Primary data were collected from the cancer patients admitted from April 2023 to February 2024 at a private hospital. Participants were selected from the health records and a face-to-face interview was conducted with a semistructured questionnaire, which included demographic details, menstrual history, existing health problems, and symptoms presented during diagnosis. The dataset included information on patients diagnosed with various gynecological cancers, such as ovarian, cervical, uterine, vaginal, and vulvar cancers, along with their presenting features.

Variable selection

We identified a set of presenting features associated with gynecological cancers, encompassing symptoms, risk factors, and demographic characteristics. Variables were selected based on their clinical relevance, previous literature, and expert consultation to ensure a comprehensive representation of gynecological cancer.

Data preparation

Data were collected by using the software Kobo-collect and then extracted in Excel (Microsoft Corporation, Washington, USA). Prior to analysis, the dataset underwent thorough cleaning and preprocessing to address missing values and outliers. The descriptive analysis was performed in SPSS version 22 (IBM, Armonk, NY, USA), and the network analysis was performed with the help of Python (Python Software Foundation, Wilmington, Delaware, USA).

Social network construction

We constructed a network graph where nodes represent presenting features denoted as S1 to S10 (Table 1), and edges represent the relationship between them. The relationship between presenting features was defined based on co-occurrence or mutual information derived from the dataset.

Table 1. Label for nodes

Nodes	Major classification	Symptoms
S1	Abdominal pain	–
S2	Abdominal distension and bloating	–
S3	Mild symptoms	Loss of appetite, weight loss, giddiness, nausea, vomiting, body ache, headache, fever, cold, fatigue, dry cough
S4	Lumps in the organs	Lumps in breast, eyelid, thigh and abdomen
S5	Pain in body parts	Ear pain, throat pain, body pain, thoracic pain, breast pain, nipple pain
S6	Abnormal discharge	Discharge in breast, vaginal discharge
S7	Abnormal bleeding	Bleeding gums, postmenopausal bleeding, abnormal menstrual bleeding, Irregular periods
S8	Blood	Blood in vomiting and stool
S9	Swelling	Swelling in tongue, breast, eyelid, and abdomen
S10	Moderate symptoms	Pleural effusion, burning sensation, skin growth, skin discoloration, pedal edema, inverted nipple, jaundice, reddish spots, difficulty in swallowing, breathlessness, and constipation

Network analysis

Quantitative metrics were calculated to characterize the network structure, including measures of centrality, weighted degree centrality, and connectivity. Visualization techniques, such as the Kamada-Kawai layout, are used to visualize the network structure and to identify the clusters or subgroups of interconnected features.

Table 2. Demographic details of study participants

Variables	Frequency	Percentage
Age		
31–40	5	8%
41–50	19	31%
51–60	27	44%
61–70	9	15%
71–80	1	2%
Marital status		
Married	48	80%
Unmarried	1	1%
Widowed	10	18%
Separated	1	1%
Religion		
Hindu	55	92%
Muslim	4	7%
Christian	1	1%
Family income		
Below 10,000	26	43%
11,000–20,000	26	43%
21,000–30,000	2	3%
31,000–40,000	1	2%
41,000–50,000	4	7%
Above 50,000	1	2%
Education		
Illiterate	21	35%
Primary School	10	17%
Middle school	16	27%
High school	8	13%
Graduate	2	3%
Professional degree	3	5%
Occupational status		
Unskilled worker	13	22%
Semi-skilled worker	1	2%
Skilled worker	0	0
Clerical/shop/farm	2	3%
Semi profession	0	0
Professional	3	5%
No work	41	68%
BMI		
Underweight	6	10%
Normal	27	45%
Overweight	18	30%
Obesity	9	15%
Periods		
Regular	47	78%
Irregular	13	22%
Flow		
Normal	45	75%
Abnormal	15	25%

Ethical considerations

All data handling and analysis have adhered to ethical guidelines and regulations to ensure patient privacy, confidentiality, and data security. Institutional Ethical Clearance (8494/IEC/2023) was obtained from the SRM Institute of Science and Technology, Kattankulathur.

Results

A total of 60 participants were included in the study (Table 2). Forty four percent of the women were between the ages of 51 to 60, followed by 31% between 41 to 50 years. Among the study participants, 80% of them were married, and 18% of them were widowed. Ninety two percent of the women were Hindu. Forty three percent of the study participant family income fell below 10000 rupees. Sixty eight percent of the study participants were not employed, and 22% were unskilled workers. Forty five percent of the study participants had normal weight, and 30% of them were overweight. Among the study participants, nearly 30% of the women had ovarian cancer, followed by breast cancer (27.8%), colon cancer (6.7%), and endometrial cancer (4.4%) (Table 3).

Table 3. Type of cancer

Type of cancer	Frequency	Percent	Type of cancer	Frequency	Percent
Breast cancer	25	27.8	Lung cancer	3	3.3
Cervical cancer	2	2.2	Multiple myeloma	2	2.2
Cholangio cancer	1	1.1	Ovarian cancer	27	30.0
Colon cancer	6	6.7	Pancreatic cancer	2	2.2
Endometrial cancer	4	4.4	Primary peritoneal cancer	4	4.4
Esophagus cancer	2	2.2	Rectal cancer	3	3.3
Eyelid cancer	2	2.2	Stomach cancer	2	2.2
Gall bladder cancer	1	1.1	Tongue cancer	2	2.2
Vulva cancer	1	1.1	Tonsil cancer	1	1.1

Figure 1 illustrates the basic connection between the symptoms of ovarian cancer. Abdominal pain, abdominal distension and bloating, lumps in the organs, mild and moderate symptoms play an important mediating role for participants having gynecological cancer.

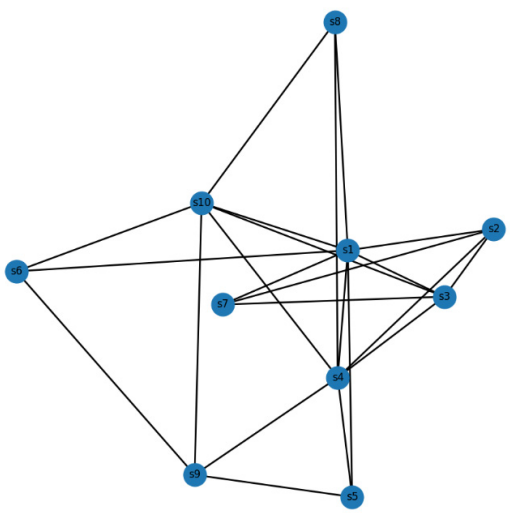

Fig. 1. Connection between the symptoms

Centrality measures

Degree centrality measures the number of edges incident upon a node. Higher values indicate nodes that are more directly connected to other nodes in the graph. Abdominal pain has the highest degree centrality at 0.89, indicating it has the most connections among the other symptoms listed (Table 4).

Table 4. Centrality measures

Nodes	Degree centrality	Betweenness centrality	Closeness centrality	Eigenvector centrality
S1	0.89	0.28	0.9	0.45
S4	0.78	0.15	0.81	0.43
S10	0.67	0.09	0.75	0.38
S3	0.56	0.04	0.69	0.35
S2	0.44	0.01	0.64	0.28
S9	0.44	0.03	0.6	0.24
S5	0.33	0.01	0.6	0.22
S6	0.33	0.01	0.6	0.21
S7	0.33	0.001	0.56	0.21
S8	0.33	0.001	0.6	0.25

Betweenness centrality measures the extent to which a node lies on the shortest paths between other nodes. Higher values suggest nodes that act as bridges between different parts of the network. Abdominal pain has a relatively high betweenness centrality of 0.28, indicating it lies on many shortest paths between other nodes.

Closeness centrality measures how close a node is to all other nodes in the graph. Nodes with higher closeness centrality values are closer to all other nodes in terms of geodesic distance. Abdominal pain has a high closeness centrality of 0.9, indicating it is very close to all other nodes in the graph.

Eigenvector centrality measures the influence of a node in the network, considering both the direct and indirect connections. Nodes with higher eigenvector centrality values are connected to other nodes that are themselves well-connected. In this study abdominal pain and lumps have a relatively high eigenvector centrality of 0.45 and 0.43, indicating it is connected to other nodes that are influential in the network.

Examining all of this data together helps researchers understand the structural significance and impact of each node in the network. Abdominal pain appears to be a highly central node according to all four measures, suggesting it plays a significant role in connecting other nodes and controlling information flow within the network. On the other hand, nodes with lower values across these centrality measures may be less influential or less central in the network structure.

Weighted centrality measures

Table 5 represents weighted degree centrality measures for different symptoms from S1 to S10 in a network, where each symptom is associated with a weighted degree value. Symptoms are the nodes in the network, each representing a symptom observed in the study participants. Weighted degree centrality measures the sum of the weights of the edge's incident upon a node. In other words, it represents the total strength of connections each symptom has with other symptoms in the network. Higher values indicate symptoms that are more strongly connected to other symptoms. Abdominal pain and lumps have the highest weighted degree centrality of 24 each, suggesting that they have the strongest connections with other symptoms in the network, with a total weight of 24. Mild symptoms follow closely with a weighted degree centrality of 21, indicating a strong connection with a total weight of 21. Abdominal distension and bloating have a weighted degree centrality of 21, indicating a moderate level of connection strength with other symptoms. Pain in the organs has a weighted degree centrality of 14, indicating a relatively lower but still significant level of connection strength. Swelling and moderate symptoms have a weighted degree centrality of 10 each, suggesting they have weaker connections compared to the above symptoms. Abnormal discharge and bleeding have a weighted degree centrality of 4, indicating they have even weaker connections. Blood in stools and vomit has the lowest weighted degree centrality of 3, suggesting it has the weakest connections in the network.

Table 5. Weighted degree of the nodes

Symptoms	Weighted degree
S1	24
S4	24
S3	21
S2	16
S5	14
S10	10
S9	10
S6	4
S7	4
S8	3

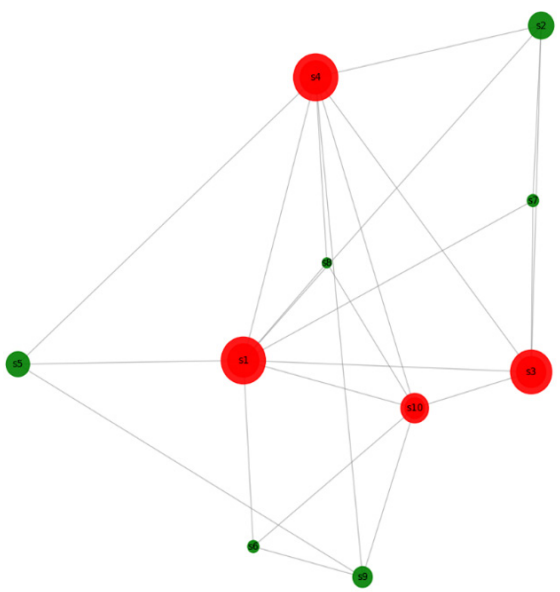

Fig. 2. Network visualization according to the weighted degree centrality

Network visualization with weighted degree centrality

The Kamada-Kawai layout algorithm is a force-directed layout algorithm commonly used for visualizing net-

works (Fig. 2). It positions the nodes in such a way that nodes with stronger connections are placed closer together, while nodes with weaker or no connections are positioned farther apart.

Node positions: Symptoms such as abdominal pain and lumps that are closer together in the visualization are likely to be more strongly connected in the network. On the contrary, nodes such as discharge, abnormal bleeding, blood in vomit, and stools that are farther apart are likely to have weaker connections or no connections. This arrangement gives an overall network structure and connectivity patterns.

Central nodes: Symptoms such as abdominal pain, lumps, mild and moderate symptoms appear centrally located in the visualization, surrounded by many other nodes, and are likely to be highly connected and influential within the network. These nodes represent key elements or hubs that play significant roles in the network's functioning.

Node community

Symptoms are referred to as nodes, and community refers to the clusters to which each symptom belongs. Nodes within the same community are more densely connected compared to nodes in other communities. Community 0 includes abdominal pain, distension and bloating, mild symptoms, and abnormal bleeding. These nodes are tightly interconnected with each other within the network and form a cohesive subgroup or cluster. They likely share similar characteristics or are involved in similar processes within the network.

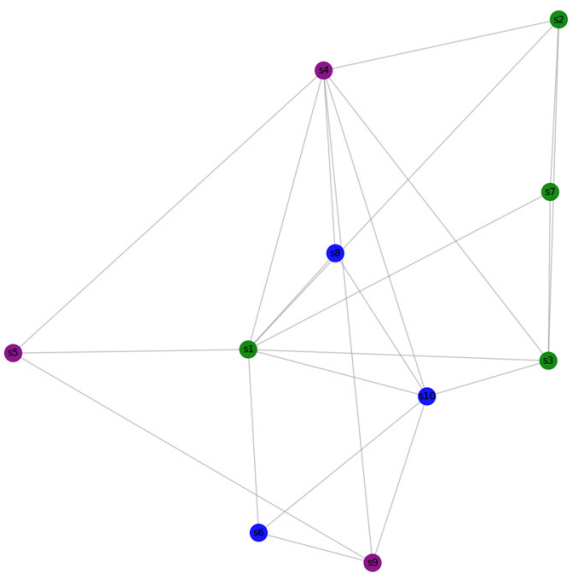

Fig. 3. Network visualization with community coloring

Community 1 includes nodes, lumps, discharge, and blood in vomit and stools. These nodes form another subgroup within the network, distinct from community 0. They are closely connected but less so to the nodes outside their community.

This community 2 includes pain, swelling, and moderate symptoms. These nodes are densely connected but have fewer connections to nodes outside their community.

Network with community coloring

The green color indicates Community 1, the blue color indicates Community 2, and the green color indicates Community 3. These clusters are groups of nodes that are densely connected but have fewer connections to nodes outside the cluster. Identifying these clusters can provide insights into the functional subdivisions within the network (Fig. 3).

Geodesic distance

The shortest path or geodesic distance gives the minimum number of edges between the two nodes in a network (Table 6). Lumps, pain, abnormal discharge, and blood in vomit and stool have the shortest path for abdominal pain.

Table 6. Geodesic distance

Symptoms	Shortest path length from S1
S1	0
S6	1
S8	1
S4	2
S5	2

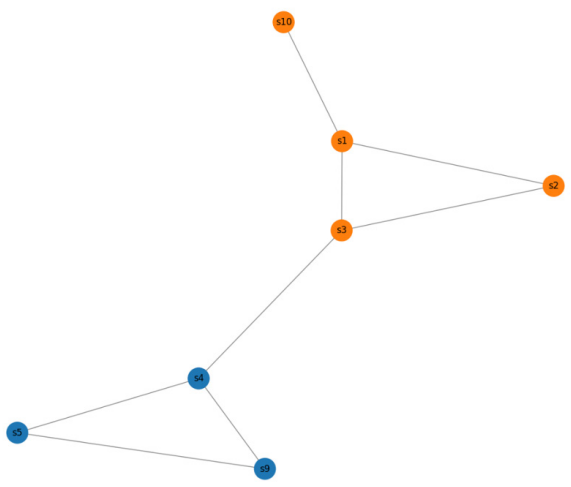

Fig. 4. Sub-network with community coloring

Sub-network weighted degree centrality with community

Using the 50 percentile of weight threshold from the descriptive statistics created a sub-network with edges that have a weight greater than the threshold. Lumps, pain, and swelling form different communities highlighted with blue color. Symptoms within the same community share common attributes or roles within the network.

Abdominal pain, abdominal distension and bloating, and mild and moderate symptoms form different communities highlighted in orange color. Mild symptoms and lump act as the bridge between these two communities (Fig. 4).

Sub-network centrality measures

Sub-network measures the weighted degree centrality of nodes within the subnetwork. The correlation between subnetwork weighted degree centrality and closeness centrality is approximately 0.93, indicating a strong positive correlation between these two measures within the subnetwork. The correlation between closeness centrality and betweenness centrality is approximately 0.93, also indicating a strong positive correlation. The correlation between closeness centrality and betweenness centrality is approximately 0.93, also indicating a strong positive correlation.

Discussion

Abdominal pain appeared to be the most common symptom among the study participants. It also demonstrates significant betweenness centrality, suggesting its importance as a bridge between different parts of the network. Moreover, its high closeness centrality underscores its proximity to all other nodes in the graph. Khan A et al. reported that abdominal pain and abdominal distension were the most common symptoms of ovarian cancer.[8] These results highlight abdominal pain as a crucial indicator and potentially a primary symptom of concern in diagnosing ovarian cancer.

A recent study by Simon et al. reported that 35% of gynecological cancer participants reported frequent symptoms and severe symptoms.[9] Abnormal bleeding in menopausal and post-menopausal women and abnormal uterine bleeding were the most commonly identified symptoms of uterine cancer.[10] These results were similar to the current study. Symptoms such as abdominal pain, abdominal distension and bloating, and mild and moderate symptoms form a cohesive subgroup, indicating potential common pathways or shared underlying mechanisms in cancer manifestation. The network analysis reveals distinct communities within the symptom network, with symptoms clustering together based on their connectivity patterns.

Weighted degree centrality provides insights into the strength of connections between symptoms. Abdominal pain and lumps exhibit the highest weighted degree of centrality, indicating strong connections with other symptoms in the network. The present study suggests that these symptoms are closely intertwined and likely co-occur frequently in patients with ovarian cancer. A study by Ebell et al. reported that abdominal mass, abdominal distension, and abdominal pain were the most common presenting symptoms during the diagnosis of ovarian cancer.[11]

The identification of subnetworks based on weighted degree centrality thresholds further delineates functional subdivisions within the network. The formation of different communities by symptoms like lump, pain, and swelling, as opposed to symptoms like abdominal pain, abdominal distension and bloating, mild, and moderate symptoms, suggests distinct patterns of symptom interactions and potential differences in disease progression or severity. Koo et al. in his study reported that breast lumps (83%) and breast pain (6%) were the frequent symptoms that occurred in breast cancer patients.[12] Another study by Goff et al. reported that back pain (45%) was the most common symptom experienced by the study participants.[13] A study by Bankhead et al. reported that abdominal distension, post-menopausal bleeding, loss of appetite, early satiety, and progressive symptoms were statistically significant variables associated with ovarian cancer.[14] Social network analysis was useful to categorize the symptoms based on their cluster.

The strong positive correlations observed between subnetwork weighted degree centrality and closeness centrality, as well as between closeness centrality and betweenness centrality within the subnetwork, indicate a cohesive structure where highly connected nodes also tend to be centrally located and play significant roles in controlling information flow.

Study limitations and recommendations

This study had a relatively small sample size, which may have limited its findings. Additionally, there is a possibility of recall bias when gathering information on symptom recognition. SNA provides valuable insights into symptom interactions and centrality within the context of gynecological cancer, further research and validation studies are warranted. Continual refinement and validation of symptom networks can enhance our understanding of gynecological cancer pathophysiology, refine diagnostic criteria, and inform the development of targeted therapeutic interventions.

Conclusion

Abdominal pain was identified as the most central symptom in this study, demonstrating its crucial function in interacting with other symptoms. The subnetwork displays a robust positive association among its centrality measurements. Given the central role of abdominal pain in the network and its strong connections with other symptoms, clinicians should prioritize the assessment and evaluation of abdominal pain in patients presenting with potential symptoms of cancer. Rapid and accurate diagnosis of abdominal pain may lead to earlier detection of gynecological cancer and improve patient outcomes. Abdominal pain, lump, abdominal distension/bloating, and moderate symptoms show a strong connection within the network. Clinicians should per-

form a thorough examination that involves analyzing these symptoms together with abdominal pain to gain a better understanding of the possible existence and development of ovarian cancer. This comprehensive method can improve the accuracy of diagnosis and facilitate timely interventions. Educating women about the significance of symptoms such as abdominal pain, lump, and abdominal distension/bloating in the context of ovarian cancer can empower them to seek timely medical attention. Increased awareness of the potential implications of these symptoms may prompt patients to undergo screening and diagnostic tests earlier, leading to improved detection rates and treatment outcomes.

Declarations

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Author contributions

Conceptualization, I.S. and G.J.; Methodology, G.J.; Software, I.S. and G.J.; Validation, G.J. and I.S.; Formal Analysis, I.S.; Investigation, I.S; Resources, G.J.; Data Curation, I.S. and G.J.; Writing – Original Draft Preparation, I.S.; Writing – Review & Editing, G.J.; Visualization, I.S.; Supervision, G.J.

Conflicts of interest

The authors declare that there is no conflict of interest.

Data availability

Data will not be available online based on ethical considerations. The datasets produced and/or examined in the present study are not accessible to the public owing to the ethical approval requirement that mandates the confidentiality of respondents' answers. On reasonable request, they will be made available by the corresponding author.

Ethics approval

Ethical clearance was obtained from the Institutional Ethics Committee of the SRM Medical College and Research Centre (Approval Number: 8494/IEC/2023).

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ORIGINAL PAPER

Effect of web-based pediatric pain management education on nursing student knowledge levels

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ABSTRACT

Introduction and aim. Pain is a common symptom in children. Studies indicate that nurses and student nurses lack knowledge about pediatric pain. This study was designed to determine the level of knowledge of pediatric pain management for nursing students and to evaluate the effectiveness of the web-based pediatric pain management education (PPME) program.

Material and methods. This study used a pre/post-test quasi-experimental design before/after the test. It was carried out with 84 pediatric nursing internship students (39 control, 45 intervention) in a nursing school. The control group received routine training, and the intervention group received web-based modules. Data were collected using the questionnaire developed by the researchers and evaluated using the paired sample t-test, independent samples t-test, Spearman's correlation and regression analysis. A structural equation model (SEM) was used.

Results. The level of knowledge of the intervention group was significantly higher than that of the control group in terms of the total score and sub-dimension scores of awareness, physiopathology and 'control' ($p<0.05$). A moderate, positive and significant correlation was observed between PPME and knowledge levels. Web-based education accounted for 56.6% of the increase in knowledge level, resulting in an improvement of 11.062 points. A notable positive correlation was observed between PPME and control scores in SEM.

Conclusion. The conclusion drawn is that the web-based PPME effectively increased student knowledge scores.

Keywords. pain management, pediatric nursing, students, web-based education

Introduction

Pain, which is one of the most common symptoms in children, is considered an important public health problem. Evidence-based research on pediatric pain management has increased the knowledge and awareness of health professionals, but pediatric patients still experience pain from illness, surgery, and medical procedures.[1,2] The World Health Organization has emphasized that pediatric pain is often not noticed, may be ignored, or even denied by health care workers in the 2012 report.[3]

8–88% of children and young people experience pain and 37–72% of hospitalized children experience clinically significant pain.[4,5] Pain in the pediatric population is difficult to realize and evaluate correctly. Many studies in the literature show that nurses experience various difficulties in assessing pain in children.[6-8] The most common reasons for this are the verbal and cognitive immaturity of young children to express their pain, the difficulties in recognizing pain symptoms from reactions such as separation from the mother, and the insufficiencies of nurses to control pain such as lack of knowledge and misunderstandings.[2]

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Studies show that nurses and student nurses lack knowledge about pediatric pain assessment and management.[6,9] This indicates a lack of structured pain education and emphasizes the need for comprehensive pain management for students before starting their professional nursing careers.[9] Several education programs have been developed to increase pain knowledge for nurses and undergraduate nursing students in the literature. Due to these studies, it was found that nurse pain knowledge, assessment, and management skills increased.[8,10]

Pain education is provided by classical learning techniques in most nursing schools. It is known that student knowledge level increases with classical learning, but the information gained may not lead to a behavioral change or application to practice.[11] As an alternative to conventional learning, web-based teaching methods have become quite popular in recent years. This method can be used to gain nursing skills and facilitate their transfer to the clinical environment.[12] This learning method can allow nursing students to meet with advanced computer technologies that they will use intensively as part of patient monitoring and care in their professional lives.[13] Students have the opportunity to self-learn by reviewing courses where and whenever they want. Students can easily communicate with instructors via messages or messages, combine material in the discussion forums, send homework, and take their exams or quizzes.[14,15]

With the use of web-based education programs, nursing students can practice their skills in a safe environment and learn the information by repeating it as often as they want before working in the clinical environment.[16] Web-based pain management education was found to improve the level of knowledge of nursing students by Keefe and Wharrad.[17] Similarly, the results of studies conducted in other health schools show that web-based pain education has been successful.[1,18] It is believed that well-equipped students with the necessary knowledge and skills in pediatric pain management, as in many other fields that require nursing expertise, will have a positive impact on clinical outcomes. It is predicted that web-based education will play an important role in successful pain management. In our country, the lack of a sufficient number of studies evaluating the effect of web-based pediatric pain management education (PPME) programs on pediatric pain management knowledge (PPMK) of nursing students has been a guiding light on the planning of this study. This study represents a significant contribution to the field of pediatric pain management, as it is one of the first studies to evaluate web-based education in this area in our country. Furthermore, it offers a novel perspective on the nursing curriculum in this field.

Aim

The purpose of this study is to determine the PPMK of the student nurse PPMK and evaluate the effectiveness of the web-based PPME program.

Material and methods

Design

This study was designed as a quasi-experiment with an unmatched group.

Setting and sample

This study was carried out in a university nursing school with the control group in the fall semester of the 2017–2018 (n=39) academic year and the initiative group (n=45) in the fall semester of 2018–2019 academic year, to prevent the sharing of information among the groups. The population of this study consisted of 84 nursing students who pursued internship education in pediatric nursing. The sample size was calculated with the Gpower 3.0 program and was based on type I error 0.05, type II error 0.20 (80% power), using the correct response rate of the study by Keefe et al. The sample size was calculated as 80 students. The study included all the students who had volunteered to participate.

Ethics approval

All participants gave their informed consent to be included before participating in the study. The study was carried out according to the Declaration of Helsinki, and the protocol was approved by the Non-Interventional Research Ethics Committee of Dokuz Eylül University (date: 05.05.2017, Number: 10-19) and the directory of Nursing Faculty (Date: 30.03.2017, number: 19396244-108.99/525).

Procedure

Development of the web-based PPME program

Educational content was developed based on the pain management guidelines of various international and national organizations, societies, universities and hospitals as well as from data from evidence-based studies.[1,3,19-28] It is stated in the literature that pain is related to awareness, physiopathology, barriers, diagnosis, evaluation and control dimensions.[29] Therefore, the modules of the educational content were created considering these dimensions (Table 1).

Expert opinion

The module's educational content was contributed by 10 faculty members who are experts in the field of child health and disease nursing. A checklist was created for expert opinion, comprising parameters such as scientific content, comprehensibility, suitability for the student's level, and recommendations. The checklist, along with the module contents, was sent to experts. The edu-

cational content was evaluated by experts to determine whether it included the criteria mentioned above. The experts provided recommendations for each module and provided updated references.

Table 1. Program modules of PPME

Modules	Content of PPME	Transferred time to the website
1.	**Pediatric Pain Awareness** - Definition of pain - Atraumatic care - Pain and empathy - Self-assessment of pain - Pain perceptions of children and factors their pain reaction - Cognitive development and perception of children - Hospitalization and perception of pain - Children's Pain Reactions - Differences between pediatric pain and adult pain	2nd week
2.	**Pediatric pain physiopathology** - Causes of pain - Classification of pain - Physiopathology of pain - Theories of pain - Dimensions of Pain - Consequences of undertreated pain in infants and children	3rd week
3.	**Barriers to effective pediatric pain management** - Conditions preventing pain management in children - Misunderstandings, fears, and realities about pediatric pain	4th week
4.	**Pediatric pain management** - **First section: diagnosis of pain** - Basic Components of Pediatric Pain Diagnosis - **Second section: assessment of pain** - Determining Pain Assessment Scale according to the developmental level of children - Reassessment of pediatric pain - Pain assessment in special children - **Third section: control of pain** - Nonpharmacologic pain control in children - Pharmacological pain control in children - Analgesia ladder method - Non-opioid analgesics - Opioid analgesics - Adjuvant analgesics - Local anesthesia in children - Patient-controlled analgesia in children - Sedation in children - Parental involvement in pain management - Nursing approach to pediatric pain management	The 5th week 6th week

For module 1:
- Explaining the subject of pain and empathy with examples from nature
- Exemplifying the factors that affect children's perception of pain
- Diversifying children's reactions to pain according to their age
- Adding the difference between child pain and adult pain

For module 2:
- Explaining the characteristics of pain according to its types
- Explaining the factors that affect chronic pain in children
- Explaining the nociception process
- Explaining Pain Transmission

For module 3:
- Adding to the topic of misconceptions about pain in children,

For module 4:
- Using local anesthetics in the effective management of procedural pain in children
- Explaining the use of adjuvant drugs among pharmacological agents
- Adding the age group in patient-controlled analgesia in children
- Adding the results of optimal pain management

The recommendations of the experts who provided feedback were collated into a single document for each module. The researchers implemented the necessary corrections and enhancements, thereby producing the final version of the educational content.

Pilot study

A pilot study was conducted with eight third-grade students who represented the sample and were not in the study group. Their feedback was received. As a result of the pilot study, it was decided to divide the fourth module into three sections because its content was quite intense.

Educational intervention

The Web-based PPME is a comprehensive evidence-based program designed for nursing students and consists of four modules focused on pediatric pain management. Modules are scheduled as follows:

1. Module: pediatric pain awareness – 2nd week
2. Module: Pediatric pain physiopathology – 3rd week
3. Module: Barriers to effective pediatric pain management – 4th week
4. Module: pediatric pain management – 5th and 6th week (Fig. 1)

The contents of the web-based pediatric pain management education program were uploaded to the web environment via the University's Distance Education Implementation and Research Center. This system has an advanced infrastructure that enables e-learning content to run in different educational software. In the system created with this infrastructure, courses can be introduced to students and registrations can be made. This system enables students to access educational resources via the Internet whenever and wherever they want. The pediatric pain management course was made available to the intervention group and the students were registered for the course. The system enabled researchers to provide web-based education through live

lessons, resource sharing, announcement, and chat room modules (Fig. 2).

Fig. 1. Flow chart of the study

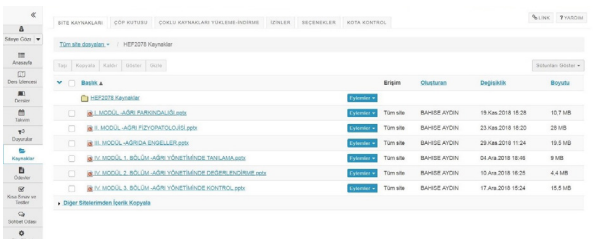

Fig. 2. An image of web-based education

Control Group

The control group underwent standardized training as part of their internship program without additional intervention. At the beginning and end of the semester, the students in this group completed a pre-test and post-test (Fig. 1).

Intervention group

The intervention group participated in a web-based PPME program. In the first week of the program, students were informed about the educational content and flow chart of the study, and the researcher introduced the website. In addition, the sociodemographic data form and pretest were applied to the students. PPME modules were uploaded to the university's existing distance education center website. The researcher interacted with students on-line and offline during education sessions following the schedule (in the second, third, fourth, fifth, sixth and seventh weeks). In the 15th week, the final stage of the study was completed by applying the post-test and satisfaction questionnaire to the students (Fig. 1).

Measurements and data collection *A* total of 84 students were enrolled in the study. The students completed data collection tools. The pre-test and post-test forms were matched with student nicknames according to the form instructions. A sociodemographic questionnaire was used to collect personal data such as age and sex. To assess the PPMK of students, the researchers developed a pediatric pain management knowledge questionnaire was developed by the researchers according to the literature consisting of multiple choice, true/false, open-ended, and matched questions.[7,30-32] The questionnaire consists of 81 questions and six sub-dimensions (pain awareness, the physiopathology of pain, barriers to effective pain management, pain diagnosis, pain assessment, and pain control) coinciding with the content of each module (Table 2). The increase in the scores of the questionnaire indicates that the students had more information about pediatric pain management. Knowledge scores were calculated by summing up the numbers of correct answers.

Table 2. Sub-Dimensions of pediatric pain management knowledge questionnaire

Module	Section	Sub-dimensions (short name)	No of questions	Score range	Content of sub-dimesions
1.		Pain awareness (awareness)	10	0–17	The students have questions about the effects of pediatric pain, perception of pain,, children's responses to pain and symptoms of pain.
2.		Pain physiopathology (physiopathology)	9	0–9	The questions include the physiological and pathological response of children to pain and the types of pain.
3.		Barriers to effective pain management (barriers)	17	0–17	There are questions about situations that prevent pediatric pain management in the clinical area and nurses' issues that prevent accurate diagnosis and treatment of pediatric pain.
4.	1	Pain diagnosis (diagnosis)	5	0–5	Questions include the time to diagnose pain and its components.
	2	Pain assessment (assessment)	5	0–8	There are questions about pediatric pain assessment and in special groups.
	3	Pain control (control)	35	0–51	Questions include pharmacological and non-pharmacological techniques.
Total			81	0–107	

Statistical analysis

Data were analyzed with SPSS 24.0 and AMOS 25.0 (IBM, Armonk, NY, USA). The number, average, and percentage values were used in the descriptive data. In comparison of the pre-test and post-test scores of the intervention and control groups, the paired samples t-test was used. The relationship between PPME and student knowledge levels was evaluated using Spearman correlation analysis. Covariance analysis was used to compare the pre/posttest difference scores of sub-dimensions of knowledge levels of the intervention and control groups. Power analysis and effect size were used to determine the effectiveness of the study. Regression analysis was performed to determine the effectiveness of PPME. Multiple correlations were evaluated using variance in-

flation factor (VIF), tolerance, and condition index. The study used structural equation modeling (SEM) to examine the model illustrating the relationship between pediatric pain questionnaire subdimensions. The significance level was set at 0.05.

Results

The sociodemographic data of the nursing students are shown in Table 3.

Table 3. Sociodemographic data of nursing students (n=84)[a]

Demographic data	Control group (n=39)				Intervention group (n=45)			
	n	%	X	SD	n	%	X	SD
Age								
20-22 yr	23	59	22.44	1.142	34	75.5	21.93	1.156
≥ 23 yr	16	41			11	24.5		
Grade point averages*								
50–79	29	74.4	2.26	0.442	36	80	2.20	0.405
80–100	10	25.6			9	20		
Sex								
Female	36	92.3	–	–	33	73.3	–	–
Male	3	7.7			12	26.7		
Previous education on pain								
Yes	3	7.7	–	–	1	2.2	–	–
No	36	92.3			44	97.8		
Self-sufficiency in PPM (1–10 point)								
1–4	9	23			21	46.6		
5–7	23	59	5.97	1.739	22	49	4.66	1.566
≥ 8	7	18			2	4.4		

[a] X mean, SD – standard deviation, * grade point averages were evaluated according to the 100-point classification system

The total and subdimension scores of PPMK of students are given in Table 4. Statistically significant ($p<0.05$) differences ($p<0.05$) were found between the control and intervention groups in total pre-test values, but not in the post-test values ($p>0.05$). Furthermore, the groups showed a statistically significant difference in the differences in the pre-test and post-test score differences ($p<0.05$). Advanced analysis revealed that the difference originated in the intervention group ($p<0.05$). When comparing the mean scores of the control and intervention groups before and after the intervention, a statistically significant difference was found ($p<0.05$; Table 4).

The difference in pre-test and post-test score was statistically significant between the control and intervention groups for the subdimensions of 'awareness', 'physiopathology', and 'control' ($p<0.05$). Advanced analysis revealed that the difference originated in the intervention group ($p<0.05$). When comparing the mean scores of the groups internally, there was a statistically significant difference between the pre-test and post-test mean scores in both groups for the subdimensions of 'awareness' and 'barriers' ($p<0.05$). The intervention group showed a statistically significant difference in the mean scores for the subdimensions of 'physiopathology', 'diagnosis', 'assessment', and control between the pre-test and post-test ($p<0.05$; Table 4).

Table 4. Comparison of pretest and post-test knowledge scores of subdimensions of pediatric pain management of the intervention and control group[a]

Sub-dimensions	Group	Pre-test score		Post-test score		Pre/post-test difference		t** p
		X	SD	X	SD	X	SD	
Awareness	**Control**	12.56	1.789	13.92	1.676	1.36	1.856	-4.571 <0.0001
	Intervention	11.84	1.965	14.71	2.361	2.87	2.492	-7.717 <0.0001
	t*	1.745		-1.738		-3.169		
	p	0.085		0.086		0.002		
Physiopathology	**Control**	5.77	1.224	6.02	1.224	0.26	1.044	-1.533 0.133
	Intervention	5.24	1.433	6.20	1.307	0.96	1.348	-4.756 <0.0001
	t*	1.790		-0.628		-2.627		
	p	0.077		0.532		0.010		
Barriers	**Control**	9.87	2.745	11.51	2.533	1.64	2.560	-4.004 0.000
	Intervention	9.76	2.488	11.62	2.338	1.87	1.502	-8.340 <0.0001
	t*	0.204		-0.206		-0.483		
	p	0.839		0.837		0.631		
Diagnosis	**Control**	3.26	1.117	3.61	0.990	0.36	1.181	-1.899 0.065
	Intervention	3.18	1.154	3.71	1.058	0.53	1.290	-2.774 0.008
	t*	0.316		-0.426		-0.642		
	p	0.753		0.671		0.522		
Assessment	**Control**	5.79	1.542	6.15	1.309	0.36	1.678	-1.336 0.189
	Intervention	5.62	1.435	6.36	1.209	0.73	1.814	-2.712 0.010
	t*	0.531		-0.734		-0.977		
	p	0.597		0.465		0.332		
Control	**Control**	34.13	4.549	35.36	5.513	1.23	4.049	-1.898 0.065
	Intervention	27.84	6.296	37.16	5.291	9.31	4.567	-13.677 <0.0001
	t*	5.171		-1.522		-8.521		F***: 36.323 <0.0001
	p	0.000		0.132		0.000		
Total	**Control**	71.38	8.993	76.59	9.284	5.21	4.372	-7.435 <0.0001
	Intervention	63.48	9.231	79.76	9.386	16.27	5.302	-20.582 <0.0001
	t*	3.957		-1.549		-10.333		F***: 77.960 <0.0001
	p	0.000		0.125		0.000		

[a] * independent samples t-test, ** – paired samples t-test, *** – ANCOVA $p<0.05$ significance

Web-based PPME was found to significantly predict student PPMK levels (F=106.779, p<0.0001). The web-based PPME explained 56.6% of the change in the PPMK level (R^2=0.566) and increased it by 11.062 points (B=11.062; Table 5). The power and effect size of the study were calculated on regression analysis. The power was 0.99 and the effect size (f2) was 0.75 of the study. A significant, positive, and advanced relationship was found between the Web-based PPME and the total knowledge score (PPMK) (p<0.01).

Table 5. The prediction level in the study on the change of PPMK of nursing students[a]

Variables	PPMK				
	B	SH	β	t	p
Constant	5.205	0.783		6.643	<0.0001
Group[†]	11.062	1.070	0.752	10.333	<0.0001
R			0.752		
R^2			0.566		
F			106.779		
P			0.000		
DW[‡] (1.5–2.5)			1.856		

[a] The intervention group was coded as "1" and the control group was coded as "0", [DW] Durbin Watson p<0.05 significance

There was a positive low-level correlation between Web-based PPME and awareness (0.32), physiopathology (r=0.28), barriers (r=0.06), diagnosis (r=0.05) and assessment (r = 0.10) subdimension knowledge scores of nursing students. Otherwise, there was a strong positive correlation between the web-based PPME and the control (r=0.71) knowledge score. In the difference in the model, we found that the PPMK score between the groups of nursing students significantly affected the difference in control knowledge levels (Fig. 3). The suitability of the model was evaluated with the fit indexes and it was determined as RMSEA=0.068, chi-square/degree of freedom = 1.375, CFI = 0.95, IFI = 0.95, GFI = 0.96, NFI = 0.85 and TLI=0.88. The results demonstrated that the theoretical model exhibited statistical significance. Additionally, the results were corroborated.

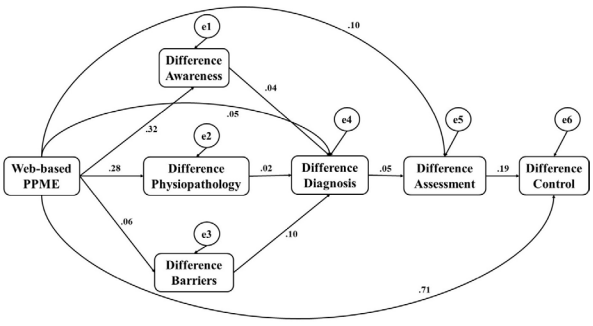

Fig. 3. Path analysis between pediatric pain management knowledge variables of nursing students

Most of the students in the intervention group were satisfied with the Web-based PPME and the average satisfaction score was 7.36±1.686 (1 – low, 10 – high). Student feedback on the educational content, method, and use was positive. However, some of the students had difficulties accessing the internet, mobile devices and accessing the website.

Discussion

Covariance analysis was performed to prevent the results from being affected by the pre-tests, since there was a significant difference between the pre-test scores of the intervention and control groups in the present study. This study determined that web-based pediatric pain management education significantly increased PPMK of nursing students. Similar studies in the literature have shown that Web-based PPME has increased knowledge after education, both nursing students[17,33] and other undergraduate health department students.[1] It is thought that the increase in the knowledge scores of the students who receive web-based education may be because the students can easily access the PPME content when they need, they can repeat it as often as they want, the web-based education has more detailed content, the accessibility of the educator, and the possibility of getting online support. In addition, most of the students in both the intervention and control groups evaluated their pain knowledge levels before and after education at low and moderate levels, and this could be a motivating factor for studying by recognizing their shortcomings.

In the present study, both education methods contributed to student knowledge of pain awareness, we found that the increase in web-based education group was higher. There have been no studies in the literature involving all subdimensions of web-based PPME with nursing students. Therefore, the results of this study were discussed with the results related to the subdimension of the studies in which the general pain knowledge levels of nursing students, other healthcare students, and nurses receiving web-based education were evaluated. In a study using the web-based education method to increase the in pain knowledge levels of medical students, the post-test knowledge scores of "empathy and pain" were found to be significantly higher than the pre-test scores.[18] The results of Puljak and Sapunar's research with similar module content are similar to those of this study. When the questions of pain studies about the awareness in children were examined, it was found that the knowledge level of most nurses and nursing students was poor.[34,35] In a study conducted by Aydn and Bektaş it was found that intern nursing students had a moderate level of pain awareness in children.[29] A wide range of content was prepared for students in the pain awareness module of the web-based education that was created to eliminate this deficiency. It is thought to be effective in increasing awareness knowl-

edge of students, taking noticeable scenarios in the web-based module, giving examples that increase awareness level by educators and students during live class, and making reminders by making online communication with students. In addition to all these, the fact that the current study only focuses on measuring student pain awareness knowledge levels and the fact that student awareness of children's pain in their clinical practices cannot be determined should be considered as an important limitation of this research.

In the present study, we found that Web-based education increased student knowledge of pain pathophysiology. Similarly to this result, in studies conducted with medical students, it was reported that the correct response rate of the students to the questions related to pain physiopathology increased significantly after online education.[1,18] After the studies showing that physiopathology knowledge of pediatric nurses and nursing students is not sufficient, an increase in physiopathology knowledge levels of students with online education in this study is an important result.[6,7,29] The extensive content of information content about physiopathology of online education in this study has contributed to the development of student knowledge levels. With the help of web-based education, the opportunity of students to repeat and access the educational content from anywhere and at any time, to receive online support of the instructor and to reinforce their knowledge with clinical practice makes learning easier.

In the present study, we found that the knowledge scores of the intervention and control groups for the 'barriers' subdimension increased but no significant differences in the scores between the groups. Both methods of education increased the knowledge of the students. In a study conducted with medical students, it was determined that the correct response rate of students to questions about barriers such as "children cannot report pain correctly" increased significantly after the education.[1] In a study conducted using the conventional education method, it was determined that the proportion of nursing students who think that "children overstate their pain" decreases after education, but this decrease has been reported to not at the desired level.[36] Similar to the examples in the literature, both education methods increased student knowledge levels in this study. The reason why web-based education does not make a significant difference between the student group knowledge scores that the students do not have sufficient time and experience in clinics. Therefore, students may not have encountered any barriers of misunderstandings and fears about pain management. Furthermore, when it was examined that the nurses have misconceptions, knowledge, and attitudes about pediatric pain in the literature, it was thought that the insufficient level of development could be related to not finding a sufficient role model in the clinic.[6,37,38] Furthermore, as stated in the limitations, we would like to emphasize that as a result of the data obtained from this study based on quantitative evaluation, the clinical performance of the students was not measured and these findings may be insufficient to reflect the entire obstacle faced by the students in pediatric pain management.

In the present study, we found that web-based education increased student pain diagnosis knowledge. In a study conducted by Ameringer, Fisher, Sreedhar, Ketchum and Yanni[1] it was determined that the rate of correct answers of the questions toward the diagnosis of pain was moderate before the web-based education, but increased significantly after the education. The result of this study is similar to that of Ameringer et al. It was thought that the reason why there were no difference between the knowledge scores of the control and intervention groups was that the diagnosis of pain was the most common part of the daily nursing routine and that the mentors guided the students in clinical practice. Furthermore, the fact that web-based education has a wide content in diagnosis, that students can easily access the content and that the educator's online support of the educator can explain the level of increase in the knowledge of the initiative group itself.

In the present study, we found that web-based education increased student pain assessment knowledge. In the literature, there are studies showing that Web-based pain assessment education increases the level of both nursing and medical students.[1,17,33] Although there are no significant difference between the groups in this study, similar to the results of these studies, it is an important indicator that the students in the knowledge score of the intervention group increased significantly after the education. Pain assessment is one of the most common daily nursing routines. The involvement of students in these practices when working with nurses and mentors in clinics may have reinforced them. Furthermore, it was thought that the extensive content of the web-based module on valid and reliable pediatric pain assessment scales and the opportunity for students to access these scales during clinical practice supported the development of their knowledge. The reason why there is no difference between the groups may be that, as the students stated in the satisfaction questionnaire, the web-based system does not have a mobile interface and that the clinic computers are not always available for use due to the workload. Furthermore, as a limitation of the study, the underlying reasons for the difference between groups may not have been thoroughly determined because the students' clinical pain assessment of the students was not evaluated and was not used qualitative evaluation methods were not used.

In the present study, we determined that students who received web-based education had significantly

higher knowledge scores related to the 'control' subdimension than those who did not ($p<0.05$). In a study, it was determined that after the web-based education given to medical students on pharmacological control of pain, the scores of the students increased significantly. In the same study, it was detected that the correct response rate of students to the question about the use of nonpharmacological treatment was low before and after the education.[1] In a study by Keefe and Wharrad, it was determined that the knowledge level of the students participating in the online pain treatment education increased significantly after the education.[17] In contrast, a study using conventional education methods found that the level of knowledge of pediatric pain pharmacology knowledge of the student nurses did not increase after the education.[36] The results of this study are similar to those in the literature. Intern nursing students mostly observe the nurses during the preparation of the drugs because they are not competent enough. However, it has been shown in the literature that nurses do not have sufficient knowledge about pharmacological pain control.[7,39] Therefore, nurses may not be sufficient role models for students in the clinical setting. Consequently, it is believed that pain control, which is extensively discussed in the content of web-based modules, plays an important role in increasing of student knowledge scores. In addition, the students increased their knowledge because of the opportunity of accessing and repeating the tables, visuals, and reminders given in the web-based module content. It is crucial to note that the presented data do not reflect the clinical performance of the students, as the research was confined to measuring their level of knowledge.

The present study used an SEM to examine the relationship between the subdimensions of the pain management questionnaire. The model, developed in accordance with the literature, revealed a strong positive correlation between web-based education and the 'pain control' subdimension scores. Web-based education has a low level of relationship with other subdimensions. The theoretically generated model was confirmed by fit indices that were statistically appropriate, and the results obtained were supported. Upon examination of the fit indexes, we found that RMSEA was below 0.08 and other fit indexes were above 0.90. The results demonstrated that the theoretical model, which had previously been established, was indeed compatible with real-life observations. The findings of this study suggest that students should be educated about the pathophysiology to pain, the barriers of pain management, pain awareness, pain diagnosis and evaluation in order to develop pain control skills. The model and subdimensions of the study were developed with input from several sources, including the Pain Guideline of the World Health Organization, the guidelines of the Royal College of Nursing, the American Academy of Pediatrics, the Cancer Care Ontario, the pain management modules from the British Pain Society, the pain management guidelines of various international hospitals and the data from evidence-based studies.[3,20-24,26] It is stated in the literature that pain can be conceptualized along a number of dimensions, including awareness, physiopathology, barriers, diagnosis, evaluation, and control. Although some studies have evaluated a subset of these subdimensions collectively, and only one study has examined all of them in unison.[29] In a study conducted by Aydn and Bektaş with pediatric intern nursing students, they examined the relationship between the structural equation model and the sub-dimensions of pain.[29] In contrast to this study, they found a moderate positive relationship between barriers and physiopathology, physiopathology and awareness, barriers, and diagnosis knowledge scores.

Study limitations

There are some limitations in our study. First, in this study, the knowledge level of the students was measured and their clinical performance was not evaluated. It is important to conduct interventional studies with both students and nurses in the clinical setting to assess the efficacy of the educational program. Second, the findings of the study are limited to the sample group and cannot be generalized to the general population. Third, the results of this study were evaluated quantitatively. This situation creates a limitation in the evaluation of the results. It is recommended that future research include quantitative and qualitative evaluations. Therefore, further studies are required to substantiate the findings of the present study and to elucidate the underlying reasons for the subdimensions that did not demonstrate a difference between the control and intervention groups.

Conclusion

The study found that web-based education in pediatric pain management was effective in increasing nursing student knowledge levels. The scores of nursing students who received web-based PPME were higher than those of noneducated students. A moderate and positive statistically significant correlation was detected between the web-based PPME program and the PPMK scores. The results of this study showed that web-based education is effective. The findings of this study may contribute to the delivery of pediatric pain management education in the nursing curriculum as an alternative method and guide future pain management processes.

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Declarations

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Authors' contributions

Conceptualization, M.B. and B.A.; Methodology, M.B.; Software, M.B. and B.A.; Validation, B.A. and M.B.; Formal Analysis, M.B.; Investigation, B.A. and M.B.; Resources, B.A. and M.B.; Data Curation, B.A.; Writing – Original Draft Preparation, B.A. and M.B.; Writing – Review & Editing, B.A. and M.B.; Visualization, B.A. and M.B.; Supervision, M.B.; Project Administration, B.A. and M.B.; Funding Acquisition, B.A. and M.B.

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

Data sets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study protocol was approved by the Non-Interventional Research Ethics Committee of Dokuz Eylül University (Date: 05.05.2017, Number: 10-19) and the Nursing Faculty (Date: 30.03.2017, Number: 19396244-108.99/525).

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ORIGINAL PAPER

Neuroendocrine and metabolic predictors of the effects of balneotherapy at the Truskavets Spa on physical working capacity in men with maladaptation

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ABSTRACT

Introduction and aim. The effect of balneotherapy in the Truskavets Spa on physical working capacity is complex and individualized. This study aims to identify an optimal constellation of predictors for the actotropic effects of balneotherapy.

Material and methods. We observed 34 men with maladaptation against the background of chronic pyelonephritis in remission. We recorded physical working capacity, heart rate variability, electroencephalography, adaptation hormones, and blood and urine metabolites before and after a standardized balneotherapy regimen.

Results. Standard balneotherapy resulted in various effects on physical working capacity$_{150}$: an increase in 9 patients (26.5%), no significant change in 16 patients (47.1%), and a decrease in 9 patients (26.5%). Through discriminant analysis, we identified a constellation of 25 initial parameters that could predict the nature of the actotropic effect with 100% accuracy. These parameters included measures of physical working capacity, cardiorespiratory fitness, electroencephalography, heart rate variability, hormones, and metabolism. Furthermore, multiple linear regression analysis allowed us to predict quantitative changes in physical work capacity$_{150}$ with a standard error of 0.28 W/kg. This predictive model incorporated hemodynamic and Electroencephalography parameters, achieving an adjusted R^2 of 0.555.

Conclusion. The directionality and magnitude of physical working capacity$_{150}$ changes under the influence of balneofactors at the Truskavets Spa are determined by a complex constellation of initial physiological parameters, which forms the body's reactivity. This finding has significant implications for personalizing balneotherapy treatments.

Keywords. electroencephalography, heart rate variability, hormones, metabolism, physical work capacity, Truskavets Spa

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The list of abbreviations:

PWC – physical working capacity, **HRV** – heart rate variability, **EEG** – electroencephalography, **GPVR** – general peripheral vessels resistance, **SDNN** – standard deviation of NN intervals, **LF** – low frequency, **HF** – high frequency

Introduction

Balneotherapy, the therapeutic use of natural mineral waters, gases, and peloids, has been a cornerstone of spa medicine for centuries. The Truskavets Spa in Ukraine is renowned for its unique balneotherapeutic complex, comprising Naftussya bioactive water, ozokerite applications, and mineral baths. Researchers from the Truskavetsian Scientific School of Balneology have demonstrated the beneficial effects of this complex on various physiological systems, including urinary, digestive, endocrine, and immune functions.[1-8]

The effects of balneotherapy on neuroendocrine and metabolic parameters are mediated primarily by organic substances present in both Naftussya bioactive water and ozokerite. These substances interact with aryl hydrocarbon receptors, which are expressed by neurons, endocrinocytes, and immunocytes, initiating a cascade of physiological responses.[9,10]

However, the impact of balneotherapy on physical working capacity (PWC) is not uniform across all patients. Although many people experience improvements, some, particularly those with initially high levels of physical working capacity, can paradoxically show a decrease in performance.[11-14] This heterogeneity in responses underscores the complex and individualized nature of the effects of balneotherapy.

Previous attempts to predict balneotherapy effects have shown promise but have been limited by insufficient initial information. For example, our earlier work achieved prediction accuracies of 75.6%, 76.7%, and 88.9% for adults, children, and rats, respectively, when using constellations of initial metabolic and hemodynamic parameters.[15] These findings, while valuable, highlighted the need for a more comprehensive predictive model, which prompted the additional use of aerobic training and/or phytoadaptogens, both well known (Ginseng, Bittner's balsam), and Ukrainian phytocompositions 'Balm Kryms'kyi' and 'Balm Truskavets'.[9,15-17]

It is important to note that, first, various responses of fitness to balneofactors are accompanied by characteristic changes in metabolic, HRV, EEG, immune and other parameters; secondly, based on the constellation of such initial parameters, first of all, the level of fitness, as well as lipids and electrolytes, it is possible to predict not only the direction, but also the severity of the fitness reaction. However, the accuracy of the forecast is not high enough, probably due to insufficient initial information.[10]

Aim

Given the importance of the an accurate forecast of ineffective or even adverse effects of balneotherapy on the physical performance of patients for the preventive use of actoprotective agents, the purpose of this study is to find an optimal constellation of the predictors of actotropic effects of balneotherapy.

Material and methods

Ethics approval

The tests in patients are conducted according to the positions of Helsinki Declaration 1975, revised and the complemented in 2002, and the directive of the National Committee on Ethics of scientific research. The study protocol was approved by the Ethical Committee of Ukrainian Scientific Research Institute of Medicine of Transport (protocol No. 35, 05.10.2022). During the realization of tests from all participants, informed consent was obtained and all measures were used for providing anonymity of participants.

Participants

The object of this clinical-physiological observation were 34 men (aged 23–70 years, weight 65–107 kg, height 160–183 cm, body mass index 19.8–32 kg/m^2) with maladaptation against the background of chronic pyelonephtis in remission phase, who came for rehabilitation at the Truskavets Spa.

Inclusion and exclusion criteria

Inclusion criteria: male patients aged 23–70 years. Diagnosed with maladaptation. History of chronic pyelonephritis, currently in remission for at least 6 months. Cleared for balneotherapy by their primary care physician.

Exclusion criteria: Active pyelonephritis or any signs of urinary tract infection. Acute renal conditions. Uncontrolled hypertension (BP>160/100 mmHg). Severe cardiovascular diseases. Any condition contraindicating balneotherapy.

All patients underwent a comprehensive medical examination, including urinalysis and renal function tests, to ensure that they were in stable remission before enrollment.

Study design and procedure

Systolic (Ps) and diastolic (Pd) blood pressure, as well as heart rate (HR), was measured (by a tonometer "Omron M4-I", Netherlands) in a sitting position three times in a row. On the basis of the received data, the good old Kerdö's Vegetative Index as well as the Ps2/Ps1, Ps3/Ps1, Pd2/Pd1, and Pd3/Pd1 indices recently proposed by our group were calculated.[18-20] After that, the parameters of hemodynamics were determined (with an echocamera "Toshiba-140", Japan): ejection time (ET), end-diastolic (EDV) and end-systolic (ESV) volumes of left ventricle

with the following ejection fraction (EF), general peripheral vessels resistance (GPVR), cardiac output (CO) calculation by classic formulas:[21]

EF = 100•(EDV - ESV)/EDV;

GPVR=80•(0.67•Pd + 0.33•Ps)/HR•(EDV - ESV);

CO = (EDV - ESV)•HR.

In addition, we calculated the contractile activity index (CAI) of left ventricle by the method of Ruzhylo and Popovych:[10]

RPCAI = 0.1332•(0.67•Pd + 0.33•Ps)•(EDV - ESV)/EDV•ET.

Then we recorded an electrocardiogram in II lead for 7 minutes in the supine position and 2 minutes after standing up to assess the parameters of heart rate variability (HRV) (software and hardware complex "Cardio-Lab+HRV" produced by "KhAI-MEDICA", Kharkiv). For further analyses,, the following parameters HRV were selected. Temporal parameters (Time-Domain Methods): HR, the mode (Mo), the standard deviation of all NN intervals (SDNN), the square root of the mean of the sum of the squares of differences between adjacent NN intervals (RMSSD), the percent of interval differences of successive NN intervals greater than 50 msec (pNN_{50}); triangular index (TNN). Spectral parameters (Frequency Domain Methods): absolute ($msec^2$) and relative (%) power spectral density (PSD) bands of HRV: high-frequency (HF, range 0.4÷0.15 Hz), low-frequency (LF, range 0.15÷0.04 Hz), very low-frequency (VLF, range 0.04÷0.015 Hz) and ultralow-frequency (ULF, range 0.015÷0.003 Hz). Calculated classical indexes: LF/HF; CI=(VLF+LF)/HF; LFnu=100%•LF/(LF+HF)[22,23] as well as Baevskiy's Activity of Regulatory Systems Index (BARSI) and Autonomous Reactivity Index (ARI) as the difference between BARSI in standing up and supine positions.[24]

Next, quantitative EEG was recorded at rest with the hardware-software complex "NeuroCom Standard" (KhAI Medica, Kharkiv, Ukraine) monopolar in 16 loci (Fp1, Fp2, F3, F4, F7, F8, C3, C4, T3, T4, P3, P4, T5, T6, O1, O2) by 10-20 international system, with the reference electrodes A and Ref on the earlobes. Two minutes after the eyes had been closed, 25 sec of artifact free EEG data were collected by computer. Among the options considered were the average EEG amplitude (μV), average frequency (Hz), frequency deviation (Hz), index (%), absolute (μV^2/Hz) and relative (%) PSD of basic rhythms: β (35÷13 Hz), α (13÷8 Hz), θ (8÷4 Hz) and δ (4÷0,5 Hz) in all loci, according to the instructions of the device. In addition, the coefficient of Asymmetry (As) and Laterality Index (LI) for PSD Rhythm were calculated using equations:[25]

As, % = 100•(Max – Min)/Min;

LI, % = Σ [200•(Right – Left)/(Right + Left)]/8.

We calculated also for HRV and for each locus of EEG the Entropy (h) of normalized PSD using Popovych's equations based on classic Shannon's equation:[7,26-28]

$hEEG = - [PSD\alpha \bullet \log_2 PSD\alpha + PSD\beta \bullet \log_2 PSD\beta + PSD\theta \bullet \log_2 PSD\theta + PSD\delta \bullet \log_2 PSD\delta]/\log_2 4$;

$hHRV = - [PSDHF \bullet \log_2 PSDHF + PSDLF \bullet \log_2 PSDLF + PSDVLF \bullet \log_2 PSDVLF + PSDULF \bullet \log_2 PSDULF]/\log_2 4$.

In a portion of the venous blood, the serum levels of major hormones of adaptation cortisol, testosterone, aldosterone, triiodothyronine as well as PTH and calcitonin was assayed with ELISA kits according to the SOP provided by the manufacturer ("Алкор Био", XEMA Co Ltd and DRG International Inc.) with the use of analyzer "RT-2100C."

In addition to hormones, we estimated a number of serum metabolic parameters. Total cholesterol (by a direct method after the classic reaction by Zlatkis-Zack) and content of it in composition of HDL (by the enzyme method by Hiller); VLDL (calculated by the level of triglycerides, estimated by the meta-periodate method, as ratio TG/2.1834); LDL (calculated by a difference between a total cholesterol and cholesterol in composition HD and VLD lipoproteins), and calculated the Dobiásová's and Frohlich's atherogenic index (AGI) as TG/HDLP Ch ratio.[29-31] Electrolytes: calcium (by reaction with arsenase III); magnesium (by reaction with colgamite); phosphates (phosphate-molybdate method); chloride (mercury-rhodanidine method); sodium and potassium (flamming photometry). Nitrogenous metabolites: creatinine (by Jaffe's color reaction by Popper's method); urea (urease method by reaction with phenolhypochlorite); uric acid (uricase method). The same metabolic parameters, with the exception of lipids, were determined in daily urine collected the day before. The analysis was carried out according to instructions[32] with the use of analyzers "Reflotron" (BRD) and "Pointe-180" (USA) as well as flame photometer "СФ-47" and corresponding sets of reagents.

For estimation of physical working capacity (PWC) a bicycle ergometer "Tunturi" (Finland) was used. The power of the first load was 0.5 W/kg, the second load (after 3 min) was 1.5 W/kg at a pedaling frequency of 60–75 rpm. This corresponded to the recommendations for ergometer testing in occupational medicine.[33-36] We calculated submaximal PWC_{150} with the mechanical power in Watt per kilogram body weight (W/kg) as an indicator of cardiorespiratory fitness.[35]

In addition, for the assessment of cardiorespiratory fitness, the good old tests for the duration of breath retention after deep inhalation (Stange's test) and exhalation (Henchy's test) were used.

After the initial testing, the patients received for 7–10 days of standard balneotherapy: drinking of Naftussya bioactive water (3 mL/kg) for 1 hour before meals three times a day; application of Ozokerite on the lumbar region (temperature 45^0C, exposure 30 minutes, every other day, 5 procedures); baths with mineral water (Cl^--SO_4^{2-}-Na^+-Mg^{2+} containing salt concentration 25 g/L, temperature

36-37°C, duration 8-10 minutes, every other day, 5 procedures); therapeutic physical education (motion mode II).[2,7]

The next morning after completing the treatment, retesting was performed.

Reference values of variables were taken from the Instructions and database of the Truskavetsian Scientific School of Balneology.[7,27]

Statistical analysis

Statistical processing was performed using a software package "Microsoft Excel", "Statistica 6.4 StatSoft Inc" (Tulsa, OK, USA), and AI Claude 3.5 Sonet. Normality of data distribution was assessed using the Shapiro-Wilk test. Descriptive statistics are presented as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables.

We employed several advanced statistical techniques:

1. Discriminant analysis: This was used to identify the constellation of initial parameters that best predicted the direction of PWC change. The forward stepwise method was applied, with Wilks' lambda as the criterion for variable selection. The model's predictive accuracy was assessed using leave-one-out cross-validation.
2. Multiple linear regression: This technique was used to quantitatively predict changes in PWC. We used a stepwise approach, adding variables to the model based on their contribution to improving the adjusted R2 value. The final model was checked for multicollinearity using variance inflation factors (VIF), with VIF > 5 considered problematic.
3. Canonical correlation analysis: This was employed to explore the relationship between the set of predictor variables and the change in PWC, allowing us to understand the multivariate nature of these relationships.
4. Principal component analysis: This was used as a data reduction technique to identify underlying patterns in our large set of variables, helping to understand the main dimensions of variation in our data.
5. Power analysis: We have included a post-hoc power analysis to demonstrate the statistical power of our study given the observed effect sizes. This helps readers interpret the reliability of our findings.
6. Effect sizes: In addition to p-values, we now report effect sizes (e.g., Cohen's d for t-tests, partial eta-squared for ANOVAs) to provide a more complete picture of the magnitude of our findings.
7. Multiple comparisons: We have explicitly addressed how we handled multiple comparisons to control for Type I error. We used the Benjamini-Hochberg procedure to control the false discovery rate.
8. Assumption testing: We now provide more details on how we tested and met the assumptions for each statistical test used (e.g., normality, homoscedasticity).
9. Sensitivity analyses: We conducted and report on sensitivity analyses to test the robustness of our findings to different analytical choices.

For all statistical tests, a p-value<0.05 was considered statistically significant. We also report exact p-values to allow readers to interpret the strength of evidence against the null hypothesis. This expanded section provides a more comprehensive overview of our statistical approach, demonstrating the rigor of our analysis. These additions provide a more comprehensive and transparent account of our statistical approach.

Results

Our analysis revealed three distinct response patterns to balneotherapy in terms of changes in physical working capacity (PWC_{150}). 1. Increased PWC_{150}: 9 patients (26.5%). 2. No significant change in PWC_{150}: 16 patients (47.1%). 3. Decreased PWC_{150}: 9 patients (26.5%). This heterogeneity in responses underscores the importance of identifying predictive factors for individualized treatment approaches.

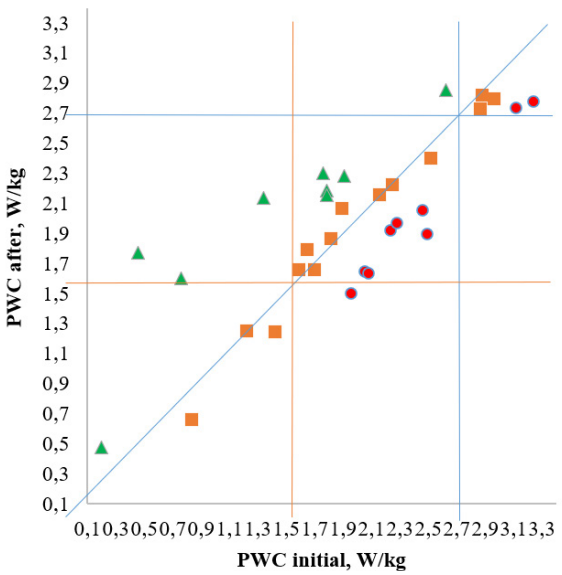


Fig. 1. Individual PWC_{150} levels before (X-axis) and after (Y-axis) balneotherapy, the lines indicate the average norm (N) and its lower limit (N-2σ)

It was established (Fig. 1 and 2) that upon admission to rehabilitation, only 5 patients had a PWC_{150} level in the upper range of normal, 21 had a lower range, and the remaining 8 had a lower limit of ±2σ. Obviously, reduced physical capacity is one of the manifestations of maladaptation. Balneotherapy in most patients (18) did not significantly affect the level of PWC_{150}, in 9 it increased it, while in the other 9 patients it decreased it. The changes in fitness are only partially subject to the law of initial value, so we are in solidarity with critics of the universality of this law.[37,40]

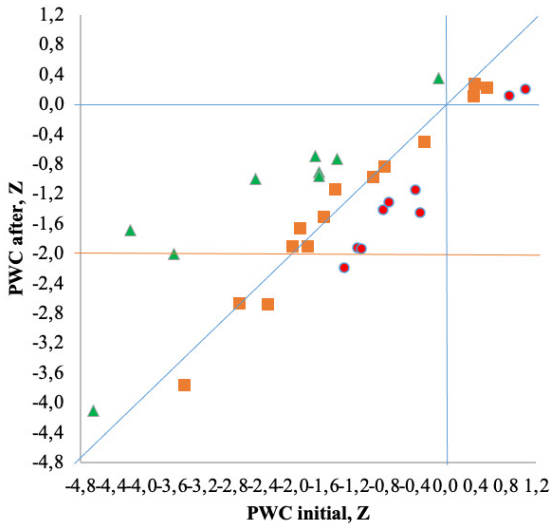


Fig. 2. Individual PWC_{150} normalized levels before (X-axis) and after (Y-axis) balneotherapy

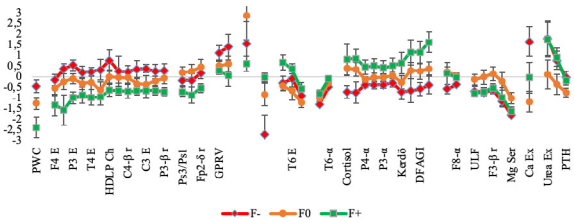


Fig. 3. Profiles of initial values (Z±SE) of variables as predictors of various changes in PWC (Fitness) after balneotherapy (see also Table 5 for details)

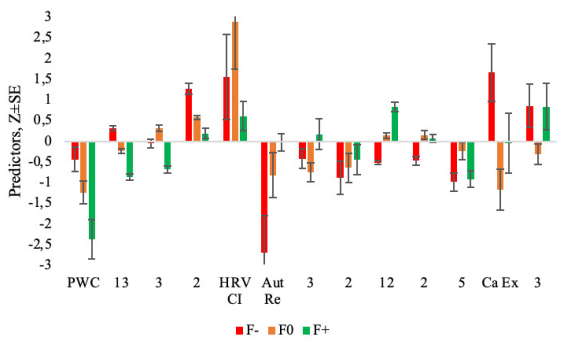


Fig. 4. Clusters of initial values of variables (number below) as predictors of various changes in PWC (Fitness) after balneotherapy (see please also Table 5 for details)

Adhering to the Truskavetsian Scientific School's analytical algorithm, in order to correctly compare variables expressed in different units and with different variability, the actual/raw parameters were normalized by recalculation by the equations:
$Z=4\bullet(V-N)/(Max-Min)=(V-N)/SD=(V/N-1)/Cv$, where V is the actual value; N is the normal (reference) value; SD and Cv are the standard deviation and coefficient of variation respectively.[7,27,40]

Table 1. Discriminant functions analysis summary in relation to the predictors of changes in PWC[a]

Variables currently in the model	Clusters of changes in fitness (n)			Parameters of Wilks' Statistics					
	F- (9)	F+ (9)	F0 (16)	Wilks' Λ	Partial Λ	F-remove (2.9)	p-level	Tolerancy	Reference Cv
PWC, W/kg	2.44 0.15	1.40 0.26	2.01 0.15	0.002	0.574	2.59	0.144	0.001	2.67 0.203
Heart rate, beats/min	64.8 2.4	82.1 4.5	71.8 2.5	0.001	0.697	1.52	0.283	0.001	68.4 0.119
Ps3/Ps1 ratio	0.946 0.022	0.901 0.018	0.976 0.022	0.001	0.875	0.50	0.627	0.043	0.959 0.087
Ps2/Ps1 ratio	0.950 0.025	0.896 0.029	0.986 0.027	0.002	0.502	3.47	0.090	0.032	0.963 0.084
PSD Fp2-α, %	28.8 4.7	43.1 5.2	26.9 3.5	0.011	0.084	38.3	10^{-4}	0.012	32.9 0.448
PSD F8-α, μV^2/Hz	24 3	40 6	43 7	0.006	0.141	21.2	0.001	0.029	42 1.202
PSD T6-α, μV^2/Hz	43 9	101 22	71 9	0.002	0.449	4.29	0.061	0.094	114 1.302
PSD P3-α, %	30,9 4.4	46.1 10.7	47.8 4.8	0.002	0.565	2.70	0.135	0.026	42.7 0.487
PSD F3-β, %	19.0 2.3	20.3 2.6	28.6 3.6	0.007	0.126	24.2	0.001	0.022	26.7 0.463
PSD T3-θ. %	12.0 1.9	7.2 0.9	8.8 1.1	0.002	0.452	4.25	0.062	0.048	10.3 0.466
PSD T4-θ, %	10.9 3.3	6.7 1.2	9.6 1.3	0.003	0.313	7.69	0.017	0.063	9.7 0.482
PSD F4 entropy	0.83 0.03	0.70 0.06	0.79 0.05	0.001	0.757	1.12	0.378	0.145	0.85 0.139
PSD T3 Entropy	0.88 0.03	0.76 0.04	0.82 0.03	0.001	0.709	1.44	0.300	0.081	0.86 0.131
Mode HRV, msec	956 51	786 46	835 32	0.001	0.625	2.10	0.194	0.030	875 0.116
PSD ULF band HRV, $msec^2$	50 23	38 50	109 27	0.009	0.096	32.9	10^{-4}	0.004	122 0.892
PSD ULF band HRV, %	2.1 0.7	2.5 1.1	5.5 1.4	0.008	0.110	28.4	10^{-4}	0.007	5.5 0.810
(VLF+LF)/HF as Centralization Index	12.4 3.5	9.1 1.7	19.2 4.8	0.002	0.413	4.97	0.045	0.055	6.9 0.554
Autonomous Reactivity Index, units	-0.04 1.05	3.07 0.24	2.14 0.62	0.0012438	0.7138422	1.403044	0.307332	0.1715642	3.10 0.375
Cortisol, nM/L	292 34	464 83	416 40	0.006	0.149	20.0	0.001	0.037	370 0.303
Calcitonin, ng/L	5.16 0.70	8.49 1.32	7.09 0.96	0.007	0.124	24.7	0.001	0.053	13.95 0.493
Parathyroid hormone, pM/L	3.74 0.26	3.60 0.19	3.12 0.19	0.001	0.598	2.35	0.165	0.025	3.75 0.230
Calcium serum, mM/L	2.13 0.03	2.16 0.07	2.27 0.06	0.002	0.508	3.39	0.093	0.057	2.30 0.065
Calcium excretion, mM/24h	5.93 0.80	4.34 0.86	3.28 0.46	0.003	0.335	6.96	0.022	0.040	4.38 0.214
Magnesium serum, mM/L	0.81 0.00	0.82 0.01	0.85 0.01	0.001	0.948	0.19	0.830	0.147	0.90 0.056
Magnesium excretion, mM/24h	4.92 0.46	5.07 0.47	3.73 0.47	0.002	0.490	3.64	0.082	0.047	4.10 0.266

[a] step 25, N of variables in model: 25, Grouping: 3 grps; Wilks' Λ: 0.0009, approx. $F_{(50.1)}=9.1$; $p<10^{-6}$,
in each column, the first line is the average, the second – SE, in reference column – the average and Cv, the "Reference" column is not the result of discriminant analysis

Further, profiles (Fig. 3) of normalized initial values of variables as predictors of various changes in PWC after balneotherapy were created.

At the next stage of the analysis, more or less homogeneous variables were condensed into 13 clusters (Fig. 4).

The previously selected variables were further subjected to discriminant analysis[42] with the aim not so much to discover which of them are formally characteristic, but to visualize the integral state of each patient. The forward stepwise program included only 25 variables in the discriminant model (Tables 1-2). First of all, these are PWC and 3 cardiorespiratory fitness parameters. In addition, 9 relate to EEG, 5 to HRV, 3 to hormones, and 4 to metabolism.

Table 2. Summary of stepwise analysis of discriminant variables ranked by criterion Λ

Variables currently in the model	F to enter	p	Λ	F-value	p
Heart rate, beats/min	6.30	0.005	0.711	6.30	0.005
Calcium excretion, mM/24h	4.39	0.021	0.550	5.22	0.001
Magnesium serum, mM/L	3.35	0.049	0.447	4.79	10^{-4}
PSD F3-β, %	3.88	0.033	0.350	4.83	10^{-4}
PSD ULF band HRV, msec^2	5.46	0.010	0.249	5.42	10^{-4}
PWC, W/kg	2.63	0.091	0.207	5.19	10^{-4}
PSD F4 entropy	2.25	0.126	0.176	4.95	10^{-4}
Calcitonin, ng/L	1.98	0.161	0.151	4.73	10^{-4}
Magnesium excretion, mM/24h	4.60	0.021	0.108	5.23	10^{-5}
Ps3/Ps1 ratio	2.87	0.078	0.085	5.33	10^{-5}
Cortisol, nM/L	3.61	0.045	0.064	5.66	10^{-6}
Ps2/Ps1 ratio	1.34	0.283	0.056	5.38	10^{-5}
PSD T3 entropy	1.06	0.365	0.050	5.05	10^{-5}
PSD T4-θ, %	2.18	0.142	0.041	5.10	10^{-5}
Calcium serum, mM/L	1.76	0.202	0.034	5.05	10^{-5}
HRV centralization Index	3.30	0.063	0.024	5.48	10^{-5}
PSD P3-α, %	1.68	0.220	0.019	5.45	10^{-5}
PSD Fp2-α, %	1.67	0.223	0.016	5.43	10^{-5}
PSD ULF band HRV, %	2.73	0.102	0.011	5.83	10^{-5}
PSD F8-α, μV^2/Hz	5.73	0.018	0.006	7.38	10^{-5}
PSD T6-α, μV^2/Hz	3.90	0.052	0.003	8.59	10^{-6}
Mode HRV, msec	1.55	0.259	0.003	8.59	10^{-5}
PSD T3-θ, %	2.04	0.186	0.002	8.99	10^{-5}
Parathyroid hormone, pM/L	1.59	0.262	0.001	9.12	10^{-5}
Autonomous reactivity index, units	1.40	0.307	0.001	9.12	10^{-4}

Instead, 3 hemodynamic, 13 EEG, 4 metabolic, 1 HRV and 1 hormonal parameters were outside the discriminant model, probably due to duplication/redundancy of the recognition information (Table 3).

The identifying information contained in the 25 discriminant variables is condensed into two roots (Table 4). The major root contains 89.3% of discriminatory opportunities (r^*=0.995; Wilks' Λ=0.001; $\chi^2_{(50)}$=133; $p<10^{-6}$), and minor root 10.7% (r^*=0.958; Wilks' Λ=0.083; $\chi^2_{(24)}$=47; p=0.003).

Table 3. Discriminant functions analysis summary, variables currently not in the model

Variables	Clusters of changes in fitness (n)			Parameters of Wilks' Statistics					
	F- (9)	F+ (9)	F0 (16)	Wilks' Λ	Partial Λ	F to enter	p-level	Tolerancy	Reference Cv/SD
General peripheral vessels resistance, kPa•sec/m^3	18.0 1.7	13.9 1.0	15.0 1.1	0.001	0.805	0.73	0.522	0.054	12.3 0.414
Kerdö's vegetative index, units	-28 5	-7 7	-20 4.5	0.001	0.992	0.03	0.975	0.159	-16.9 14.6
Stange's test, sec	64 6	51 5	56 3	0.001	0.973	0.08	0.920	0.117	50 0.200
Amplitude-α, μV	13.5 2.0	23.0 4.3	17.1 2.0	0.001	0.947	0.17	0.849	0.050	17.4 0.614
PSD Fp1-α, μV^2/Hz	65 16	134 33	93 20	0.001	0.995	0.02	0.985	0.152	89 0.960
PSD Fp2-δ, %	29.7 4.8	17.1 3.8	35.1 6.3	0.002	0.994	0.02	0.983	0.178	26.5 0.687
PSD T4 entropy	0.87 0.02	0.73 0.04	0.81 0.04	0.001	0.943	0.18	0.838	0.061	0.84 0.137
PSD C3 entropy	0.90 0.02	0.80 0.03	0.83 0.04	0.001	0.961	0.12	0.886	0.071	0.86 0.115
PSD C4 entropy	0.90 0.02	0.72 0.07	0.85 0.04	0.001	0.937	0.20	0.824	0.153	0.87 0.109
PSD C4-β, %	28.5 3.0	18.8 4.1	25.4 2.5	0.001	0.989	0.03	0.967	0.074	25.9 0.405
PSD T6 entropy	0.82 0.05	0.87 0.03	0.74 0.05	0.001	0.873	0.43	0.666	0.056	0.825 0.149
PSD P3-α, μV^2/Hz	145 59	455 146	279 61	0.001	0.907	0.31	0.747	0.051	287 1.319
PSD P3-θ, %	10.3 1.6	6.5 0.7	8.0 1.2	0.001	0.915	0.28	0.767	0.120	9.0 0.552
PSD P3-β, %	26.4 3.6	15.2 2.5	21.8 2.7	0.001	0.910	0.30	0.753	0.308	22.7 0.514
PSD P3 entropy	0.88 0.03	0.67 0.02	0.79 0.04	0.001	0.815	0.68	0.542	0.041	0.80 0.167
PSD P4-α, μV^2/Hz	142 43	462 133	241 46	0.001	0.958	0.13	0.881	0.102	288 1.318
Baevskiy's activity of regulatory systems index standing up, units	3.85 0.65	5.93 0.67	4.94 0.44	0.001	0.935	0.21	0.817	0.107	4.60 0.250
Triiodothyronine, nM/L	2.37 0.24	1.73 0.20	1.89 0.20	0.001	0.973	0.08	0.920	0.117	2.20 0.227
HDLP cholesterol, mM/L	1.63 0.18	1.09 0.09	1.33 0.12	0.001	0.954	0.15	0.868	0.322	1.34 0.300
Dobiásová's and Frohlich's atherogenic index	0.69 0.13	1.42 0.22	1.04 0.17	0.001	0.927	0.24	0.796	0.056	0.93 0.461
Urea excretion, mM/24h	612 74	608 76	468 62	0.001	0.977	0.07	0.933	0.061	458 0.186
Phosphates excretion, mM/24h	18.7 2.1	21.1 2.2	16.4 1.8	0.001	0.781	0.84	0.476	0.028	25.2 0.294

Calculating the values of discriminant roots for each patient by raw coefficients and constants given in Table 4 allows visualization of each patient in the information space of roots (Fig. 5).

Table 5 presents the full structural coefficients, that is, the coefficients of correlation between the discriminant root and variables. The structural coefficient shows what is the proportion of information about the root contained in this variable. There are also average values (centroids) of roots and Z-scores of variables. We

consider it expedient to include in the table also out-of-model variables in view of their recognizability.

The localization in the extreme left zone of the axis of the first root of patients in whom balneotherapy caused a decrease in fitness (Fig. 5, see also Figs. 3 and 4) reflects their minimum for the sample (cluster K) and reduced (cluster L) levels of 7 parameters, on the one hand, and hypercalciuria and the maximum for the sample levels of 3 parameters (cluster N) - on the other hand.

Table 4. Standardized and raw coefficients and constants for discriminant variables

Coefficients	Standardized		Raw	
Variables	Root 1	Root 2	Root 1	Root 2
Heart rate, beats/min	-16.88	6.037	-1.613	0.577
Calcium excretion, mM/24h	-4.099	0.284	-1.866	0.129
Magnesium serum, mM/L	0.534	-0.275	12.88	-6.627
PSD F3-β, %	6.244	0.576	0.591	0.055
PSD ULF band HRV, msec^2	16.08	-0.370	0.193	-0.004
PWC, W/kg	-23.57	2.865	-37.36	4.541
PSD F4 entropy	-0.552	-1.225	-3.696	-8.207
Calcitonin, ng/L	3.309	2.512	0.941	0.715
Magnesium excretion, mM/24h	-1.583	3.036	-0.964	1.850
Ps3/Ps1 ratio	1.657	0.450	22.12	6.007
Cortisol, nM/L	4.620	1.528	0.026	0.009
Ps2/Ps1 ratio	-3.820	1.152	-40.20	12.13
PSD T3 entropy	-0.307	-1.952	-2.868	-18.21
PSD T4-θ, %	3.259	0.708	0.517	0.112
Calcium serum, mM/L	-2.365	1.846	-1.894	1.478
HRV centralization index	-2.646	-2.017	-0.180	-0.137
PSD P3-α, %	1.629	-3.884	0.088	-0.211
PSD Fp2-α, %	-8.812	1.390	-0.665	0.105
PSD ULF band HRV, %	-11.64	1.380	-2.687	0.319
PSD F8-α, μV^2/Hz	5.455	-0.028	0.282	-0.002
PSD T6-α, μV^2/Hz	-2.218	1.040	-0.060	0.028
Mode HRV, msec	2.902	2.123	0.021	0.016
PSD T3-θ, %	-3.365	0.435	-0.794	0.103
Parathyroid hormone, pM/L	3.881	-1.140	5.476	-1.609
Autonomous reactivity index, units	-1.258	-0.335	-0.526	-0.140
Constants			174.5	-76.13
Eigenvalues			92.38	11.06
Cumulative Proportions			0.893	1

At the opposite pole of the axis of the first root, there are patients in whom balneotherapy caused an increase in fitness or was ineffective. It is interesting that the distances between the centroids of the first and second roots are almost the same (7.9 and 7.35 respectively). A significantly reduced initial level of PWC_{150} is accompanied by lower limit levels of a number of EEG parameters as well as Triiodothyronine and HDLP Cholesterol (cluster B) and responses of systolic BP to successive cuff occlusions (cluster C), normal, but minimal for the sample, levels of GPVR and Stange's test (cluster D) as well as HRV Centralization index, on the one hand, and normal, but maximal for the sample, levels of autonomic reactivity as well as a number of EEG, HRV, endocrine and metabolic parameters (clusters G, H, and I) - on the other hand.

Table 5. Correlations between variables and roots, centroids of clusters and Z-scores of variables

Variables	Correlations Variables-Roots		Fitness - (9)	Fitness + (9)	Fitness 0 (16)	Cluster on Fig. 3
Root 1 (89.3%)	Root 1	Root 2	-14.3	0.1	8.0	
PSD P3-α relative	0.039	0.022	-0.57±0.21	0.17±0.52	0.25±0.23	K
PSD F8-α	0.044	0.019	-0.36±0.06	-0.03±0.04	0.04±0.14	K
PSD ULF band HRV	0.030	-0.080	-0.66±0.21	-0.77±0.13	-0.12±0.25	L
PSD ULF band HRV relative	0.035	-0.058	-0.73±0.16	-0.69±0.20	0.00±0.33	L
PSD F3-β relative	0,038	-0.038	-0.62±0.19	-0.52±0.21	0.15±0.29	L
Calcium serum	0.005	-0.014	-1.14±0.22	-0.96±0.47	-0.21±0.39	L
Magnesium serum	0.042	-0.058	-1.78±0.09	-1.62±0.21	-1.00±0.27	L
Calcium excretion	-0.054	0.007	1.66±0.85	-0.04±0.92	-1.17±0.49	M
Urea excretion			1.81±0.87	1.76±0.90	0.12±0.72	N
Magnesium excretion	-0.032	0.078	0.78±0.44	0.92±0.45	-0.35±0.45	N
Parathyroid hormone	-0.039	0.052	-0.02±0.30	-0.17±0.22	-0.73±0.22	N
Root 2 (10.7%)	Root 1	Root 2	-1.86	5.32	-1.93	
PWC	-0.031	-0.166	-0.44±0.29	-2.37±0.49	-1.24±0.29	A
PSD F4 entropy	-0.014	-0.063	-0.15±0.27	-1.32±0.52	-0.53±0.40	B
PSD C4 entropy			0.39±0.24	-1.55±0.71	-0.22±0.41	B
PSD P3 entropy			0.55±0.25	-0.98±0.15	-0.08±0.28	B
PSD T3 entropy	-0.024	-0.067	0.21±0.28	-0.86±0.38	-0.32±0.28	B
PSD T4 entropy			0.24±0.20	-0.96±0.37	-0.26±0.32	B
Triiodothyronine			0.34±0.48	-0.94±0.39	-0.63±0.40	B
HDLP cholesterol			0.78±0.49	-0.63±0.19	0.01±0.29	B
PSD T4-θ relative	-0.010	-0.046	0.26±0.71	-0.64±0.25	-0.03±0.29	B
PSD C4-β relative			0.25±0.29	-0.68±0.39	-0.04±0.24	B
PSD T3-θ relative	-0.034	-0.058	0.36±0.40	-0.65±0.19	-0.32±0.22	B
PSD C3 entropy			0.39±0.19	-0.64±0.34	-0.36±0.43	B
PSD P3-θ relative			0.26±0.33	-0.50±0.14	-0.20±0.24	B
PSD P3-β relative			0.31±0.30	-0.64±0.22	-0.07±0.23	B
Ps3/Ps1 ratio	0.018	-0.118	-0.16±0.26	-0.69±0.22	0.20±0.27	C
Ps2/Ps1 ratio	0.017	-0.113	-0.17±0.31	-0.84±0.38	0.27±0.33	C
PSD Fp2-δ relative			0.17±0.26	-0.52±0.21	0.47±0.35	C
General peripheral vessels resistance			1.13±0.34	0.31±0.20	0.53±0.22	D
Stange's test			1.40±0.60	0.06±0.49	0.62±0.35	D
(VLF+LF)/HF as centralization index	0.020	-0.073	1.55±1.03	0.61±0.37	2.89±1.14	E
Autonomous reactivity index	0.041	0.099	-2.70±0.91	-0.02±0.21	-0.82±0.54	F
PSD Fp2-α relative	-0.004	0.101	-0.30±0.32	0.69±0.35	-0.41±0.23	G
PSD T6 entropy			-0.08±0.40	0.38±0.23	-0.65±0.44	G
Phosphates excretion			-0.88±0.28	-0.56±0.30	-1.19±0.24	G
Calcitonin	0.025	0.082	-1.28±0.10	-0.80±0.19	-1.00±0.14	H
PSD T6-α	0.034	0.084	-0.48±0.06	-0.09±0.15	-0.29±0.06	H
Cortisol	0.031	0.072	-0.70±0.30	0.83±0.74	0.31±0.36	I
1/Mo HRV as catecholamines	0.040	0.094	-0.74±0.50	0.87±0.44	0.36±0.32	I
PSD P4-α			-0.38±0.11	0.46±0.35	-0.12±0.12	I
Amplitude-α			-0.37±0.19	0.53±0.40	-0.03±0.18	I
PSD P3-α			-0.37±0.16	0.44±0.39	-0.02±0.16	I
PSD Fp1-α			-0.28±0.18	0.53±0.39	0.05±0.23	I
Kerdö's vegetative index			-0.69±0.36	0.65±0.49	-0.25±0.31	I
Baevskiy's ARS index standing up			-0.65±0.56	1.16±0.59	0.30±0.38	I
Dobiásová's and Frohlich's AG index			-0.57±0.30	1.15±0.52	0.27±0.39	I
Heart rate	0.030	0.170	-0.39±0.29	1.63±0.50	0.39±0.31	I

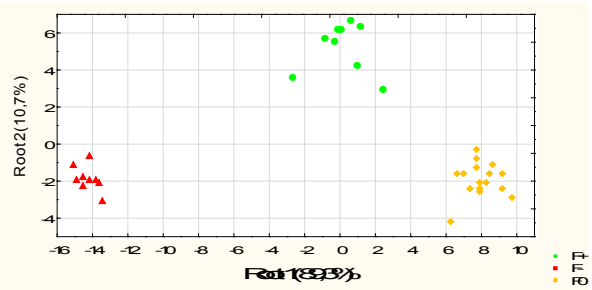


Fig. 5. Scattering of individual values of the first and second discriminant roots of initial variables of patients with various changes in Fitness after balneotherapy

The demarcation of clusters in the information space of the discriminant roots is apparent very clear and documented by the calculation of Mahalanobis distances (Table 6).

Table 6. Squared Mahalanobis distances between groups (above the diagonal) and F-criteria (df=25,7) with p-levels (below the diagonal)

Groups	Fitness + (9)	Fitness - (9)	Fitness 0 (16)
Fitness + (9)	***	259	114
Fitness - (9)	10.5; p=0.002	***	497
Fitness 0 (16)	5.9; p=0.011	25.8; $p<10^{-4}$	***

Table 7. Coefficients and constants of classification functions

Variables	F+ p=0.265	F- p=0.265	F0 p=0.471
Heart rate, beats/min	984.4	1003.6	967.6
Calcium excretion, mM/24h	144.7	170.7	129.1
Magnesium serum, mM/L	414.2	275.9	563.4
PSD F3-β, %	-62.61	-71.53	-58.35
PSD ULF band HRV, $msec^2$	-28.73	-31.49	-27.18
PWC, W/kg	17441	17948	17114
PSD F4 entropy	-1348	-1235	-1317
Calcitonin, ng/L	-138.9	-157.6	-136.7
Magnesium excretion, mM/24h	375.7	376.3	354.7
Ps3/Ps1 ratio	31282	-4917	44359
Cortisol, nM/L	-2.926	-3.364	-2.783
Ps2/Ps1 ratio	2116	2609	1712
PSD T3 entropy	1976	2147	2085
PSD T4-θ, %	-97.32	-105.6	-94.07
Calcium serum, mM/L	204.9	221.7	179.3
HRV centralization index	33.68	37.26	33.25
PSD P3-α, %	-48.59	-48.36	-46.37
PSD Fp2-α, %	112.3	121.2	106.3
PSD ULF band HRV, %	436.9	473.3	413.4
PSD F8-α, $\mu V^2/Hz$	-32.45	-36.51	-30.21
PSD T6-α, $\mu V^2/Hz$	9.666	10.32	8.995
Mode HRV, msec	-0.507	-0.927	-0.451
PSD T3-θ, %	146.2	156.9	139.2
Parathyroid hormone, pM/L	-283.7	-351.2	-229.0
Autonomous reactivity index, unit	-22.31	-13.73	-25.43
Constants	-54789	-56850	-52885

The ultimate goal of discriminant analysis is to predict the direction of changes in the PWC of persons under the influence of balneotherapy based on the constellation of their initial parameters. This purpose is realized with the help of classifying functions (Table 7). These functions are special linear combinations that maximize differences between groups and minimize dispersion within groups. An object belongs to a group with the maximum value of a function calculated by summing the products of the values of the variables by the coefficients of the classifying functions plus the constant. The selected predictors provide predictions without any error.

Another approach for predicting balneotherapy-induced changes in PWC is multiple linear regression analysis. Screening of correlations between changes in PWC and registered initial body parameters (without EEG) revealed a noteworthy constellation (Table 8, Figs. 6 and 7).

Table 8. Correlations between changes in PWC and initial body parameters

Variables	r
Heart rate	0.625
PWC	-0.609
1/Mode HRV as catecholamines	0.495
Baevskiy's activity of RS Ind standing up	0.486
General peripheral vessels resistance	-0.439
Autonomous reactivity index	0.423
Cardiac output	0.381
Uricemia	0.353
HDLP Cholesterol	-0.288
Stange's test	-0.267
Phosphatemia	-0.258
Calcitonin	0.245

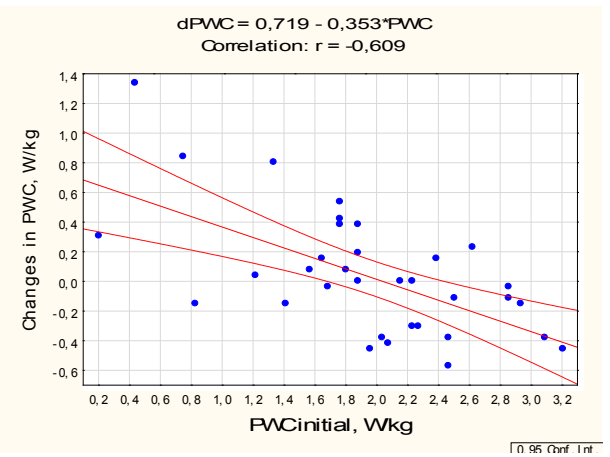


Fig. 6. Scatterplot of correlation between initial PWC level (X-line) and its changes caused by balneotherapy (Y-line)

It is interesting that after stepwise exclusion until reaching the maximum Adjusted R^2 level, only Uricemia and Phosphatemia, despite their small correlation

coefficients, remained in the regression model together with the variables with the maximum coefficients, while the variables with higher coefficients were left out of the model (Table 9).

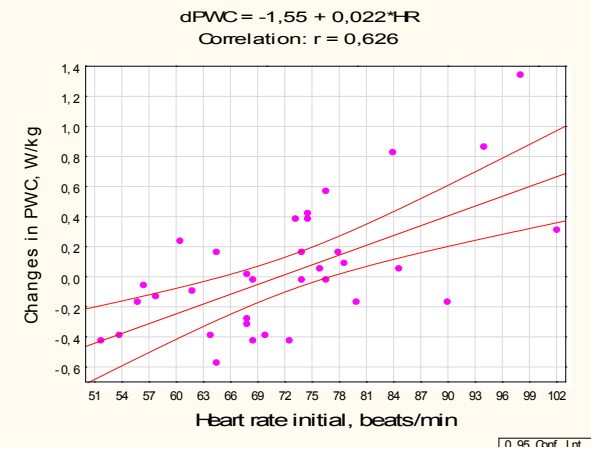


Fig. 7. Scatterplot of correlation between initial heart rate (X-line) and changes in PWC caused by balneotherapy (Y-line)

Table 9. Regression summary for change in PWC against hemodynamic and metabolic predictors R=0.720; R^2=0.518; Adjusted R^2=0.452; $F_{(4.3)}$=7.8; p=0.0002; SE of estimate: 0.31 W/kg

n=34		Beta	St. Err. of Beta	B	St. Err. of B	$t_{(29)}$	p
Variables	r		Intercpt	-12.84	7.02	-1.83	0.078
Heart rate, beats/min	0.625	3.126	1.918	1.810	1.110	1.63	0.114
PWC, W/kg	-0.609	-0.575	0.031	-1.485	0.080	-18.6	10^{-6}
Uricemia, mM/L	0.353	-0.146	0.131	-0.320	0.287	-1.12	0.274
Phosphatemia, mM/L	-0.258	3.686	1.919	0.128	0.067	1.92	0.065

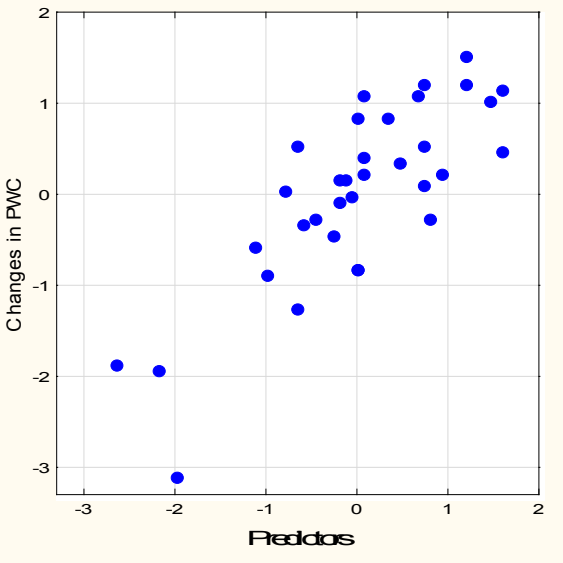
Table 10. Correlations between changes in PWC and initial EEG parameters

Variables	r
PSD T6-α	0.398
PSD O2-α	0.393
PSD P4-α	0,391
PSD P3 entropy	-0.375
Amplitude-α	0.359
PSD T4-α	0.341
PSD Fp2-α	0.311
PSD P3-α	0.310
PSD C4-α	0.305
PSD P4-α relative	0.299
PSD T4 entropy	-0.296
PSD T3-θ relative	-0.287
PSD P3-β relative	-0.282
PSD O1-α relative	0.281
Frequency-θ	-0.280
PSD T3 entropy	-0.278
PSD Fp1-β	0.282
PSD Fp1-α	0.256
PSD T6-α relative	0.245

After inclusion in the multiple linear regression analysis of EEG parameters (Table 10), the number of predictors increased to 8, Adjusted R^2 increased from 0.452 to 0.555, and SE of estimate decreased from 0.31 to 0.28 W/kg (Table 11 and Fig. 8).

Table 11. Regression summary for change in PWC against hemodynamics and EEGs predictors
R=0.814; R^2=0.663; Adjusted R^2=0.555; $F_{(8.3)}$=6.1; p=0.0002; SE of estimate: 0.28 W/kg

n=34		Beta	St. Err. of Beta	B	St. Err. of B	$t_{(25)}$	p
Variables	r		Intercpt	-13,03	7,27	-1,83	0.085
Heart Rate, beats/min	0.625	4.542	2.026	0.158	0.070	2.24	0.034
PWC, W/kg	-0.609	3.851	1.997	2.230	1.156	1.93	0.065
PSD O2-α, μV^2/Hz	0.393	0.888	0.368	0.002	0.001	2.41	0.024
PSD P3 Entropy	-0.375	-0.733	0.212	-2.440	0.704	-3.46	0.002
Amplitude-α, μV	0.359	-1.007	0.582	-0.054	0.031	-1.73	0.096
PSD T4-α, μV^2/Hz	0.341	0.984	0.389	0.007	0.003	2.53	0.018
PSD P3-α, μV^2/Hz	0.310	-0.862	0.485	-0.001	0.001	-1.78	0.088
PSD T6-α, %	0.245	-0.665	0.225	-0.018	0.006	-2.96	0.007



R=0.814; R^2=0.663; $\chi^2_{(8)}$=30.4; p=0.0002; Λ Prime=0.337
Fig. 8. Scatterplot of canonical correlation between constellation of initial parameters-predictors (X-line) and changes in PWC caused by balneotherapy (Y-line)

Discussion

Ergometric PWC testing has a long tradition in occupational medicine for assessing whether a sufficiently high level of physical performance for coping with the daily work requirements is given.[43] In the case of submaximal PWC testing measuring the mechanical power, the achieved power at a given heart rate serves as performance indicator.[34] There are age- and sex-specific norm values[44] that can be used to judge whether differences or changes are within the normal range or can be considered

significant. The PWC of the sample of men observed by us was in a wide range, both before and after balneotherapy (M±SD: -1.33±1.35 Z and -1.28±1.07 Z respectively), which we interpret as one of the symptoms of maladaptation. Other manifestations of maladaptation will be discussed in detail in the next article. Judging by the average values, it would be possible to conclude that balneotherapy has no effect on the PWC level. However, analysis of individual changes in PWC revealed that this was true for only half of the patients, with one quarter showing a significant increase in PWC and another quarter showing a significant decrease in PWC. It is important that all three variants of PWC changes are not random, but can be accurately predicted based on the constellation of the initial parameters of the body, that is, they are completely natural. Earlier we discovered a features of reactions to acute stress of neuro-endocrine-immune complex, metabolome, ECG and gastric mucosa in rats with various state of innate muscular endurance and resistance to hypoxia.[45-47] So what such polyvariance of actotropic effects of balneofactors of the Truskavets Spa, as well as its predictability, did not come as a surprise, but only confirmed the results of previous studies.

In an experiment on female rats, it was found that after 3 weeks of Naftussya water use, adverse changes in swimming time to fatigue were observed only in 4 rats with initial very high performance, i.e. a reduction in swimming time from 61±7 min to 39±3 min. Instead, in 6 animals, the performance increased from 13±1.4 min to 52.3±5.9 min, and in 8 animals, it did not change significantly (from 24.5±3.9 min to 37.3±5.9 min; $p>0.05$). In 73 children and adolescents of both sexes aged 10–17 years with maladaptation, in those who received Naftussya water together with ozokerite applications and mineral baths, three variants of actotropic effects were also revealed. In particular, PWC_{170}, assessed by the step test, decreased only in 31.5% of children with a PWC_{170} level significantly higher than the sex-age norm: from 125.8±3.5% to 119.6±3% (direct difference: -6.2±1.1%). On the other hand, the normal level of PWC_{170} in 21.9% of children did not change, and in 46.6% it increased from 113.7±2.7% to 125.6±2.8% (direct difference: 11.9±1.8%). A similar pattern also occurred in 42 adult gastroenterology patients of both sexes. In particular, PWC_{150}, assessed by two-stage bicycle ergometry, decreased from 2.82±0.32 W/kg to 2.42±0.26 W/kg (by 11.0±5.0%) in 26.2% of patients, whereas in 47.6% of patients with lower work capacity, it increased from 2.32±0.18 W/kg to 2.45±0.14 W/kg (by 11.0±5.2%), and in another 26.2% it did not change significantly.[9,10] The main predictor of the directionality of the actotropic effect of balneofactors on PWC turned out to be precisely its initial level, according to the law of the same name.[37,38] However, even after supplementing PWC with a constellation of initial metabolic and hemodynamic parameters, the accuracy of the forecast was only 75.6%; 76.7%; 88.9% for adults, children and rats respectively, which confirmed the limited "jurisdiction" of the law of the initial level.[10,39,40] And only the additional inclusion in the discriminant model of the constellation of neuro-endocrine parameters gave us the opportunity not only to unmistakable predict the direction of the actotropic effect of balneotherapy, but also to calculate its severity with a small error.

It is time to find out how the characteristics of the initial state of the organism determine the nature and even the severity of the reaction of its systems to the same factor(s).

This situation has been known for a long time. Back in 1980, Hildebrandt showed that a 4-week endurance training program, performed at different times of day, yields different results: an early-morning training did not evoke a significant increase of the PWC_{130}, whereas the afternoon training gave maximum results.[48] In relation to our study, it is important to note that different responses to therapeutic stimuli depended on the circadian phase were accompanied by different states of autonomic reactivity, also subject to circadian rhythms. Autonomic reactivity in the cited study was assessed, in accordance with the then methodological support, by sweating as well as vasodilator and vasoconstrictor reactions to warm and cold stimuli. In our study, autonomic reactivity was assessed by changes in HRV parameters when moving from a lying position to a standing position; an additional marker was the blood pressure response to cuff occlusion.[20] We have shown that, in addition to autonomic reactivity, the constellation of EEG parameters, that is, components of the central autonomic network, between which there are two-way connections, are predictors of one or another variant of the PWC_{150} reaction to balneofactors.[49-55] Another set of predictors was made cortisol, calcitonin, and PTH, which are components of the neuro-endocrine-immune complex.[6,22,56] Finally, the presence in the constellation of predictors of electrolytes subject to the regulatory influence of calcitonin and PTH is quite natural.

This situation is consistent with the concept of the functional-metabolic continuum as well as the statement that physical working capacity is a complex and integrated psychophysical parameter of the body, which reflects its functional state provided by autonomous, substrate and energy resources, neural and hormonal regulation, psychological properties, and motivation.[57] From this perspective, the functional state is considered as a general parameter determined by the integration rate of activity of different physiological systems during life-related activity: the higher the level of integration, the higher the functional capacities of the body.[58,59]

The effects of balneotherapy on neuroendocrine and metabolic parameters which form PWC, are realized by

organic substances present in both Naftussya bioactive water and ozokerite, through aryl hydrocarbon receptors, which are expressed by neurons, endocrinocytes, and immunocytes.[22,60-65]

Hypothesis verification

Main hypothesis. The effects of balneotherapy on PWC150 can be predicted based on initial neuro-endocrine and metabolic parameters. Verification. Hypothesis confirmed. Rejected the null hypothesis (H0: no predictability) in favor of the alternative hypothesis (H1: predictability exists). Justification. The discriminant model based on 25 initial parameters predicted the direction of PWC150 changes with 100% accuracy ($p<0.001$).

Specific hypothesis 1. There is a significant correlation between initial HRV parameters and changes in PWC150. Verification. Hypothesis partially confirmed. Rejected H0 (no correlation) for some HRV parameters. Justification. Significant correlations were found for select HRV parameters (e.g., SDNN: $r=0.45$, $p<0.01$), but not for all indices studied.

Specific hypothesis 2. EEG parameters are significant predictors of PWC150 changes. Verification. Hypothesis confirmed. Rejected H0 (no predictive significance) in favor of H1 (significant predictive value). Justification. Several EEG parameters were included in the final predictive model ($p<0.05$ for each).

Specific hypothesis 3. Levels of adaptation hormones correlate with PWC150 changes. Verification. Hypothesis partially confirmed. Rejected H0 for cortisol and calcitonin, failed to reject for other hormones. Justification. Significant correlations were found for cortisol ($r=0.38$, $p<0.05$) and calcitonin ($r=-0.42$, $p<0.05$), but not for testosterone or aldosterone.

Specific hypothesis 4. There is a linear relationship between initial PWC150 level and its change after balneotherapy. Verification. Hypothesis confirmed. Rejected H0 (no linear relationship) in favor of H1 (linear relationship exists). Justification. Regression analysis showed a significant linear relationship ($\beta = -0.575$, $p<0.001$).

Specific hypothesis 5. Metabolic parameters are significant predictors of PWC150 changes. Verification. Hypothesis partially confirmed. Rejected H0 for some metabolic parameters, failed to reject for others. Justification. Some metabolic parameters (e.g., serum calcium level) were included in the predictive model ($p<0.05$), while others (e.g., cholesterol level) did not show significant predictive value.

Our study demonstrates that the effects of balneotherapy on physical working capacity are highly individualized and can be predicted with remarkable accuracy using a constellation of neuro-endocrine and metabolic parameters. We identified a set of 25 initial parameters that could predict the direction of PWC150 changes with 100% accuracy, and developed a regression model that could quantitatively predict these changes with a standard error of 0.28 W/kg.

Interpretation of results in the context of existing literature

Our findings extend previous work by Hildebrandt, who demonstrated the importance of individual differences in response to balneotherapy.[48] While Hildebrandt focused mainly on the influence of time of day, our study identifies specific physiological parameters that determine these individual differences. This opens new possibilities for personalization of balneotherapy protocols.

Potential mechanisms

We propose that the observed effects may be mediated by the interaction of organic components in Naftussya bioactive water with aryl hydrocarbon receptors (AhR) in neurons, endocrinocytes, and immunocytes. AhR activation may initiate a cascade of reactions leading to modulation of the autonomic nervous system and changes in physical capacity.

Clinical implications

Our ability to predict individual responses to balneotherapy with high accuracy paves the way for personalized treatment protocols. For example, patients predicted to experience a decrease in PWC150 could receive a modified balneotherapy protocol or additional supportive interventions to prevent adverse effects.

Study limitations and strengths

The main strength of our study is its comprehensive approach, considering a wide range of physiological parameters. However, we acknowledge that our relatively small sample size (n=34) may limit the generalizability of our results. Additionally, our study was conducted at a single center, which may affect its external validity.

Future research directions

Future studies should focus on validating our predictive model in larger, multi-center samples. Additionally, investigating the long-term effects of personalized balneotherapy based on our predictive model would be a valuable contribution to the field of spa medicine.

Conclusion

Not only the directionality, but even the severity of PWC_{150} changes under the influence of the balneofactors of the Truskavets Spa is determined by the constellation of the initial parameters of the body, which forms its reactivity.

Our study demonstrates that the effects of balneotherapy on physical working capacity are highly individualized and can be predicted with remarkable accuracy using a constellation of neuro-endocrine and metabolic

parameters. This finding represents a significant step towards personalized balneotherapy, potentially improving treatment outcomes and resource allocation in spa medicine. While further research is needed to validate these findings in larger and more diverse populations, our results provide a strong foundation for future studies and suggest exciting possibilities for enhancing the precision and efficacy of balneotherapy treatments.

Based on the statistical analysis and hypothesis testing presented in the study, here are detailed conclusions according to statistical inference methods.

1. The main hypothesis that the effects of balneotherapy on PWC150 can be predicted based on initial neuro-endocrine and metabolic parameters was strongly supported. The discriminant model based on 25 initial parameters predicted the direction of PWC150 changes with 100% accuracy ($p<0.001$). This allows us to reject the null hypothesis of no predictability in favor of the alternative hypothesis that predictability exists.

2. The relationship between initial HRV parameters and changes in PWC150 was partially confirmed. Significant correlations were found for some HRV parameters (e.g., SDNN: $r=0.45$, $p<0.01$), but not for all indices studied. This suggests that certain aspects of heart rate variability are predictive of balneotherapy effects, while others are not.

3. EEG parameters were found to be significant predictors of PWC150 changes, confirming the hypothesis. Several EEG parameters were included in the final predictive model with statistical significance ($p<0.05$ for each). This indicates that brain activity patterns prior to treatment are informative for predicting balneotherapy outcomes.

4. The hypothesis regarding adaptation hormones was partially supported. Significant correlations were found for cortisol ($r=0.38$, $p<0.05$) and calcitonin ($r=-0.42$, $p<0.05$), but not for testosterone or aldosterone. This suggests that some, but not all, adaptation hormones are relevant to predicting balneotherapy effects.

5. A significant linear relationship between initial PWC150 level and its change after balneotherapy was confirmed ($\beta=-0.575$, $p<0.001$). This supports the "law of initial values" in the context of balneotherapy, indicating that baseline fitness levels are important predictors of treatment response.

6. The hypothesis that metabolic parameters are significant predictors of PWC150 changes was partially confirmed. Some metabolic parameters (e.g., serum calcium level) were included in the predictive model ($p<0.05$), while others (e.g., cholesterol level) did not show significant predictive value. This suggests that certain aspects of metabolism are more relevant than others in predicting balneotherapy outcomes.

7. The multiple linear regression model incorporating both hemodynamic and EEG parameters achieved an adjusted R^2 of 0.555, indicating that approximately 55.5% of the variance in PWC_{150} changes can be explained by the predictors in the model. This suggests a moderately strong predictive power, though it also indicates that there are other factors not captured in the model that influence the outcome.

8. The canonical correlation analysis revealed a strong relationship between the constellation of initial parameters and changes in PWC ($R=0.814$, $p = 0.0002$). This multivariate approach supports the overall conclusion that a combination of physiological parameters can effectively predict balneotherapy outcomes.

The statistical analyses provide strong evidence for the predictability of balneotherapy effects on physical working capacity based on a constellation of initial physiological parameters. The findings support a personalized approach to balneotherapy, where treatment outcomes can be forecasted with high accuracy using pre-treatment assessments of neuro-endocrine, metabolic, and electrophysiological parameters. The partial confirmation of some hypotheses suggests that the relationships are complex and that further research may be needed to fully understand all the factors influencing balneotherapy outcomes.

Declarations

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Author contributions
Conceptualization, I.P.; Methodology, I.P.; Software, I.P. and W.Z.; Validation, I.P. and W.Z.; Formal Analysis, I.P. and W.Z.; Investigation, OM, VF, HK, IB, OV, IK, OL, TS, MK, OM, DP; Resources, OM, VF, HK, IB, OV, IK, OL, TS, MK, OM, DP; Data Curation, I.P. and W.Z.; Writing – Original Draft Preparation, I.P. and W.Z.; Writing – Review & Editing, I.P. and W.Z.; Visualization, I.P. and W.Z.; Supervision, I.P. and W.Z.; Project Administration, I.P. and W.Z.; Funding Acquisition.

Conflicts of interest
The authors declare no competing interests.

Data availability
The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethics approval
The study protocol was approved by the Ethical Committee of Ukrainian Scientific Research Institute of Medicine of Transport (protocol No. 35; 05.10.2022).

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ORIGINAL PAPER

Prevalence and pattern of abnormalities of cervical smear examination in women attending the fertility clinic at Uniosun Teaching Hospital, Osun state, Nigeria

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ABSTRACT

Introduction and aim. Infertility is described as the failure to conceive after one year of unprotected sexual intercourse. One of the causes of female infertility is cervical abnormalities that may be due to bacterial, parasitological, and hormonal imbalances. The purpose of this study was to determine the prevalence and Pattern of Abnormalities of cervical smear examination in women attending fertility clinic at the University of Osun Teaching Hospital, Osun State, Nigeria.

Material and methods. This study was conducted in the fertility clinic of University of Osun Teaching Hospital, Osun State. The study population consisted of 50 infertile (case group) and 50 fertile participants (control group) who are attending the gynecology clinic of the University of Osun Teaching Hospital, Osogbo. A questionnaire was used to obtain sociodemographic information and other relevant data. Cervical samples were collected using Ayre's spatula, two smears were made from each subject and stained with Papanicolaou, hematoxylin, and eosin staining techniques. The results were analyzed using a frequency table.

Results. Cervical smears revealed atypical squamous cells of undetermined significance in 15 cases (30%), while only 3 (6%) were observed among controls. Cervical cervicitis 19 (38%), *Candida* spp. (10%), *Trichomonas vaginalis* (16%), *Gardnerella vaginalis* (8%), inflammatory cell infiltrate (72%) and increased nucleo-cytoplasmic ratio (26%) were observed between cases and were significantly higher compared with the controls.

Conclusion. Abnormal pap smears in this study was significantly more often found in the case group when compared with the controls.

Keywords. infertility, estrogen, Pap smears, progesterone

Introduction

Papanicolaou smear is a useful screening tool to identify cellular alterations and anomalies in the cervix that can lead to cervical cancer and infertility.[1] Numerous factors, including hormonal fluctuations, prolonged use of birth control pills (five years or more) and infection with the Human papillomavirus (HPV), can cause abnormal changes in the cervix.[2] After the birth of three or more children, the Pap test is recommended every three years for all women between the ages of 21 and 65 years, while the HPV test is performed every five years.[3] Preinvasive cervical lesions range in prevalence from 6 to 12%, according to studies conducted in Nigeria.[4] However, women who tested positive for HIV had a prevalence of 12%, and it is well recognized that they have a higher chance of acquiring cervical cancer.[5] The current recommended screening methods in Nigeria include liquid-based cytology (LBC), visual inspection

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with acetic acid (VIA), conventional (Pap-smear) tests and HPV testing for high-risk HPV types.[6] Sub-Saharan Africa has a pervasive infertility problem, according to a recent issue of Nature.[7]

In our society, infertility is stigmatized, and as a result, marital (or domestic) discord and eventual divorce are common. According to the World Health Organization.[8] It is a social public health issue. In Nigeria, the prevalence of primary infertility is 5% and secondary infertility is 8%.[8] Numerous factors including genetics, work-related stress, the environment and infectious diseases.[9] Ovulation problems, endometriosis, fallopian tube damage or obstructions, primary ovarian failure (early menopause), pelvic adhesions, uterine or cervical abnormalities, and endometriosis are some of the causes of infertility in women.[9]

The cervix is part of the female reproductive system that connects the vagina to the uterus. It has a significant impact on fertility, menstruation, pregnancy, and labor. Infertility in humans is the inability to conceive after one year of unprotected and regular intercourse between a man and a woman.[10] Infertility has a variety of causes, some of which are treatable with medications.[11] Around 5% of heterosexual couples worldwide were thought to be experiencing unresolved infertility issues in 1997. However, a significant number of couples between 12% and 28% experience infertility for at least a year.[12] It was in 1974 when a scientist named Zur Hausen established the link between HPV and cervical anomalies. Cervical cancer has been explicitly linked to the HPV virus through ongoing studies throughout subsequent years.[13] As a result, the primary risk factor for cervical cancer is high-risk HPV infection. The virus spreads through sexual activity. Studies have identified about 40 strains classified as high-risk. It has been shown that having several sexual partners and making early sexual debuts enhance the risk of HPV infection.[8] Compared to women who have only had one partner, those who have had three or more sexual partners during their lives are almost 94% more likely to have HPV.[14]

Up to 186 million people worldwide are believed to be affected by infertility. Although male infertility accounts for almost 50% of all cases of childlessness globally, infertility nonetheless places a social cost on women. Unfortunately, infertility rates tend to be higher in areas of the world with limited access to assisted reproductive technologies (ARTs).[8] In addition to not being able to conceive, the primary symptom of infertility is irregular or nonexistent menstruation, caused by hormonal dysregulation or deficiency in the proliferative follicular phase of the pre-ovulatory cycle and the secretory luteal phase of the postovulatory cycle.[15] Additionally, the sex hormones progesterone and estrogen ensure that proper anatomical and physiological conditions for ovulation and fertilization occur. After a year of unprotected sex, infertility can be indicated by unsuccessful attempts to conceive, requiring testing possibilities for both men and women. The most popular tests for female infertility are the Papanicolaou cervical test, luteinizing hormone (LH), and follicle stimulating hormone (FSH).[16]

Aim

The aim of this study was to determine the prevalence and Pattern of Abnormalities of cervical smear examinations in women attending the fertility clinic at the University of Osun Teaching Hospital, Osun State, Nigeria. The objective of the study was to examine and compare the pattern of cervical smears in women presented with infertility with those with fertility.

Material and method

Study area

This study was conducted in Osun state, specifically in the fertility clinic of the University of Osun Teaching Hospital in Osogbo, Osun State. Osun state is a state in southwestern Nigeria; bordered to the east by Ekiti and Ondo state for 84 km and for 78 km respectively, to the north by Kwara State for 73 km, to the south by Ogun State for 84 km, and to the west by Oyo State, mostly across the River Osun. Osun state is named after the River Osun – a vital river that flows through the state. Of the 36 states of Nigeria, Osun is the ninth smallest in area and 25th most populous state with an estimated population of approximately 4.7 million as of 2016.[17] Osun State is primarily inhabited by the Yoruba people, mainly of the Ibolo, Ifẹ, Igbomina, Ijesha, and Oyo. Economically, Osun state is largely based around agriculture, mainly in cocoa, cassava, millet, maize, potato and yam crops.[18] Other key industries are services, especially in urban areas, along with artisanal mining and livestock herding. Osun state is additionally noted for having the second highest literacy rate in Nigeria.[19] Yoruba and English are the official languages. People of Osun State practice Christianity, Islam, and the traditional faith. Currently, Osun State has almost 30,000 estimated people living with HIV, 13,500 of which are yet to be identified and placed on treatment. Hepatitis E virus infection is prevalent among some vulnerable groups, such as immunosuppressed individuals, pregnant women, and HBV coinfected individuals in Osun state.[20]

Uniosun Teaching Hospital (formerly known as Lautech Teaching Hospital) is a state owned medical teaching hospital located in Osogbo, Osun State, Nigeria, to provide tertiary health care and support undergraduate medical students from Osun State University, Osogbo; Adeleke University, Ede, and Fountain University, Osogbo, Osun State. Services offered at Uniosun Teaching Hospital include; clinical services,, medical laboratory services and subclinical services, it is a 400

bed hospital situated in the Idi-Seke area of station road, Osogbo. The fertility clinic is available every Tuesday.

Study population

The study population was made up of infertile women (case group) who visited the Fertility Unit of University of Osun Teaching Hospital's fertility center and fertile women (controls) who are staff of Uniosun Teaching Hospital, Osogbo, and those who have their private businesses within the premises of the University of Osun Teaching Hospital, Osogbo, Osun State. The fertile participants recruited for this study served as the control because the study design is a case control.

Study duration

This study was carried out for a period of 6 months, between January 3, 2023 to June 30, 2023.

Inclusion criteria

This study comprised participants who met the following requirements:

1. Participants diagnosed with primary infertility attended the fertility clinic at Uniosun Teaching Hospital.
2. Participants diagnosed with secondary infertility attended the fertility clinic at Uniosun Teaching Hospital.
3. Participants who had conceived and delivered 2 or more children and had never had a delay were used as controls.
4. Participants who are 20 years old and above
5. Participants who are not on any contraceptive drug
6. Participants in the case group must have had regular and unprotected sex with their spouse (male) for at least 1 year.

Exclusion criteria

The following criteria led to the exclusion of research participants:

1. Participants who did not participate.
2. Participants that were menstruating
3. Participants who just had a colposcopy
4. Participants who recently douched
5. Participants that are under 20 years of age
6. Participants in the test group who are on contraceptives

Method of sampling

A convenient sampling method was used.

Ethical approval

The protocol for this study was sought and approved by the Ethics and research committee of UNIOSUN Teaching Hospital, Osun State, with the approval number UTH/EC/2023/03/746 dated 2 March 2023.

The confidentiality and privacy of the participants was strictly respected during and after the period of data collection and collection. Serial numbers were used instead of the names of the participants to ensure confidentiality. Participants received written inform consent written in English. Participants were allowed to read their informed consent and appended their signatures before samples were collected from them.

Sample size determination

P – prevalence of abnormal Pap smear from a study done in Zaria, Nigeria (6%)

The sample size for this research work will be determined using:

$$n = \frac{Z^2P(1-P)^{21}}{d^2}$$

n – required sample size

Z confidence level at 95% (standard value 1.96)

P – prevalence of abnormal Pap smear from a study done in Zaria, Nigeria (6%)

d – accepted error

$$n = \frac{1.96^2 \times 0.06(1-0.06)}{0.05^2}$$

n=87 (minimum sample size)

A total of 100 participants that consisted of 50 cases and 50 controls.

This is a case-control study, the cervical smears obtained from controls were compared with cases.

Collection of samples

Every participant (case and control) recruited for this study was individually called into a consulting room for confidentiality purposes. Each participant was educated about the study and those who gave their verbal consent received a questionnaire and informed consent in English language to fill in privately inside the consulting room. Each question in the questionnaire was explicitly explained to the participants. The acronyms were also fully interpreted and explained to them. Participants were properly guided on how to fill in the questionnaire. The completed questionnaire and the informed consent forms were collected from the participants and kept private by the researchers. Proper care was taken to ensure names or other related personal data that could be used in tracing the completed questionnaire, and the cervical smears collected from participants were completely avoided. After this, the participants were prepared for sample collection and for those who were eligible (that is, not menstruating or not having douched for 3 days), cervical smears were collected from them. Participants were asked to sit in a lithotomy position, a disposable single use plastic spatula was used to dilate the cervix to collect sample from the participants. The Ayre spat-

ula was inserted into the cervix at the squamo-columnal junction, until only the bottom fibers was exposed. It was slowly rotated at 360° in one direction. The sample and collected was transferred immediately onto two different slides where smears were made and fixed immediately.

Laboratory procedure

Participants (infertile) for this study were recruited from individuals who visited the fertility unit of the Uniosun Teaching Hospital, Osogbo. Participants were privately called into a consulting room for verbal education and explanation of what the study entails. Verbal consent was obtained prior to the questionnaire and inform consent forms were privately given to each of the participants in the consulting room.

The sample collection was carried out by the medical personnel team (Medical Doctors, Nurses and Medical Laboratory Scientists) at the fertility center of the University of Osun Teaching Hospital and laboratory analyzes were exclusively done by the Cytoscientists at the Cytopathology Laboratory of the University of Osun Teaching Hospital, Osun State. The collected samples were smeared onto clean slides and immediately fixed with 95% alcohol. A portion of the smears were stained with the Papanicolaou staining technique, while the second portion of the smears were stained with hematoxylin and eosin staining techniques. The stained smears were viewed, analyzed, and captured on a Brunel light microscope, 20 mega pixels (Brunel SP35 Digital Trinocular).

Papanicolaou staining technique

Slides were fixed in 95% alcohol for 30 minutes and rinsed with tap water. The smears were flooded with Harris Hematoxylin solution for 4 minutes before being briefly differentiated in 1% acid alcohol, then rinsed in running tap water for 10 seconds. The smears were briefly dipped in 70% alcohol briefly, then 95% alcohol for 10 seconds. The smears were flooded with Orange G 6 for 1 minute, then briefly dipped in 95% alcohol briefly. Smears were stained with Eosin Azure 50 for 2 minutes. Thereafter, briefly dipped into two changes of 95% alcohol. The smears were dipped in absolute alcohol (100%) for 1 minute, cleared in Xylene, covered with a slip and examined microscopically.[22] The stained cervical smears were viewed and captured on a Brunel light microscope, 20 mega pixels (Brunel SP35 Trinocular).

Hematoxylin and eosin staining technique (H&E)

Cervical smears were fixed in 95% alcohol for 30 minutes before adding water. Hydrated smears were stained in Harris hematoxylin for 4 minutes, rinsed in tap water and briefly differentiated in 0.5% acid alcohol. The smears were rinsed in water and blued in tap water for 10 minutes. Stained smears were counterstained with 1% Eosin for 2 minutes, rinsed in water and dehydrated in ascending grades of alcohol, cleared in xylene and mounted with DPX.[18] The stained cervical smears were viewed and captured on a Brunel light microscope, 20 mega pixels (Brunel SP35 Trinocular).

Data Analysis

Statistical analysis for Social Sciences (SPSS, IBM, Armonk, NY, USA) version 25 was the statistical package used to analyze all data obtained from the questionnaire and cervical smears. Data obtained from this study were captured from the questionnaire and cervical smears obtained from cases and controls. The statistician handled the statistical analysis of this study. The Student's t test and Pearson's correlation were used to compare the mean of the different analytes with $p < 0.05$ statistical significance.

Variables captured from the questionnaire administered prior to sample collection in this study include: the age of the participants, educational level, years of marriage, occupation, days of menstrual flow, intercourse frequency, knowledge of ovulation, timing of ovulation, history of past pregnancy, previous results obtained from pap smears and family history of infertility. The variables captured from the cervical smears collected included distribution of cell morphology in the smears of both the case group and controls, distribution of cytomorphological characteristics, and infections among the participants.

Results

Approximately 100 participants consisting of 50 in the case group and 50 controls were recruited for this study, with ages ranging from 20 years to 60 years and older. Among the cases recruited for this study, there were 8 participants between the ages of 20 to 29 years (16%), 15 between 30 and 39 years(30%), 16 between 40 and 49 years (32%), 8 between 50 and 59 years (16%), and 3 participants that were 60 years and above (6%). Among the controls recruited for this study, there were 6 participants between the ages of 20 to 29 years old (12%), 29 between the age of 30 and 39 years (58%), 12 between the age of 40to 49 years (24%), 2 between 50 and 59 years (4%), and 1 participant that was 60 years old (2%). The number of years that the participants have been married differs and are listed in Table 1.

The educational level of the participants recruited for this study differs between the cases and controls. About 5 (10%) participants in the case group have no formal education, 17 (34%) have a primary school leave certificate; 15 (30%) have a secondary school certificate while 13 (26%) have a tertiary certificate. Among the control group, 3 (6%) have no formal education; 6 (12%) have a primary school leaving certificate; 17 (34%) have

a secondary school leaving certificate, and 24 (48%) had a tertiary certificate. However, a significant difference was revealed between the age of participants (p=0.042), educational status (p=0.0034) and the years of marriage (p=0.014) between the case group and controls.

Table 1. Sociodemographic characteristics of study participants

Variables	Case group n=50, frequency (%)	Control group n=50, frequency (%)	p
Age (years)			
20–29	8 (16)	6 (12)	
30–39	15 (30)	29 (58)	0.042
40–49	16 (32)	12 (24)	
50–59	8 (16)	2 (4)	
60 and above	3 (6)	1 (2)	
Years of marriage			
≤5	8 (16)	12 (24)	
6–15	37 (74)	22 (44)	0.014
16–25	4 (8)	11 (22)	
26 and above	1 (2)	5 (10)	
Education			
None	5 (10)	3 (6)	
Primary	17 (34)	6 (12)	0.0034
Secondary	15 (30)	17 (34)	
Tertiary	13 (26)	24 (48)	
Occupation			
Artisan	5 (10)	8 (16)	
Civil servants	10 (20)	23 (46)	
Public servants	3 (6)	1 (2)	0.06
Traders	15 (30)	8 (16)	
Unemployed	14 (28)	8 (16)	
Others	3 (6)	2 (4)	

The occupations of the participants recruited for this study are different, with 5 (10%) being artisans, 10 (20%) civil servants; 3 (6%) public servants; 15 (30%) traders; 14 were unemployed (28%), while others were 3 (6%) the least among the cases. Among the controls, 8 (16%) were artisans, 23 (46%) civil servants, 1 (2%) public servants, 8 (16%) traders, 8 (16%) were unemployed while those who did not disclose their occupation and were placed under "others" were 2 (4%). However, no significant difference observed in occupation (p=0.06) between the case group and controls (Table 1).

Note: Not all variables on socio-demographic status detailed in the answered questionnaire were included in the table above. The variables above are very important and relevant to this study.

Among the cases recruited for this study, 42 (84%) have their menstrual flow for 4-6 days, 7 (14%) have menstrual flow for 13 days, while only 1 (2%) had menstrual flow for 7 days and more. For controls, 49 (98%) always observed menstrual flow between 1–3 days, while only 1 (2%) observed 4–6 days of menstrual flow and none had menstrual flow for 7 days and above.

Among the cases recruited for this study, 42 (84%) have their menstrual flow for 4-6 days, 7 (14%) have menstrual flow for 13 days, while only 1 (2%) had menstrual flow for 7 days and more. For controls, 49 (98%) always observed menstrual flow between 1–3 days, while only 1 (2%) observed 4–6 days of menstrual flow and none had menstrual flow for 7 days and above.

Table 2. Distribution of days of menstrual flow and number of times of sexual intercourse

Variables	Case group n=50, frequency (%)	Control group n=50, frequency (%)	p
Menstrual flow			
1–3 days	7 (14)	49 (98)	0.0002
4–6 days	42 (84)	1 (2)	
7 days and above	1 (2)	0 (0)	
Numbers of intercourse			
Daily	2 (4)	1 (2)	
Once a week	5 (10)	28 (56)	0.0002
2–5 times weekly	41 (82)	20 (40)	
6 times weekly	2 (4)	1 (2)	

Regarding the number of times of sexual intercourse among the case group, 41 (82%) always had intercourse with their spouse 2–5 times a week, 2 (4%) always had intercourse daily and 6 times a week, respectively. For the control group, 28 (56%) always had sexual intercourse once a week, 20 (40%) had 2–5 times a week while 1 (2%) had sexual intercourse daily and 6 times a week, respectively. Significant differences were observed on the days of menstrual flow (p=0.0002) and number of sexual intercourse instances (p=0.0002) between the case and control group (Table 2).

Table 3. Knowledge of ovulation, timing of ovulation, history of previous pregnancy, and previous results of Pap smear among participants

Variables	Case group n=50, frequency (%)	Control group n=50, frequency (%)	p
Knowledge of ovulation			
Yes	21 (42)	39 (78)	0.0005
No	29 (58)	11 (22)	
Ovulation timing			
Yes	17 (34)	29 (58)	0.0273
No	33 (66)	21 (42)	
History of past pregnancy			
Yes	11 (22)	50 (100)	0.0006
No	39 (78)	0 (0)	
Previous Pap smear result			
Yes	4 (8)	12 (24)	0.0462
No	46 (92)	38 (76)	
Family history of infertility			
Yes	18 (36)	8 (16)	0.0402
No	32 (64)	42 (84)	

Among the cases, 29 (58%) of the participants had no knowledge of ovulation while 21 (42%) had knowledge of

ovulation. Among the controls, 39 (78%) of the participants had knowledge of ovulation, while 11 (22%) had no knowledge. A significant difference (p=0.0005) was observed in ovulation knowledge of ovulation among the case group and controls. Among the case group, only 17 (34%) knew their ovulation time, while 33 (66%) did not know their ovulation periods. Among the controls, 29 (58%) knew their ovulation time, while 21 (42%) did not knowledgeable about their ovulation time. About 11 (22%) had a history of previous pregnancy, while 39 (78%) had no history of previous pregnancy among the case group. For the controls, all participants had history of pregnancy 50 (100%). Only 4 (8%) out of the participants had a Pap smear test previously, while the majority 46 (92%) of the participants had no history of a Pap smear test among the cases. For the controls, approximately 12 (24%) had a previous Pap smear test, while 38 (76%) had no previous Pap smear test. A majority of the cases, 32 (64%) had no family history of infertility, while 18 (36%) have a family history of infertility. For the controls, 8 (16%) had a history of infertility, while 42 (84%) had no family history of infertility. Significant differences in ovulation timing were observed (p = 0.0273), history of past pregnancy (p=0.0006), previous results of Pap smear results (p=0.0462) and family history of infertility (p=0.0402) between the case group when compared with the controls (Table 3).

Table 4. Distribution of cell morphology among participants*

Variables	Case group n=50, frequency (%)	Control group n=50, frequency (%)
Normal cell	34 (68)	47 (94)
ASC-US	15 (30)	3 (6)
LGSIL	1 (2)	0 (0)
HGSIL	0 (0)	0 (0)
SQC	0 (0)	0 (0)
AGC	0 (0)	0 (0)
AIS	0 (0)	0 (0)

* ASC-US – atypical squamous cells of unknown significance, LGSIL – low-grade squamous, intraepithelial lesion, HGSIL – high-grade squamous intraepithelial lesion, SQC – squamous cell carcinoma, AGC – atypical glandular cells, AIS – adenocarcinoma in situ

The morphology of cervical smears among the case group revealed that 34 (68%) of the participants had normal cell morphology, 15 (30%) of the participants had atypical squamous cells of unknown significance, and only 1 (2%) had low-grade squamous intraepithelial lesion. Although the morphology of the control group revealed that 47 (94%), 3 (6%) and 0 (0%) had normal cell morphology, atypical squamous cells of undetermined significance, and low grade squamous intraepithelial lesions, respectively.

Table 5. Distribution of cytomorphological features and infections among participants

Variables	Case group n=50		Control group n=50		p
	Yes (%)	No (%)	Yes (%)	No (%)	
Cervicitis	19 (38)	31 (62)	8 (16)	42 (84)	0.0243
Candida spp.	5 (10)	45 (90)	1 (2)	49 (98)	0.2065
T. vaginalis	8 (16)	42 (84)	1 (2)	49 (98)	0.036
G. vaginalis	4 (8)	46 (92)	2 (4)	48 (96)	0.674
Infiltrate of inflammatory cells	36 (72)	14 (28)	12 (24)	38 (76)	0.00001
Increased nucleo-cytoplasmic ratio	13 (26)	37 (74)	2 (4)	48 (96)	0.034

The distribution of cytomorphological characteristics and infections among the case group was observed as follows; cervicitis 19 (38%), yeast cell (*Candida* spp.) 5 (10%), *T. vaginalis* 8 (16%), *G. vaginalis* 4 (8%), infiltrate of inflammatory cells 36 (72%) and increased nucleocytoplasmic ratio 13 (26%). On the other hand, cervicitis 8 (16%), yeast cell 1 (2%), *T. vaginalis* 1 (2%), *G. vaginalis* 2 (4%), infiltrate of inflammatory cells 12 (24%) and increased nucleocytoplasmic ratio 2 (4%) were observed among the control group (Fig. 1–10). Infection with bacteria, fungi, and protozoa may be one of the significant causes of infertility in women (Table 5).

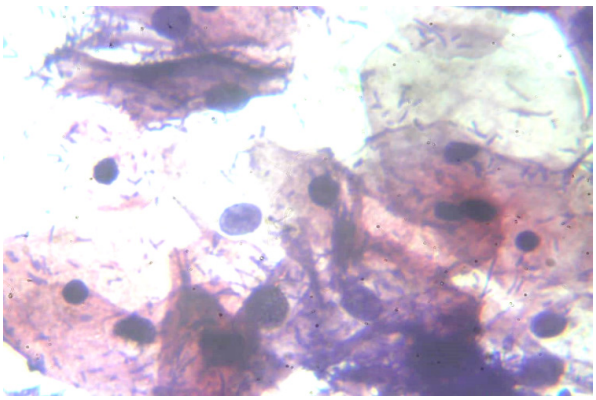

Fig. 1. Cervical smear from a participant (control group) (6-15 years of marriage) revealing *Candida* spp. and slight increase in nuclear-cytoplasmic ratio (Pap stain, 400X)

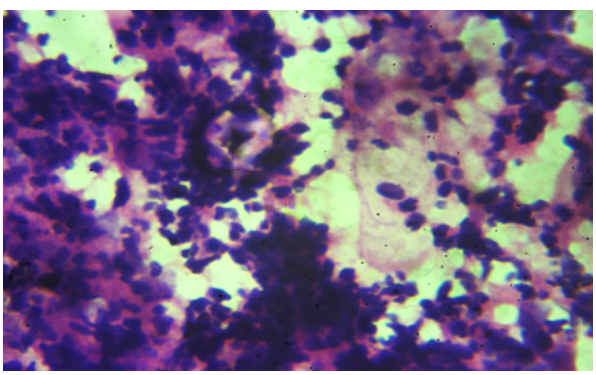

Fig. 2. Cervical smear from a participant (case group) (6–15 years of marriage) revealing LGSIL (Pap stain, 400×)

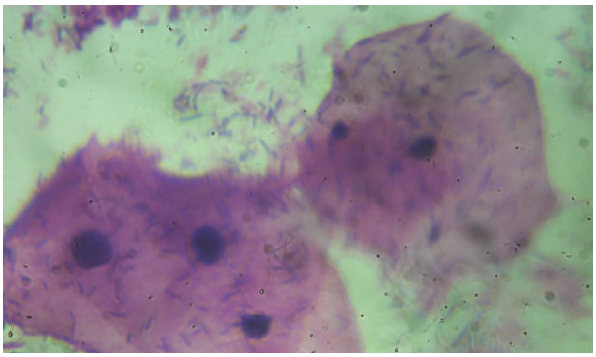

Fig. 3. Cervical smear from a participant (case group) (<5 years of marriage) revealing *Candida* spp. (Pap stain, 400×)

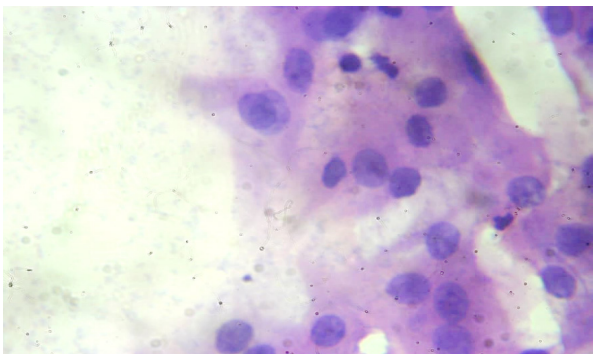

Fig. 4. Cervical smear of a participant (case group) (16-25 years of marriage) revealing LGSIL (Pap stain, 400×)

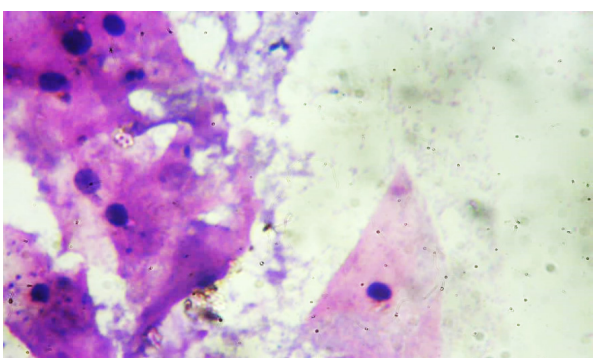

Fig. 5. Cervical smear from a participant (control group) (6–15 years of marriage) revealing normal squamous cells (H&E, 400×)

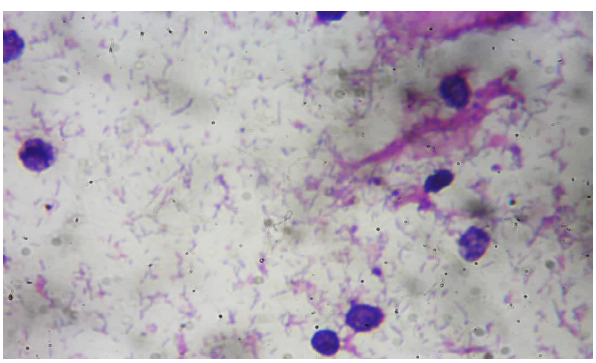

Fig. 6. Cervical smear from a participant (case group) (6–15 years of marriage) revealing *Candida* spp. and cytolysis (H&E, 400×)

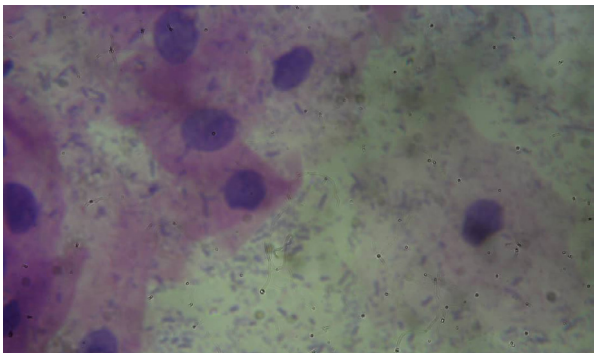

Fig. 7. Cervical smear from a participant (case group) (6–15 years of marriage) revealing ASC-US also with *Candida* spp. (H&E, 400×)

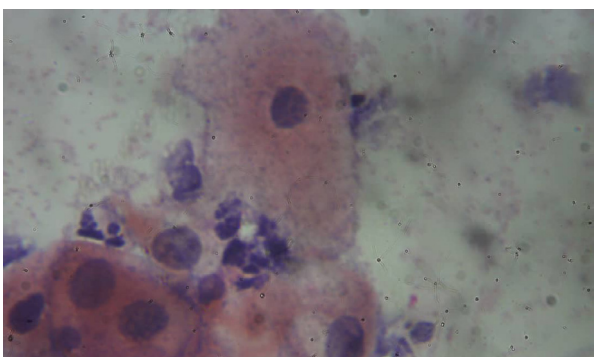

Fig. 8. Pap smear of a participant (case group) (26 years and older of marriage) revealing LGSIL (H&E, 400×)

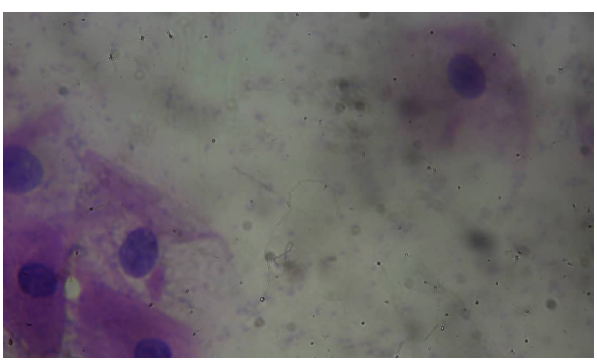

Fig. 9. Cervical smear from a participant (case group) (16–25 years of marriage) revealing ASC-US also with *Candida* spp. (H&E, 400×)

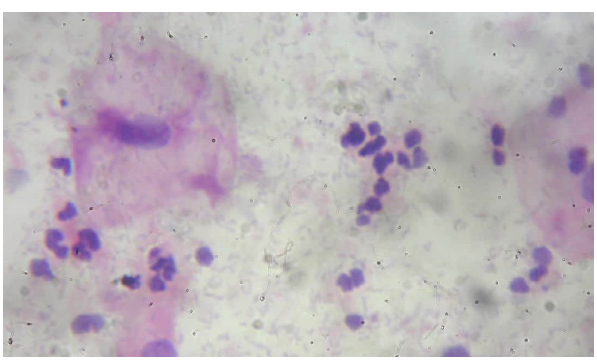

Fig. 10. Cervical smear from a participant (case group) (6–15 years of marriage) revealing cervical cervicitis (H&E, 400×)

Discussion

Infertility is a global problem that affects between 8–10% of couples.[23] Infertility can be primary or secondary. Primary infertility is when a person has never been achieved by a pregnancy, and secondary infertility is when at least one prior pregnancy has been achieved.[24] Generally, pelvic inflammatory disease, which is frequently brought on by sexually transmitted infections (STDs), particularly Chlamydia trachomatis (CT) is more common than infertility.[23] The idea that women experiencing infertility are at increased risk of developing cervical intraepithelial lesions or even carcinoma, was reinforced by the fact that these women are typically treated with exogenous hormones and are exposed to sexually transmitted infections (STDs) and HPV.[25,26] Ovulation failure is the most frequent reason for infertility in women.[23] Cervical cell abnormalities can be detected early with Pap smear screening, which also triggers intervention according to established criteria. It has been demonstrated that screening is associated with a considerable decline in the prevalence of invasive cervical cancer.[27]

In this study, about 50 cases and 50 controls were screened for Pap smear test using Papanicolaou and hematoxylin and eosin staining techniques. Participants between the ages of 40–49 were observed to be the highest number of cases randomly recruited for this study, while participants between the ages of 30 39 were observed to be the highest number of controls randomly recruited for this study. This study observed a significant association on the age of the participants (p=0.042) and years of marriage (p=0.014) between the case and control group. Among the cases recruited for this study, most had a menstrual flow for 4–6 days, while among the controls, majority had a menstrual flow for 1–3 days. This is in parallel with the study carried out in Sudan by Almobarak et al., who revealed that bad menstrual flow among Sudanese is the main cause of infertility among women.[28] In terms of the number of times sexual intercourse with their spouses in the cases, majority always had intercourse 25 times a week. Regular intercourse among cases may be due to the eagerness of having conception. This finding is in tandem with the study conducted in Saudi Arabia by Al-Jaroudi and Hussain.[26]

This study revealed that most cases had no previous Pap smear result, as evident by the fact that many of them did not know what the test entails. Most of those who had heard about it were unaware of the recommended frequency of Pap smear tests within a year. This research contradicts the findings conducted in Niger and Iran by Owoeye and Ibrahim and Tran et al. which found that only 50.6% and 44.3% of the participants, respectively, were aware of cervical cancer screening tests.[29,30] This discrepancy may be due to low health awareness in the study area.

The results of this study observed that some of the cases did not know about ovulation, this could be as a result of a low level of education among the participants. Only 34% of the cases in this study had a good understanding of when they usually ovulate. This low percentage is consistent with a study by Wolde et al. that found that 23% of Ethiopian women of childbearing age know about their fertile period and its determinants.[31] The study was based on a multilevel analysis of data from the Ethiopian demographic and health survey of 2016.

Among the cases recruited for this study, there was a higher prevalence of abnormal cervical smear compared to controls. This abnormal prevalence of Pap smear is consistent with the 29.5% of subfertile participants in the study conducted in Saudi Arabia by Al-Jaroudi and Hussain who examined the prevalence of abnormal cervical cytology among subfertile Saudi women.[26] Also, in the study by Lundqvist et al. on the cytological screening and human papilloma virus test in women undergoing artificial fertilization.[32] Their findings revealed different results, reporting aberrant cytology in 4.1% of the control and 2.3% of the infertile participants. However, this study had numerous drawbacks, including a small sample size of 100 participants.

Cervical cervicitis was observed in cervical smears of the case group. However, cervical cytology is meant to be one of the diagnostic tools to examine gynecological infections, additional microbiology and immunology research is usually advised in microbiology and immunology. This study observed that 30% of the case group had atypical squamous cells of unknown significance; 2% had low-grade squamous intraepithelial lesion, while none of the case group had high grade squamous intraepithelial lesion. These findings contrast with the study by Pushp et al. who reported ASCUS to be 2.9% of screened women, LSIL 5.09% and HSIL to be 0.48%.[33] These findings are similar to those of Verma et al. who found LSIL in 5.5% and HSIL in 2.5% of their women screened at King Georges Medical University, Lucknow, UP, India.[34] In addition, Padmini et al. conducted a study on the cytological and colposcopic evaluation of unhealthy cervix in women who attended the gynecological outpatient department of Sri Siddartha Medical College, India.[35] The study revealed that 5% of the participants had LSIL and 3% had HSIL. A study by Nayani and Hendre reported a higher percentage of LSIL (8.6%) and HSIL (3.8%) lesions.[36] Cytologists disagree on the threshold for the ASC-US diagnostic category, which characterizes cellular abnormalities more marked than those caused by reactive alterations, but which do not quantitatively or qualitatively support a conclusive diagnosis of low-grade squamous intraepithelial lesion. The high prevalence of ASCUS cytological abnormalities seen in this study may be caused by variations in age, in-

cidence of associated infections, awareness of screening, and the existence or nonexistence of cervical screening programs across the nation. The epithelial pathological diagnosis of SIL has a 4.9% detection rate, according to a study conducted in Saudi Arabia by Magdy et al.[37] Lack of screening programs and low awareness of screening contributed to the low SIL rate. According to Saha et al.[38] ASCUS is the most prevalent cytological anomaly, which is consistent with these findings. Infiltrate of inflammation cells were seen in 72% of the study participants, while 26% of Pap smears revealed an increased nucleocytoplasmic ratio. This finding is contrary to the studies conducted by Atilgan et al. and Kulkarni et al., both observed a higher prevalence (95%) of infiltrates of inflammatory cells among Turkish and Indian women.[39,40] The low prevalence observed in this study suggests that participants had less genital tract infections, which are common in women of reproductive age and have a significant financial impact. Many women with cervicitis or vaginitis have been found to not exhibit any symptoms. According to studies of Bhutia et al. and Barouti et al., women who have prolonged inflammation should receive the proper care because failing to do so raises the risk of developing intraepithelial cervical lesions.[41,42]

Conclusion

The need to screen for cervical cytology in infertile participants is emphasized by the high frequency of abnormal cervical cytology among this group of participants. However, compared to participants who are fertile, this study also found that infertile participants had abnormal pap smears far more frequently. Governments and non-governmental organizations should encourage the general awareness among women of regular cervical screening should be encouraged by governments and non-governmental organizations.

Declarations

Funding

The three authors provided funding for this study.

Authors' contributions

Conceptualization, A.B.A., B.S.O. and A.O.A; Methodology, A.B.A., B.S.O. and A.O.A.; Software, A.B.A., B.S.O. and A.O.A.; Validation, A.B.A., B.S.O. and A.O.A.; Formal Analysis, A.B.A.; Investigation, A.B.A., B.S.O. and A.O.A.; Resources, A.B.A., B.S.O. and A.O.A.; Data Curation A.B.A. and A.O.A.; Writing – Original Draft Preparation, A.B.A. and A.O.A.; Writing – Review & Editing, A.B.A.; Visualization, A.B.A. and B.S.O.; Supervision, A.B.A. and B.S.O.; Project Administration, A.B.A., B.S.O. and A.O.A.; Funding Acquisition, A.B.A., B.S.O. and A.O.A.;

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

All data and materials collected during this study are available with the corresponding author upon reasonable request.

Ethics approval

The protocol for this study was sought and approved by the Ethics and research committee of UNIOSUN Teaching Hospital, Osun State, with the approval number UTH/EC/2023/03/746 dated 2 March 2023.

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ORIGINAL PAPER

Analysis of cervical mucosal epithelium proliferation during the postmenopausal period

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ABSTRACT

Introduction and aim. This study explores the proliferative growth of cervical mucosal epithelial cells during the postmenopausal period, offering new insights on age-related changes in cervical tissues, a topic previously under-examined in postmenopausal health research. By employing histologic analysis, immunostaining techniques, and Ki-67 mitotic index assessment, this research provides novel data on the alterations in cervical epithelial cells during and after menopause. The findings enhance our understanding of the biological processes affecting cervical health in the postmenopausal phase, which is increasingly significant as the global population ages and emphasizes the need for tailored healthcare approaches. The primary goal was to investigate the proliferative growth of cervical mucosal epithelial cells in postmenopausal women by assessment of Ki-67 gene activity in progesterone-positive cells in normal and pathological postmenopausal periods (PMPs) in the presence or absence of autonomic symptoms, manifested by mood instability, headaches and dizziness.

Material and methods. This research involved analyzing tissue samples from 149 postmenopausal women with suspicion of malignancy using histologic and immunostaining methods to evaluate epithelial cell proliferation markers, with the Ki-67 mitotic index as a key measure. The average activity of the Ki-67 gene was evaluated using the global scoring method, with Ki-67 percentages below 2.5% considered low. Statistical analysis included both parametric and non-parametric methods, specifically Student's t and Wilcoxon's tests.

Results. The study found a reduction in the proliferative activity of cervical mucosal epithelial cells during the normal postmenopausal period, indicated by a significant decrease in Ki-67 expression in the exocervical zone and during the first year in the transformation zone and the endocervix ($p<0.01$).

Conclusion. Understanding the proliferative growth of cervical mucosal epithelial cells during the postmenopausal period helps to understand the dynamics of age-related cervical tissue. These results can lead to better prevention and monitoring strategies for cervical health in postmenopausal women.

Keywords. Cervix, Ki-67, postmenopausal period, proliferative growth

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Introduction

According to the global scientific literature, it is known that the outside of the cervix is covered with a pink-colored multilayer squamous epithelium.[1-3] Four main layers of keratinocytes have been identified in the multilayer epithelium:

- the first layer - basal cells (characterized by large nuclei and high nuclear-cytoplasmic ratios),
- the second layer parabasal cells,
- the third layer -intermediate cells,
- the fourth layer - superficial cells with low nuclear--cytoplasmic ratios, containing glycogen.

At the external cervix, the squamous epithelium, which consists of multiple layers, interfaces with cylindrical epithelial cells.[4,5] Occasionally, cylindrical epithelium can extend to the cervix's outer surface and around the external os, a phenomenon known as ectopia. This is a common occurrence in newborn girls, where the cylindrical epithelium gradually transitions into multilayered squamous epithelium as they age. This transformation is facilitated by reserve cells located between cylindrical epithelial cells, which give rise to patches of multilayered squamous epithelium.[6,7]

Ectopia may also result from the body's response to hormonal contraceptives or pregnancy and can occur due to hormonal treatments during the postmenopausal period to extend menopause and prevent rapid involution of the genitourinary organs.[8,9] In women of reproductive age, the boundary between the squamous and cylindrical epithelium is typically aligned with the external os, while in younger women, it is located on the endocervix and in older women, it is found within the cervical canal. This boundary, known as the transformation zone, is crucial and warrants meticulous examination by specialists.[10-12] The cervical epithelium is a complex multilayered system that interacts intricately both internally and with the adjoining cylindrical epithelium.

Menopause significantly impacts various physiological processes, including the structure and function of cervical tissues. The postmenopausal period is characterized by a decline in estrogen levels, leading to thinning of the cervical epithelium and a reduction in its regenerative capacity. This hormonal change contributes to a decreased proliferative activity of cervical mucosal (CM) epithelial cells, as indicated by lower Ki-67 expression, and a diminished ability to respond to environmental and physiological challenges. Furthermore, menopause is associated with changes in the microenvironment of the cervix, such as altered immune responses and increased susceptibility to infections and neoplastic transformations.[13] The impact of menopause on cervical health is further complicated by infections by human papillomavirus (HPV). HPV, particularly high-risk strains like HPV-16 and HPV-18, disrupt normal cellular processes by inactivating tumor suppressor proteins p53 and Rb via its oncoproteins E6 and E7. This leads to uncontrolled cell proliferation, impaired apoptosis, and genomic instability. HPV-induced changes can transform normal endocervical epithelium into precancerous lesions, increasing the risk of cervical cancer.[14,15] Therefore, understanding the combined effects of menopause and HPV infection is crucial for developing tailored medical approaches to manage cervical health in postmenopausal women.

The cervix has three distinct sections characterized by unique morphological features and varying immunological activities. The physiological regeneration of these different epithelial layers involves cambium-like cells and follows diverse signaling pathways for differentiation and specialization. This process necessitates an understanding of not just cellular plasticity but also the patterns of neurohormone systems, known as keilons.[16] The intermediate area, known as the transformation zone, is a critical site for microbial contamination and the localization of cells prone to malignancy.[17] Research has yet to fully explore the phenotypes of innate and adaptive immune cells and their interactions under conditions of papillomavirus infection. Studies by the authors have revealed that the quantitative and qualitative characteristics of the differon system, including local mucosal immunity, differ significantly from those in more proximal regions.

Aim

The research aim is to investigate the proliferative growth of cervical mucosal epitheliocytes by assessment of Ki-67 gene activity in progesterone-positive cells during normal and pathological postmenopausal periods (PMPs).

Material and methods

The main aim of this research was to explore the proliferative growth of cervical mucosal epithelial cells in patients from the Primorsky Region across various age groups. The study involved analyzing 149 cervical mucosa biopsy specimens prepared at the Medical complex of Far Eastern University that were collected from patients with clinical indications for malignancy in accordance with the Declaration of Helsinki (2000, 2013) and approved by the Local Ethics Committee of Far Eastern Federal University, Vladivostok (protocol No. 6 dated April 19, 2023).

The study compared the clinical material of women (in equal proportions, 74 and 75, respectively) with the normal and pathological course of the postmenopausal period (depending on the presence or absence of persistent symptoms in the form of vegetative-vascular manifestations with emotional instability, headaches and dizziness). Observed samples were obtained from differ-

ent patients for 5 years. During the first year of study biopsies of 25 patients were analyzed, the second year 30 patient samples were analyzed, the third year 42 patient samples were analyzed, the fourth year 33 patient samples were analyzed, and the 5th year 29 patient samples were analyzed. Patients did not have high HPV activity, surgical interventions, or hormonal medications in the past 10 years before visiting a gynecologist. The normal postmenopausal period occurred in women without signs of cellular atypia and malignancy (those women in whom immunohistochemistry (IHC) did not confirm the presence of factors predisposing to tumor growth). In the pathological postmenopausal period, signs of cellular atypia with high Ki-67 activity were found.

Progesterone-positive cells were identified in routine IHC diagnosis. As a marker, a monoclonal antibody clone of the progesterone receptor (PgR 636), specifically targets the progesterone receptor (PR), was applied. This antibody binds to the PR protein in formalin-fixed paraffin-embedded tissue sections, allowing the detection of PR-expressing cells. Following primary antibody binding, a secondary antibody conjugated with an enzyme or fluorescent marker was applied, enabling visualization under a microscope, typically as a brown chromogenic signal.

General morphological characteristics were identified using hematoxylin and eosin staining, while proliferative activity in the epithelial lamina was assessed by the Ki-67 marker, counting mitoses per 100 cells. Progesterone-positive cells were quantified by IHC assessment of level protein expression over a 4 mm² area, with ≥13 positive cells distinguishing normal course from pathological course of the postmenopausal period. Average Ki-67 gene activity was evaluated using the global scoring method, with Ki-67 percentages below 2,5% considered low. Statistical analysis employed both parametric and non-parametric methods, including Student's and Wilcoxon's tests, facilitated by standard software packages like LYSYS II ("Becton Dikinson Immunocytometry system"). Visual documentation was captured using a digital camera with DP×25 software.

Results

Analysis of proliferative activity at different stages of a the postmenopausal period showed differences in various sections of the cervical mucosa (Figs. 1 and 2).

Thus, we observed that in postmenopausal women there is an increase in progesterone receptors, a decrease in estrogen levels is associated with a decrease in estrogen receptors and a sharp decrease in proliferative activity in the epithelial lamina of the CM (Fig. 3).

The number of Ki67-positive cells in the pathologic course during the postmenopausal period increases to a greater extent in the exocervical CM compared to the norm, which can be attributed to pathogen contamination and adaptive sloughing of the epithelium on the epithelial surface, these data for norm and pathology are presented in Tables 1 and 2.

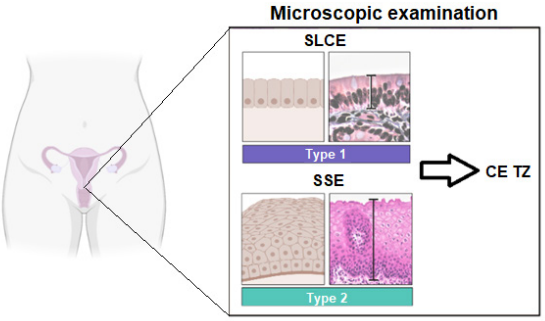

Fig. 1. Scheme of the cervical epithelium, SSE stratified squamous epithelium of the exocervix, CE TZ columnar epithelium of the transformation zone, SLCE single-layered cylindrical epithelium of the endocervix

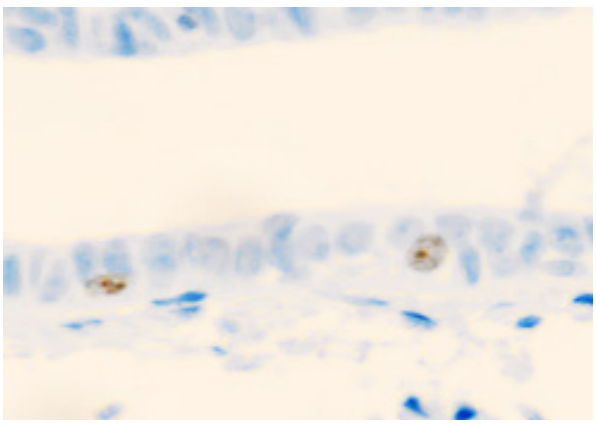

Fig. 2. 63-year-old woman, localization of Ki67 gene protein with low expression (dim brown markers in image) in the structures of the cervical mucosa in normal postmenopausal period (Immunohistochemistry with hematoxylin, 400×)

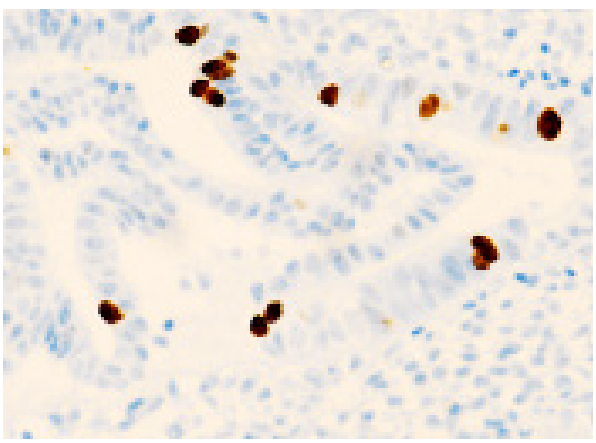

Fig. 3. Localization of the Ki67 gene protein with high expression (bright brown markers in image) in the structures of the cervical mucosa in the presence of polyps in the cervical mucosa (Immunohistochemistry, 200×)

When statistical analysis was performed to compare the average using Student's test and the difference in the distribution of the data using the Wilcoxon test, it was determined that there were statistically significant differences in the exocervix in any period (average over the next 5 years after the onset of expression at 2.34% at normal vs. 2.78% at pathology), and in the transformation zone (2.4% at normal vs. 3.2% at pathology) and endocervix (1.8% at normal vs. 2.5% at pathology) in the first year of postmenopause as a function of PMP normality (p-value < 0.01) and increased mean Ki-67 expression level at pathology; no statistically significant differences were observed in the transformation zone and endocervix in subsequent years after the onset of postmenopause with respect to the progression of PMP.

Table 1. Evaluation of the location and quantification of Ki-67-positive cell activity in the CM of patients*

CM structures control	Average Ki-67 gene activity in normal postmenopausal period (number of mitoses per 100 cells in %)					
	1 year PMP	2 year PMP	3 year PMP	4 year PMP	5 year PMP	Total average for 5 years
Exocervix	3.2	2.4	2	2.9	1.2	2.34
Transformation zone	2.4	2.4	2.1	2.6	1.1	2.12
Endocervix	1.8	2.4	1.9	1.5	1.1	1.74

* CM – cervical mucosa, PMP – postmenopausal period

Table 2. Evaluation of location and quantitative characterization of Ki-67 - positive cells activity in CM of patients with pathological course of postmenopausal period*

CM structures in the observation group	Average activity of the Ki-67 gene in the pathological course of the postmenopausal period (number of mitoses per 100 cells in %)					
	1 year PMP	2 year PMP	3 year PMP	4 year PMP	5 year PMP	Total average for 5 years
Exocervix	4.2	2.9	2.5	2.6	1.7	2.78
Transformation zone	3.2	2.5	2.2	1.8	1.3	2.2
Endocervix	2.5	2.4	1.9	1.6	0.8	1.84

* CM – cervical mucosa, PMP – postmenopausal period

Discussion

The observed variations in the activity of the Ki-67 gene in different sections of the CM reveal complex patterns of regenerative potential during the postmenopausal period. Our findings contribute novel insights into the dynamic nature of cervical tissue regeneration, expanding the existing literature.[8,9]

A particularly notable result is the significantly higher activity of the Ki-67 gene observed in the exocervix, corroborating and extending previous research that reported elevated regenerative potential in this outermost cervical layer.[6,7] This robust regenerative activity, marked by increased cell proliferation, highlights the exocervix's essential role as a protective barrier against environmental challenges, suggesting that constant exposure to external factors requires active tissue renewal and repair. In contrast, other studies have indicated no significant decrease in Ki-67 gene activity in the transformation zone compared to the exocervix.[12]

This discrepancy could arise from differences in study populations, methodologies, or age group definitions. Our findings suggest a nuanced transition in the balance between cell proliferation and turnover as the cervical mucosa progresses from the exocervix to the transformation zone. More research on hormonal signaling, microenvironmental factors, and molecular mechanisms specific to this region is needed to clarify these differences.

Our findings regarding the endocervix, which exhibited the lowest Ki-67 gene activity, align with previous data indicating a reduced regenerative potential in this innermost cervical layer.[16] This diminished proliferative activity offers valuable insights into the cellular mechanisms associated with age-related changes in cervical tissues. Investigating the molecular pathways that regulate the endocervix's regenerative potential could reveal novel therapeutic targets for age-related cervical health issues, underscoring the importance of our study in advancing a broader scientific understanding of postmenopausal cervical dynamics.

HPV infection, particularly with high-risk strains such as HPV-16 and HPV-18, significantly affects the regenerative and transformation mechanisms of the endocervix by disrupting normal cellular processes. The viral oncoproteins E6 and E7 inactivate tumor suppressor proteins p53 and Rb, respectively, leading to uncontrolled cell proliferation and impaired apoptosis. This interference alters the regenerative potential of endocervical cells, initially increasing proliferation but eventually causing genomic instability. Furthermore, HPV-induced changes can promote the transformation of normal squamous epithelium into precancerous lesions, increasing the risk of cervical cancer. Studies have shown that HPV infection can also result in chronic inflammation, further exacerbating cellular damage and dysregulation.[14] Additionally, the integration of HPV DNA into the host genome can disrupt key regulatory regions and contribute to the carcinogenic process.[15] The persistent infection and the resulting cell changes highlight the critical need for monitoring and managing HPV infections to mitigate their impact on cervical health.

The comparative analysis with existing research offering new perspectives on the regenerative potential of various cervical mucosal sections during the postmenopausal period. These insights contribute to a more

comprehensive understanding of age-related changes in cervical tissues and establish a foundation for potential therapeutic interventions. Notably, the ectocervix's low Ki-67 gene activity suggests a relatively diminished regenerative capacity, likely due to natural aging, hormonal changes, or other postmenopausal physiological factors. This observation highlights the cellular mechanisms behind age-related changes in cervical tissues. Further exploration of the molecular pathways involved could lead to novel therapeutic approaches for managing age-related cervical health issues.

Conclusion

In this study, we elucidated the regenerative potential of distinct sections of the CM by examining Ki-67 gene activity across PMP age groups. The findings reveal a nuanced age-related dynamic in the proliferative activity of cervical mucosal epithelial cells, aligning with the research objectives.

Our investigation demonstrated significantly higher Ki-67 gene activity in the exocervix, indicating robust regenerative potential and active tissue renewal and repair mechanisms. This emphasizes the exocervix's crucial role in maintaining a healthy cervical barrier, especially against environmental challenges.

Conversely, the transformation zone showed a notable decrease in Ki-67 gene activity compared to the exocervix, suggesting a shift in the balance between cell proliferation and turnover as the mucosa transitions from the exocervix to the transformation zone. This dynamic may be influenced by hormonal signaling, microenvironmental factors, or other molecular mechanisms specific to this region.

Additionally, the study revealed the endocervix's lowest Ki-67 gene activity values, indicating a relatively diminished regenerative potential in this innermost layer. This observation provides critical insights into the cellular mechanisms underlying age-related changes in cervical tissues, aligning with our objective to explore the postmenopausal period's impact comprehensively.

The practical significance of these findings lies in their potential to inform tailored medical approaches for postmenopausal women, enabling improved prevention and monitoring strategies for cervical conditions. Understanding the regenerative dynamics of different cervical sections contributes to the broader landscape of women's health, guiding healthcare professionals in addressing age-specific concerns. However, this study has limitations, since it was performed in a single medical institution, and there was no more complete information about the patients' medical history and their further treatment.

Looking forward, future research endeavors should delve deeper into the molecular pathways governing the regenerative potential of each cervical section. Investigating specific factors influencing Ki67 gene activity and exploring potential therapeutic interventions based on these insights could enhance our ability to address age-related cervical health issues more effectively. Additionally, expanding the study to include a broader demographic and considering longitudinal assessments could provide a more comprehensive understanding of the age-related dynamics in cervical mucosal epitheliocytes.

In conclusion, our study advances the understanding of age-related changes in cervical tissues, providing valuable insights into the regenerative potential of different cervical mucosal sections. These findings contribute to the evolving field of women's health, with implications for personalized medical approaches and avenues for further in-depth exploration.

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Declarations

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Author contributions

Conceptualization, D.P.P. and G.V.R.; Methodology, D.P.P. and B.O.S.; Software, B.O.S.; Formal Analysis, B.O.S., and D.P.P. and I.V.R.; Investigation, I.P.K. and S.N.S.; Writing – Original Draft Preparation, B.O.S., D.P.P. and G.V.R.; Writing – Review & Editing, I.V.R., S.N.S. and V.V.U.; Visualization, D.P.P. and B.O.S.; Supervision, K.V.S. and M.B.K.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

Data not available due to legal restrictions.

Ethics approval

The research was approved by the Local Ethics Committee of Far Eastern Federal University, Vladivostok in the protocol No. 6 dated April 19, 2023.

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ORIGINAL PAPER

Comparative evaluation of morphological and chemical characteristics of bone after performing osteotomy with a piezoelectric device, hard tissue laser, and low-speed handpiece

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ABSTRACT

Introduction and aim. Osteotomy procedures play a crucial role in achieving the desired osseous contour and elimination of the pocket. Traditional instruments such as chisels, files, and rotating burs have limitations including heat generation and tissue damage. Novel instruments like piezoelectric and hard tissue lasers offer potential advantages in terms of precision and reduced tissue trauma. The aim of this study was to compare the chemical and morphological characteristics of bone surfaces after osteotomy procedures performed with three different instruments: piezoelectric, hard tissue laser, and low-speed handpiece.

Material and methods. Fifteen fresh cadaver bone specimens were randomly assigned to three groups: group A (piezoelectric), group B (hard tissue laser) and group C (low-speed handpiece). Osteotomy procedures were performed according to standardized protocols. The specimens were determined under an energy-dispersive X-ray spectroscopy.

Results. Analysis of morphological and chemical characteristics revealed significant differences in bone surface characteristics between groups. Groups A and B exhibited the smoothest surface with minimal tissue damage and microfractures. Group C showed the roughest surface with prominent microfractures and tissue damage.

Conclusion. Hard tissue laser and piezosurgery have shown better results due to greater precision as they preserved the bone morphology, with less microfracture and chemical demineralization after osteotomy preparation compared with low-speed handpiece.

Keywords. hard tissue laser, osteotomy, piezoelectric device

Introduction

Osseous surgery, particularly in periodontics, involves osteotomy and osteoplasty to remove periodontal pockets and reshape the bone to achieve a more physiological osseous contour. Traditional methods employ tools such as rongeurs, files, chisels, and rotating instruments such as steel bursts, diamond bursts, and carbide burs. However, despite their widespread use, these methods present significant challenges, including excessive heat generation, mechanical stress, and the potential for tissue necrosis, which can compromise clinical outcomes.[1,2]

The generation of heat during bone cutting with rotary instruments is a major concern, as it can lead to thermal injury of bone tissue. This risk persists even

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with the incorporation of internal cooling mechanisms into these instruments. Several studies have shown that elevated temperatures during osseous surgery can cause irreversible damage to the bone, leading to delayed healing, bone resorption, and, in some cases, implant failure.[3,4] Furthermore, the mechanical forces exerted by these tools can increase the risk of microfractures in the bone, particularly in situations involving brittle or compromised bone structures.[5]

In addition to thermal and mechanical challenges, conventional osseous surgery techniques carry the risk of injuring critical anatomical structures. For example, inadvertent damage to the Schneiderian membrane during maxillary sinus procedures or the inferior alveolar nerve during mandibular surgery can result in significant complications, such as chronic pain, altered sensation, or even permanent nerve damage.[6,7]

To address these limitations, advanced technologies such as piezoelectric devices and hard tissue lasers have been introduced into dental surgery. Piezoelectric surgery, first introduced by Vercellotti in the early 2000s, has gained popularity due to its ability to perform precise bone cuts with minimal damage to surrounding soft tissues. This technique operates through the ultrasonic vibration of a piezoelectric crystal, which generates micro-movements at the surgical tip, enabling selective bone cutting while sparing vital structures.[8] Studies have shown that piezoelectric surgery not only reduces the risk of thermal injury, but also enhances surgical precision, reduces postoperative pain, and accelerates healing compared to traditional methods.[9,10]

Laser technology has also revolutionized dental surgery and offers several advantages over conventional methods. Since the development of the ruby laser by Theodore Maiman in 1960, lasers have been increasingly used in various dental applications, including osseous surgery. Lasers provide improved visibility, hemostasis, and a relatively dry surgical field, which are particularly beneficial in periodontal procedures. The interaction of laser energy with biological tissues is wavelength-dependent, with specific tissue components, known as chromophores, absorbing wavelengths more efficiently. For example, carbon dioxide (CO_2) lasers, which operate in the infrared spectrum, are highly absorbed by water, making them effective for soft tissue procedures. However, its application in hard tissue surgery, such as bone, is limited due to the risk of excessive heat generation and subsequent thermal damage.[11,12]

Erbium-doped lasers such as the Er: Cr: YSGG lasers have emerged as viable alternatives for hard tissue applications. These lasers operate at wavelengths that are highly absorbed by both water and hydroxyapatite, the primary mineral component of bone. This selective absorption allows for precise ablation of bone tissue with minimal thermal damage, making them suitable for procedures such as osteotomy and osteoplasty. Research has shown that erbium lasers can effectively cut bone with less collateral damage, reduced postoperative inflammation, and faster healing times compared to traditional rotary instruments.[13,14]

Despite the advantages of piezoelectric devices and lasers, further research is still needed to fully understand their effects on bone morphology and chemical characteristics after osteotomy. Previous studies have focused primarily on clinical outcomes, such as healing times and complication rates, but there is a lack of comprehensive data on the microscopic and chemical changes that occur in bone tissue after these advanced surgical techniques.[15] Understanding these changes is crucial to optimize surgical protocols and improve patient outcomes in periodontal osseous surgery.

Aim

This study aims to address this gap in the literature by conducting a comparative evaluation of the morphological and chemical characteristics of bone surfaces following osteotomy using a low-speed handpiece, a hard tissue laser, and a piezoelectric device. By analyzing differences in bone surface morphology and mineral content, this research will provide valuable information on the effectiveness of these advanced technologies in preserving bone quality and promoting successful periodontal regeneration.

Material and methods

The experimental analysis was conducted on 15 newly obtained sternal sections of porcine ribs (Fig. 1), which were divided into 2 halves with sagittal osteotomy. Porcine bone is frequently utilized as a biomechanics model due to its cancellous density and cortical thickness comparable to human bone. The outer section of these bone blocks is densely cortical, while the interior portion is mainly composed of medium-density (D2–D3) trabecular bone. Before the osteotomy procedures, the bone blocks were cut into the desired size of the measurement using a hard tissue microtome (Isomet®1000 Precision Saw) with a speed of 150 Rpm (Fig. 2).

After the initial preparation of the porcine bone, they are divided into three groups for osteotomy procedures. Each group consists of 5 samples, which will be split into two halves.

- ***Piezosurgery:*** The Piezosurgery system with insert tip B1 (WandH Piezomed). The cuts were made in a horizontal direction in the bone blocks (Fig. 3A).
- ***Hard tissue laser:*** Er:Cr: YSGG (Waterlase – iPlus) was the hard tissue laser system that was used. The laser parameters were as follows: Wavelength (λ): 2780nm; Power: 3W; Frequency: 30Hz; Air: 80%; Water: 70%; Handpiece: Turbo Mx7 with a spot diameter of 700 μm and the mode used was H mode (hard tis-

sue mode) and the Waterlase turbo handpiece functioned in a non – contact mode at an optimal distance between 3 to 5 mm from the bone block (Fig. 3B).

– ***Low-speed handpiece:*** The low-speed handpiece system was used with 702 carbide burs with an rpm of 35,000 with the appropriate coolant (Fig. 3C).

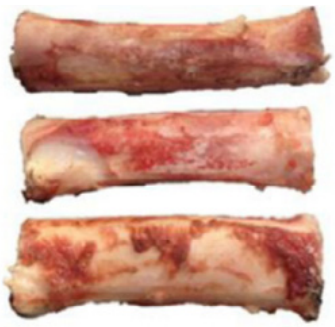

Fig. 1. Freshly obtained sternal sections of porcine ribs

Fig. 2. Isomet® Precision saw

Fig. 3. A: WandH piezomed with B1 tip, B: Waterlase – iPlus (Turbo Mx7 handpiece with a spot diameter of 700 µm), C: Low–Speed Handpiece with 702 carbide bur

For every sample, a cross-sectional surface was selected. Scanning electron microscopy (SEM) was used in this study to analyze the surface morphology of bone tissues after osteotomy. SEM is highly effective in providing high-resolution images that reveal microstructural details of bone, such as surface roughness, porosity, and the impact of different surgical instruments on bone integrity. The technique allows for detailed visualization of how osteotomy methods alter bone surfaces, thus contributing valuable insights into the effects of surgical interventions on bone morphology. SEM has been widely used in similar studies to examine the microstructure of bone and other mineralized tissues, allowing for a complete understanding of the material's properties of the material at the microscopic level.[16]

Energy SEM combined with energy-dispersive X-ray spectroscopy (EDS) is a powerful analytical technique used to examine the surface morphology and elemental composition of materials at high resolution. When applied to bone tissue, this method allows for a precise analysis of calcium and phosphate, the primary components of hydroxyapatite, which is the mineralized matrix of bone. By detecting the characteristic X-rays emitted from these elements during SEM imaging, EDS provides quantitative and qualitative data on their distribution, enabling a detailed study of bone mineralization and composition.[17] Calcium and phosphate analysis was performed. All statistical procedures were performed using the Statistical Package for Social Sciences (SPSS, IBM< Armonk, NY, USA) 20.0. Statistical analysis was performed using one-way ANOVA test. Ethical approval was acquired from the institutional ethical committee of Vinayaka Mission Sankarachariyar Dental College IRC/24062022/S/1.

Results

Figure 4 presents the SEM images.

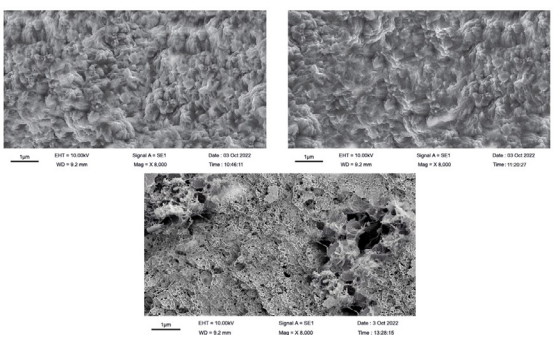

Fig. 4. SEM images of A: group I (piezoelectric), B: group II (hard tissue laser), C: group III (low-speed handpiece)

There are no morphological changes in group I (piezoelectric) and group II (hard tissue laser); group III (low-speed handpiece) shows significant changes in the bone morphology. Figure 5 shows SEM with EDS.

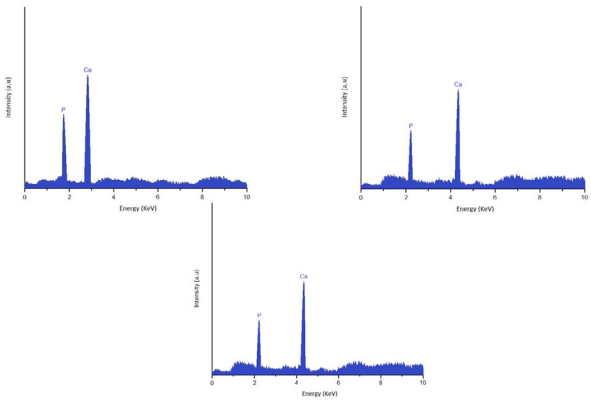

Fig. 5. EDS of A: group I, B: group II, C: group III

Table 1 denotes that the calcium particles were significantly decreased in group III (mean value=21.46) than in other groups, which signifies more calcium destruction. Both groups I and II do not have a significant reduction in calcium levels after osseous preparation.

Table 1. Comparison of calcium levels among group I(piezoelectric), group II (hard tissue laser), and group III (low-speed handpiece)

Group	n	Calcium analysis			ANOVA	p
		Mean	SD	SE		
Group I	10	24.94	0.542	0.171		
Group II	10	25.02	0.315	0.100	232.99	0.001
Group III	10	21.46	0.373	0.118		
Total	30	23.81	1.736	0.317		

Table 2 denotes that phosphate levels were significantly decreased in group III (mean value=12.51) compared to group I and II. Both groups I and II do not have a significant reduction in calcium levels after osseous preparation.

Table 2. Comparison of phosphate levels among group I (piezoelectric), group II (hard tissue laser) and group III (low-speed handpiece)

	n	Phosphate analysis			ANOVA	p
		Mean	SD	SE		
Group I	10	17.20	0.495	0.157		
Group II	10	17.27	0.624	0.197	223.61	0.001
Group III	10	12.51	0.602	0.190		
Total	30	15.66	2.331	0.426		

Discussion

This in vitro analysis assessed the ultrastructural and morphological properties of bone tissue after performing osteotomy with a piezoelectric device, hard tissue laser, and low-speed handpiece. Bone is composed of apatite crystals surrounded by an organic matrix. The EDX-SEM study exhibited that Er:Cr: YSGG ("Waterlase – iPlus") with parameters -Wavelength (λ): 2780nm; Power: 3W; frequency: 30Hz; Air: 80%; Water: 70%; handle: The 700 μm spot diameter of Turbo Mx7 is precise, with regular boundaries and sharp edges that are clearly defined for the surrounding tissue. It also shows no signs of thermal degradation. The same results were noted by Sasaki et al.[18] Furthermore, when exposing bone tissue to radiation using the Er:Cr:YSGG (Waterlase – iPlus) laser, they did not discover any regions of melting or carbonization. The microirregularities on the surface and the absence of a smear layer are the consequence of thermomechanical ablation, which is highly dependent on the energy used during radiation. Dental or bone tissues absorb nearly all the energy applied due to the 'high absorption coefficient of the Er: Cr: YSGG wavelength (2940 nm) in water along with hydroxyl ions of hydroxyapatite.[19,20] This results in an instantaneous increase in local temperature.

Water is vaporized by heat, and tissue ablation and microexplosions are produced by internal positive pressure.

Safer osteotomies and several other treatments, including ridge splitting, bone harvesting, and orthognathic and neurological surgeries, can now be carried out with the help of piezosurgery. It cuts bone selectively and does not harm soft tissue.[21] The piezosurgery device is made up of a unique piezoelectric ultrasonic transducer that can drive a variety of resonant cutting inserts. It is powered by an ultrasonic generator. With control over surgical operations in all anatomical scenarios, the piezoelectric drill's cutting action is the consequence of linear microvibrations of an ultrasonic nature, with a range between 60 μm to 200 μm in a longitudinal direction. Panduric et al. conducted a study to analyze morphological, chemical, and crystallographic changes in bone tissue after osteotomy performed with an erbium:yttrium-aluminum-garnet (Er:YAG) laser and a low-speed pilot drill. They concluded that the Er:YAG laser ablation did not cause any chemical or crystallographic changes of the bone tissue. Compared to the drill, Er:YAG laser created well-defined edges of the preparations, and the cortical bone had no smear layer.[22]

Many studies have been performed that compare the results of osteotomy performed using conventional drill systems and piezoelectric systems. Esteves et al. studied the variations between osteotomies performed with piezosurgery and a traditional drill in terms of "histomorphometrical, immunohistochemical and molecular analysis" and found that aside from a slightly greater amount of recently formed bone found 30 days after the use of the piezosurgery device, there were no differences between the bone healing from a histological and histomorphometric perspective.[23] However, in different research, Esteves et al. compared the effectiveness of a piezoelectric device with a frequently used diamond bur (DB) or carbide bur (CB), showing the healing rate of a postoperative wound in a dog model after osteoplasty

and osteotomy.[24] The surgical sites treated with CB or DB showed a loss of bone relative to baseline measures, while the surgical sites treated by PS showed a degree of bone growth by the 14th day. Ercoli et al. examined the wear, heat production, durability, and cutting efficiency of implant drills in environments that simulate implant placement.[25] They concluded that cutting durability and efficiency are highly influenced by the design, material, and mechanical characteristics of drills. Implant drills can be used repeatedly without raising bone temperature to potentially dangerous levels. Deep osteotomies can develop localized temperatures that could be detrimental to the bone if the drilling is carried out continuously. Rashad et al. conducted research to assess metal attrition residues inside irrigation fluid and the structural integrity of the bone.[26] Newer sonic and ultrasonic techniques challenge traditional bur reduction. They concluded that an improvement in bone architecture was observed in sono and piezo surgery, while metal attrition was inconclusive. Piezo and sono surgery turned out to be less invasive, whereas attrition features remained the same.

In this study, it was shown that there was no significant reduction in bone calcium and phosphate levels of bone after osteotomy with piezosurgery. It is not feasible to avoid heat injury to surrounding bone tissue, even if mechanically rotating tools are equipped with an internal cooling mechanism during the process. In this study, it has been shown that the amount of calcium and phosphate is reduced in the samples in which osteotomy has been performed using a low-speed handpiece.

Conclusion

The bone did not undergo any chemical or crystallographic alterations as a result of the "Er:Cr: YSGG" laser after osteotomy. In contrast to the drill, Er:Cr: The YSGG laser produced precisely defined preparation boundaries, and the cortical bone lacked a smear layer. A relatively new surgical method called piezosurgery could be used in many different surgical procedures to supplement traditional oral surgical techniques and, in certain situations, to replace traditional procedures. It does not lead to a loss of calcium and phosphorous compared to conventional drill systems. Future research should focus on long-term healing, integration, and advanced imaging to further validate these findings. These techniques offer promising alternatives for safer and more precise bone surgery.

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Declarations

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The authors have clearly stated that they have any commercial interest and financial interest. The researchers easily covered by the researchers.

Author contributions

Conceptualization, V.K. and J.D.; Methodology, V.K., J.D. and S.N.; Writing – Original Draft Preparation, V.K., J.D. and P.K.; Writing – Review & Editing, V.K., S.G., and K.R.; Supervision, J.D., S.N. and P.K.; Project Administration, J.D. and V.K.

Conflicts of interest

All authors clearly stated that they have any conflicts of interest.

Data availability

Usually, data sets are created during and/or analyzed throughout the entire study and are available from the corresponding author on reasonable request.

Ethics approval

Ethical approval was acquired from the institutional ethical committee of Vinayaka Mission Sankarachariyar Dental College IRC/24062022/S/1.

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ORIGINAL PAPER

Fall risk and avoidance behavior due to fear of falling in elderly nursing home residents

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ABSTRACT

Introduction and aim. Falls in the elderly affect their daily activities, causing a decrease in their quality of life and may even lead to death. This study aims to examine the risk of falling and the relationship between fear of falling and avoidance behaviors in elderly nursing home residents.

Material and methods. Data were obtained using the "Fall Risk Assessment Scale (FRAS)" and the "Fear of Falling Avoidance Behavior Questionnaire (FFABQ)".

Results. The average age of the participants was 70.70±5.23 years. Total mean scores of FRAS and FFABQ were significantly higher in participants who could partially meet their daily needs on their own, had chronic diseases, used continuous medication, had problems with walking or balance, had vision or hearing problems, used walking aids, had fear of falling, and had experienced falls in the last three months. It was found that their average was significantly higher. It was determined that there was a strong and significant positive relationship between the FRAS and FFABQ total score averages.

Conclusion. It was determined that elderly residents of nursing homes have a high risk of falling and that increased risk is associated with an increase in avoidance behaviors due to fear of falling.

Keywords. elderly, fall risk, fear of falling

Introduction

The increase in the elderly population worldwide and the accompanying increasing health problems have led scientists to pay increasing attention to aging and the elderly population.[1,2] The World Health Organization reports that the number of people aged 60 and over will increase day by day and reach approximately 2.1 billion in 2050.[3]

With aging, a decrease in physical and cognitive functions and a decline in muscle strength, coordination, and balance may occur. This situation makes individuals prone to falls.[4] Around 30% of people over 65 experience falls annually, and approximately half of these cases repeat.[5]

Falls in the elderly cause problems such as fractures, permanent disabilities, head injuries, disability, decreased mobility, chronic pain, and loss of independence. It is a public health problem that requires long-term and expensive treatments and early hospitalization.[2,6,7] The severity of injuries resulting from falls varies significantly, from minor skin injuries to major fractures and, in some cases, fatal trauma.[2] In addition to physical health problems, although falls do not result in serious injuries, they cause elderly people to experience fear of falling.[6]

Fear of falling causes elderly people to feel unsafe performing daily life activities and leads to an inactive

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lifestyle.[8] In addition, unnoticed fear of falling significantly affects the individual's life.[6] Fear of falling causes negative effects in the elderly, such as fear, anxiety, avoidance of activities, not leaving the house, social isolation, depression, and a decrease in quality of life.[6] A study found that 84.4% of community dwelling elderly individuals have a fear of falling and 31.9% have a high risk of falling.[9] In another study, it was observed that 71.8% of the elderly living in nursing homes experienced fear of falling, and as the fear of falling increased, individuals' independence levels in daily life activities decreased.[10]

It is seen that the assessment of fall risk is strongly recommended in the clinical practice guideline, which includes a total of 79 randomized controlled trials on fall prevention.[11] Considering the negative health consequences experienced by elderly people due to falling and fear of falling, it is very important to examine the risk of falling and fear of falling in these individuals. When the literature was examined, to our knowledge, no study was found that examined the risk of falling and avoidance behaviors due to fear of falling in elderly people living in nursing homes.

Research questions:

Elderly staying in nursing home;

- What are the fall risk levels and affecting factors?
- What are the avoidance behaviors due to fear of falling and the influencing factors?
- Is there a relationship between the risk of falling and avoidance behaviors due to fear of falling?

Aim

This study aimed to examine the risk of falling and avoidance behaviors due to fear of falling of elderly people living in nursing home.

Material and methods

Study population and procedure

This study is a cross-sectional descriptive study. The study was conducted as a single center study. The research was carried out in the nursing home with the largest number of residents in Türkiye, located in Istanbul, between October and November 2023. The appropriate permissions were obtained from the institution where the study was conducted. Ethic Committee approval was obtained from the University Institutional Review Board (IRB date and number: 12.10.2023/2023.122). The study conforms to the ethical principles outlined in the Declaration of Helsinki. Both verbal and written informed consent approval was obtained from participants who met the study criteria. Participants were assured that their responses would remain anonymous and confidential. Surveys were conducted face-to-face with individuals and took an average of 10 to 15 minutes.

Participant selection

The population consisted of individuals 65 and over living in a nursing home during the study period. The study was completed with 220 volunteer participants who met the inclusion criteria of the study (65 years and older, no communication problems, no cognitive impairment such as dementia, Alzheimer's, etc.) and agreed to participate in the study. Individuals with a diagnosed psychiatric disease (schizophrenia, depression, etc.), bedridden, cognitive impairment, and individuals who did not agree to participate in the study were excluded from the study. In the posthoc power analysis performed using the G-Power 3.1.9.4 program to determine that the sample size was sufficient, it was determined that the effect size of the study was 0.67 and the power was 1.00 at a 95% confidence interval, 0.05 significance level.

Measures

Data gathering form

The form includes 15 questions describing the sociodemographic and fall-related characteristics (age, sex, marital status, education level, income level, chronic diseases, continuous use of medications, history of falls, and the presence of situations that can cause a fall).

Fall Risk Assessment Scale (FRAS)

The validity and reliability of the Turkish form of the Fall Risk Assessment Scale (FRAS), prepared by the Delmarva Foundation and adapted by CIMRO of Nebraska in collaboration with the Centers for Medicare & Medicaid Services (CMS), were evaluated by Tekin et al. It was carried out in 2013.[12] The scale evaluates the fall risk of patients with nine different parameters (subdimensions), and when the scores obtained as a result of the evaluation are added, the patient's fall risk score is obtained. Total score received by the patient: a score between 0 and 5 indicates a low risk of falling; a score between 6 and 9 indicates a medium risk; and a score of 10 and above indicates a high risk of falling.

Fear of Falling Avoidance Behavior Questionnaire (FFABQ)

Fear of falling avoidance behavior questionnaire (FFABQ) was developed by Landers et al.[13] (2011) to evaluate the avoidance behavior of elderly individuals living in the community. The validity and reliability of the scale in Turkish were determined by Açaröz-Candan et al.[14] (2020). The scale is calculated with a five-point Likert-type scoring system and consists of 14 items and two subscales ("challenging balance demands in daily life" and "instrumental activities of daily life and socialization"). The minimum score on the scale is 0, and the maximum score is 56. A high score indicates limitation of activity and participation restriction due to fear of falling.

Statistical analysis

Continuous variables were expressed as mean±SD, and categorical variables were expressed as percentages. The suitability of the data for a normal distribution was checked with the Kolmogorov-Smirnov test. Data were evaluated with chi-square, student t test, one-way variance (ANOVA), and Pearson correlation analyzes. For all tests, two-sided *P* values <0.05 were considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Table 1. Sociodemographic and descriptive characteristics of participants (n = 201)*

		Mean±SD	Min.–max. (Median)
Age (year)		70.7±5.23	65–91
Number of falls		0.25±0.5	0–3
		n	%
Gender	Female	85	38.6
	Male	135	61.4
Marital status	Single	100	100
Education status	Illiterate	12	5.5
	Literate	28	12.7
	Primary education	117	53.2
	High school	54	24.5
	University	9	4.1
Regular exercise	Yes	117	53.2
	No	103	46.8
Meeting daily needs on one's own	Yes	108	49.1
	Partially	102	50.9
Has a chronic illness	Yes	160	72.7
	No	60	27.3
Chronic illness[a]	Hypertension	122	55.5
	Diabetes Mellitus	64	29.1
	COPD	30	13.6
	CAD	9	4.1
Constantly use medication	Yes	170	77.3
	No	50	27.2
Have walking or balance problems	Yes	137	62.3
	No	83	37.7
Have vision or hearing problems	Yes	135	61.4
	No	85	38.6
Use assistive devices for walking	Yes	94	42.7
	No	126	57.3
Fear of falling	Yes	144	65.5
	No	76	34.5
Does fear of falling affect daily living?	Yes	122	84.7
	No	22	15.3
Fall in the last three months	Yes	49	22.3
	No	171	77.7

* [a] – more than one option has been selected, CAD – coronary artery disease, COPD – chronic obstructive pulmonary disease

Results

The average age of the participants was 70.70 years, and the majority of the participants were men (61.4%), single (100%), and high school graduates (53.2%). Furthermore, 53.2% of the participants exercise regularly, 50.9% can partially meet their daily needs, 72.7% have a chronic disease, 77.3% use medication, 62.3% had walking or balance problems, 61.4% had vision or hearing problems, 65.5% had a fear of falling, 84.7% had an existing fear of falling that affected their daily activities, and 77.7% had a fear of falling. It was determined that none of them had a fall in the last three months (Table 1).

When the of the participants on the FRAS scale responses were examined, it was determined that the lowest mean score was taken from the "level of consciousness/mental state" parameter, while the highest mean score was taken from the "gait and balance" parameter. When the FRAS risk classification was evaluated, it was determined that the majority of participants (39.5%) were in the high-risk group (Table 2).

Table 2. FRAS and FFABQ scores of participants

	Mean±SD	Min.-Max. (Median)
FRAS		
Consciousness level	0±0	0–0
History of falling (last three months)	0.45±0.86	0–4
Ambulation / toileting	0.03±0.3	0–4
Vision	0.94±1	0–2
Walking and balance	2.27±2.11	0–6
Orthostatic hypotension	0.27±0.69	0–2
Medication	2.09±1.54	0–5
Illness	0.98±1.11	0–4
Use of assistive device	0.48±0.53	0–2
Total score	7.51±4.78	0–18
FFABQ		
Walking	1.77±1.66	0–4
Lifting and carrying objects (eg, cup, child)	1.84±1.69	0–4
Going up and downstairs	2.18±1.66	0–4
Walking on different surfaces (eg, grass, uneven ground)	1.93±1.71	0–4
Walking in crowded places	1.86±1.72	0–4
Walking in dimly lit, unfamiliar places	1.97±1.67	0–4
Leaving home	1.52±1.68	0–4
Getting in and out of a chair	1.68±1.69	0–4
Showering or bathing	2.18±1.66	0–4
Exercise	1.95±1.76	0–4
Preparing meals (eg, planning, cooking, serving)	1.72±1.69	0–4
Doing housework (eg, cleaning, washing clothes)	1.69±1.66	0–4
Work or volunteer work	1.64±1.68	0–4
Recreational and leisure activities (eg, play, sports, arts and culture, crafts, hobbies, socializing, traveling)	1.74±1.71	0–4
	n	%
FRAS degree of risk		
Low risk	80	36.4
Medium risk	53	24.1
High risk	87	39.5
FFABQ		
Challenging balance demands in daily life	16.70±14.28	0–36
Instrumental activities of daily living and socialization	8.96±7.98	0–20
Total score	25.66±22.03	0 - 56

When the participants' responses on the FFABQ scale were examined, it was determined that the lowest mean score was taken from the parameter "leaving

Table 3. Comparison of the sociodemographic and clinical characteristics with the FRAS and FFABQ scores*

	FRAS						FFABQ					
	Low risk n (%)	Medium risk n (%)	High risk n (%)	Z; p	Total	t/F; p	Challenging balance demands in daily life	t/F; p	Instrumental activities of daily living and socialization	t/F; p	Total	t/F; p
Gender												
Female	33 (41.2)	19 (35.8)	33 (37.9)	0.42;	7.50±4.90	-.019;	17.62±13.56	0.764;	9.40±7.21	0.667;	27.02±20.43	0.748;
Male	47 (58.8)	34 (64.2)	54 (62.1)	0.81	7.52±4.71	0.98	16.11±14.74	0.44	8.69±8.43	0.52	24.80±23.02	0.46
Education status												
< High school	54 (67.5)	37 (69.8)	66 (75.9)	0.08;	7.73±4.73	1.073;	17.44±14.00	1.221;	9.41±7.81	1.326;	26.85±21.56	1.271;
≥ High school	26 (32.5)	16 (30.2)	21 (24.1)	0.47	6.97±4.88	0.70	14.84±14.92	0.22	7.84±8.31	0.18	22.68±23.07	0.20
Regular exercise												
Yes	45 (56.2)	20 (62.3)	39 (44.8)	4.49;	7.09±4.63	-1.392;	15.36±14.80	-1.483;	7.95±8.25	-2.040;	23.31±22.83	-1.694;
No	35 (43.8)	33 (37.7)	48 (55.2)	0.10	7.99±4.91	0.16	18.21±13.59	0.13	10.12±7.51	0.04	28.33±20.88	0.09
Meeting daily needs on one's own												
Yes	62 (77.5)	25 (47.2)	21 (24.1)	47.58;	5.13±4.34	-8.326;	8.37±10.93	-10.340;	4.39±6.08	-10.111;	12.76±16.62	-10.410;
Partially	18 (22.5)	28 (52.8)	66 (75.9)	<0.001	9.81±4.00	<0.001	24.72±12.45	<0.001	13.37±7.04	<0.001	38.10±19.32	<0.001
Chronic illness												
Yes	39 (48.8)	38 (71.7)	83 (95.4)	47.76;	8.94±4.36	8.252;	18.18±14.09	2.551;	9.84±7.79	2.714;	20.02±21.67	2.636;
No	41 (51.2)	15 (28.3)	4 (4.6)	<0.001	3.72±3.65	<0.001	12.73±14.14	0.01	6.62±8.01	0.007	19.35±21.92	0.009
Constantly use medication												
Yes	41 (51.2)	43 (81.1)	86 (98.9)	54.36;	8.90±4.27	11.135;	18.63±14.00	3.828;	10.10±7.76	4.034;	28.73±21.55	3.943;
No	39 (48.8)	10 (18.9)	1 (1.1)	<0.001	2.80±3.10	<0.001	10.10±13.35	<0.001	5.10±7.48	<0.001	15.20±20.58	<0.001
Have walking or balance problems												
Yes	17 (21.2)	37 (69.8)	83 (95.4)	99.23;	10.09±3.59	14.227;	24.47±11.43	15.912;	13.15±6.66	13.538;	37.62±17.76	14.434;
No	63 (78.8)	16 (30.2)	4 (4.6)	<0.001	3.26±3.20	<0.001	3.85±7.76	<0.001	2.06±4.31	<0.001	5.91±11.83	<0.001
Have vision or hearing problems												
Yes	24 (30.0)	34 (64.2)	77 (88.5)	60.39;	9.55±4.13	9.472;	20.34±14.06	5.145;	10.96±7.90	5.065;	31.30±21.70	5.172;
No	56 (70.0)	19 (35.8)	10 (11.5)	<0.001	4.27±3.85	<0.001	10.90±12.70	<0.001	5.79±7.03	<0.001	16.69±19.54	<0.001
Use assistive devices for walking												
Yes	16 (20.0)	19 (35.8)	59 (67.8)	40.29;	10.16±4.02	8.157;	22.48±12.23	5.648;	12.53±6.78	6.333;	35.01±18.72	5.963;
No	64 (80.0)	34 (64.2)	28 (32.2)	<0.001	5.54±4.33	<0.001	12.38±14.22	<0.001	6.30±7.77	<0.001	18.68±21.79	<0.001
Fear of falling												
Yes	26 (32.5)	39 (73.6)	79 (90.8)	64.70;	9.43±4.02	9.816;	24.87±10.65	25.150;	13.38±6.29	23.538;	38.26±16.53	25.237;
No	54 (67.5)	14 (26.4)	8 (9.2)	<0.001	3.88±3.92	<0.001	1.20±2.73	<0.001	0.59±1.27	<0.001	1.79±3.80	<0.001
Fall in the last three months												
Yes	3 (3.8)	12 (22.6)	34 (39.1)	30.05;	10.69±3.40	6.750;	22.47±10.03	4.058;	12.28±6.31	3.900;	34.75±15.87	4.086;
No	77 (96.2)	41 (77.4)	53 (60.9)	<0.001	6.60±4.73	<0.001	15.04±14.90	<0.001	8.01±8.15	<0.001	23.05±22.88	<0.001

* Z – chi square, T – students t test, F – ANOVA

the house," while the highest mean score was taken from the parameters "going up and down stairs" and shower and/or bath." When looking at the participants' average FFABQ subscale scores, it is seen that he received 16.70±14.28 points from the "challenging balance demands in daily life" subdimension and 8.96±7.98 points from the "instrumental activities of daily life and socialization" sub-dimension. The total FFABQ score was determined to be 25.66±22.03 (Table 2).

Both the FRAS and the FFABQ total scores of participants who can partially meet their daily needs on their own, have a chronic disease, constantly use medication, have walking or balance problems, have vision or hearing problems, use an assistive device for walking, have fear of falling, and have experienced a fall in the last three months. Their average scores were significantly higher compared to the average scores of other participants ($p<0.05$) (Table 3).

A significant positive and strong relationship was found between the FFABQ subscales "challenging balance demands in daily life" and "instrumental activities of daily living and socialization" and the FRAS total score. Furthermore, a significant positive and strong relationship was detected between FFABQ and FRAS total scores (Table 4).

Table 4. Correlation analysis between DENN and FFABQ scores*

			FRAS Scale
FFABQ Scale	Challenging balance demands in daily life	r	0.674
		p	<0.001
	Instrumental activities of daily living and socialization	r	0.648
		p	<0.001
	Total score	r	0.671
		p	<0.001

* r – correlation coefficient, using Pearson's correlation analyses

Discussion

Falls are the most common type of accident among the elderly, and most of them are preventable. Additionally, older adults have several risk factors that may lead to

avoidance behaviors resulting from fear of falling.[15] For this reason, it is very important to evaluate the risk of falling, fear of falling, and related avoidance behaviors of people. In this study, it was determined that elderly residents of nursing homes had a high risk of falling and that increased risk was associated with increased avoidance behaviors due to fear of falling.

Gender is stated among fall risk factors for the elderly.[16] When the literature is examined, different results are found regarding the relationship between gender and fall risk. In the Shao et al. meta-analysis study, which included 18 prospectively designed studies, it is emphasized that the gender is associated with falls.[17] In the Smith et al. study (n = 240), female gender of women was mentioned among the factors that increase the risk of falling.[18] In different studies conducted in Turkey, the presence of women has been reported as one of the factors that increase the risk of falling.[10,19-20] Gürler et al. state in their study that there is no difference between the genders in terms of fall risk.[21] In this study, it was found that there was no difference between the genders in terms of fall risk. It is thought that the variability in the results may be due to the number of genders in the population.

In the study, the average FFABQ score was found to be 25.66±22.03. According to this result, it can be said that the participants' fear of falling avoidance behavior was at a moderate level. While Öztürk and Özer found in their study that the fear of falling avoidance behavior of the elderly was low,[22] similar studies also stated that the fear of falling avoidance behavior levels of the elderly were low.[23-25] These results made us think that, in relation to the fact that the study took place in a nursing home, this emerged as a result of the fact that nursing homes impose different restrictions on the elderly than the environment they are used to and the sedentary lifestyle in the nursing home. The fact that the most frequently stated avoidance behaviors by the participants were "going up and downstairs" and "showering or bathing" activities confirms this idea.

The most important preventable risk factors in the elderly are psychiatric drug use, polypharmacy, environmental hazards, decreased vision, lower extremity strength, impairment of balance, and daily living activities.[26] Fall history, chronic diseases, gait and balance disorders, visual impairment, and cognitive disorders are some of the risk factors for falls.[16] In Shao et al.'s meta-analysis study, history of falls, impaired daily living activity performance, use of assistive devices, polypharmacy, unbalanced gait, and hearing problems were found to be associated with falls.[17] Lee et al. in a study, age-related decreases in vision and the resulting fear of falling and avoidance behaviors were found to be associated with an increase in the potential risk of falling.[23] In parallel with the literature, this study also included patients who constantly use medication, have walking or balance problems, have vision or hearing problems, and have walking aids. The fall risk of participants who were driving was found to be significantly higher.

Fear of falling can be considered protective to some extent in individuals with a high risk of falling.[27] Studies have stated that the fear of falling increases with age, that women experience more fear of falling than men, and that fear of falling is greater in elderly people who use assistive devices.[10,28] In the study by Chen et al. (n=5599), falls experienced by older adults in the previous month or the previous year were significantly associated with fear of falling.[29] In addition to physical consequences, falls also have psychological and social consequences. Fear of falling, the most common psychological consequence, leads to a reduction in physical and social activities.[30] When avoiding daily activities due to fear of falling is excessive, this can lead to deconditioning and a decrease in physical functions in individuals.[31] In this study, it is seen that the increase in the risk of falling increases in direct proportion to the increase in avoidance behaviors. Therefore, it is quite likely that elderly people who have avoidance behaviors due to fear of falling will also experience limitations in their daily activities.

Study limitation

It should be taken into consideration that the data obtained in the study based on the statements of the participants may be subjective and open to reporting errors. Furthermore, the generalizability of the results is limited by the characteristics of the study sample. In addition, the study was conducted in a single center and the study sample was relatively small, which constitutes the limitations of the study.

Conclusion

In this study, it was determined that elderly people living in a nursing home had a high risk of falling and that the increase in risk was associated with an increase in avoidance behaviors due to fear of falling. Total score on both FRAS and FFABQ for participants who can partially meet their daily needs on their own, have a chronic disease, constantly use medications, have walking or balance problems, have vision or hearing problems, use assistive devices for walking, have fear of falling, and have experienced falls in the last three months.

There are multiple risk factors in the elderly, which can lead to avoidance behaviors related to fear of falling. In addition to creating programs to prevent falls, it is recommended to identify avoidance behaviors related to the fear of falling in performing daily life activities and plan appropriate interventions. Rehabilitation and technological rehabilitation can help prevent falls. Fur-

thermore, it should not be forgotten that avoidance behaviors related to fear of falling may lead to restrictions on movement and activities for a long time and thus have harmful effects on the musculoskeletal system.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Data availability
The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval
Istanbul Kültür University Institutional Review Board approved this study (IRB date and number: 12.10.2023/2023.122).

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ORIGINAL PAPER

Predictors of family burden in parents of children with intellectual disabilities and their children's sexual development characteristics

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ABSTRACT

Introduction and aim. Although the existing literature includes both quantitative and qualitative studies that examine the sexual characteristics of children with intellectual disabilities and the challenges they face, these studies have not addressed the impact of these characteristics on the burden of the family. Our objective was to examine the predictors of sexual development of family burden among parents of children with intellectual disability in this cross-sectional study.

Material and methods. We collected data from 815 parents with children aged 10–18 years with intellectual disabilities between May 2021 and March 2022. Data collection tools were 'Descriptive Characteristics Form", "Family Burden Rating Scale for families with Mentally Handicapped Children" and 'Sexual Development Characteristics scale of Adolescents with Intellectual Disability". A logistic regression analysis was performed.

Results. The predictive model for the burden of the family explained 60% of the variance in this sample of parents ($p < 0.001$). The sexual predictors with the strongest effect on the model were previous sexual development education ($\beta=0.74$; $SE=0.16$, $p \leq 0.001$), followed by sexual harassment ($\beta=-0.56$, $SE=0.26$, $p \leq 0.001$).

Conclusion. Education on sexual development in children with intellectual disability can facilitate management of challenges in adolescence.

Keywords. child, family burden, intellectual disability, logistic models, sexual development.

Introduction

Having a child with an intellectual disability influences family life, causes insufficiency and care burden to be at the center of a family's life, changes family roles, duties, private living spaces, social situations, desires, and plans. Some components such as family economy, parental instruction, calling, conjugal alteration, culture, diminished social skills, communication challenges, illness, child age, weakened family cycle, needs for therapeutic assistance, and monetary healthcare burden influence guardians of children with intellectual disability.[1] Challenges and uneasiness in families of children with intellectual disabilities cause adaptation issues and expand family burden. Families with children having intellectual disability by and large have lower vitality due to the increased reliance of their children on them.[2] They may have social confinement and depression, decreasing their quality of life.[3]

Children with intellectual disabilities are more likely than their peers to experience emotional and behavioral problems.[4] These problems can include anxiety disorders, oppositional defiant disorders, attention deficit/hyperactivity disorders, and peer troubles.[5] Among children with intellectual disabilities, aggressiveness,

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impatience, kicking, and socially inappropriate actions are troublesome.[6] Families of these children can view and treat their impaired children negatively as a result of the behavioral issues, high care demands, and stigma associated with them.[7] Particularly, children with intellectual disabilities exhibit some behaviors brought on by adolescence, increasing family burden and making it more difficult to cope with challenges.[8]

Impaired sexual development in adolescents is linked to behavioral issues.[9] For people with intellectual disabilities, regardless of age, sexuality and sexual development are challenging concerns.[10] Due to hormonal changes that accompany sexual development, children with intellectual disabilities may require assistance with cleanliness and behavior control.[11] Children who have intellectual disabilities may view their actions as typical and/or may be unable to comprehend and analyze the methods used by others. Children with intellectual disabilities are unable with communicate to their classmates, family, and friends regarding sexual development or why they find it difficult to control their sexual activity.[12]

During puberty, changes happen within the hormonal structure and bodies of children. These changes are related, for the most part, to sexuality and sexual improvement. Both sexuality and sexual desires of children with intellectual disabilities vary essentially in agreement with their insights remainder level.[13] Children with gentle intellectual disability have psychosocial and sexual behaviors comparable to their peers. They investigate and control sexual inclinations and driving forces and react to a verbal shape of sexual instruction.[14] The advancement of auxiliary sex characteristics may be deferred in children with direct intellectual disability. As they work generally by compensating and fortifying, operant conditioning and social learning-based behavior adjustment techniques may be required for sexual instruction to be successful in children with direct intellectual disability. Parents may be required to alter behaviors in children with extreme intellectual disability.[15] Families of children with intellectual disability attempt to clarify these circumstances to their children and to oversee them viably.[16] They may have trouble with respect to their children's social environment due to a few reasons such as expanded intrigued within the inverse sex, need of division between genders and outsiders, and running absent from domestic.[17,18]

Parents of children with intellectual disabilities may disregard their children's sexual improvement.[16] Besides parents who disregard the sexual advancement of their children, there may also be parents who make this issue a cause for concern.[17,18] The existing literature includes quantitative and qualitative studies on the sexual characteristics and problems experienced by children with intellectual disabilities.[17,18] However, these studies do not examine the effect of these characteristics and problems on families, particularly in terms of family burden. Given that children are more likely to engage in these sexual behav iors during adolescence, it is crucial to investigate the impact on family burden.

Aim

This current study aimed to examine the predictors of sexual development of family burden among parents of children with intellectual disabilities.

Research hypotheses:

H_0: The presence of sexual characteristics does not predict the family burden experienced by their parents.

H_1: The presence of sexual characteristics predicts the family burden experienced by their parents.

Material and methods

Study design and participants

This is a descriptive cross-sectional study. The study population constituted a total of 1131 parents of different socioeconomic backgrounds who were enrolled in six special education and rehabilitation centers affiliated with the National Directorate of Education. A convenience sample was used in the study.[19] The parents were informed about the purpose of the study. The study inclusion criteria were as follows: being the mother of a child with intellectual disability, having a child with intellectual disability aged between 10–18 years, agreeing to participate in the study. The study sample consisted of 815 parents (participation rate=72%) who were enrolled at these centers and agreed to participate in the study.

The mean age of the children with intellectual disability was 15.06±1.88 years, 56.07% of them were girls, 43.93% were boys, 24.29% had low intellectual disability, 45.16% moderate intellectual disability, 30.55% severe intellectual disability, and 46.63% had an additional disability. Of their mothers, 52.51% were primary school graduates and their mean age was 42.29±1.96 years. Of their fathers, 40.74% were primary school graduates and their mean age was 47.45±2.43 years. Of their families, 51.04% had an income lower than expenses, 65.64% had nuclear families, and 34.36% had large families. Furthermore, 81.97% of children with intellectual disabilities had not receive sexual health and development education prior to (Table 1).

Data collection

Data were collected between October 2021 and July 2022. The researcher went to six special education and rehabilitation centers and invited parents of children with intellectual disability to participate in the study. This time was used to ensure that the data collection tools were filled out as authentically and correctly as possible; The purpose of the study was explained to the participants in detail. It took the parents about 30 min to complete the questionnaires. Data collection tools were

"Descriptive Characteristics Form", "Family Burden Assessment Scale for Families with Mentally Handicapped Children" and "Sexual Development Characteristics Scale of Adolescents with Intellectual Disability".

Table 1. Participant characteristics (n=815)

	Mean±SD	Low-high score
Age of child	15.06±1.88	10–18
Age of mother	42.29±1.96	34–49
Age of father	47.45±2.43	35–52
	n	**%**
Gender		
Girl	457	56.07
Boy	358	43.93
Intellectual disability level		
Mild	198	24.29
Moderate	368	45.16
Severe	249	30.55
Having another disability		
Yes	380	46.63
No	435	53.37
Education level of mother		
Primay school	428	52.51
High school	257	31.54
University	101	12.39
Postgraduate	29	3.56
Education level of father		
Primay school	332	40.74
High school	294	36.07
University	147	18.04
Postgraduate	42	5.15
Family income		
The income is less than the expenditures	416	51.04
Income is equal to the expenditures	261	32.02
The income is more than the expenditures	138	16.94
Family type		
Nuclear	535	65.64
Large	280	34.36
Having previous sexual education		
Yes	147	18.03
No	668	81.97

Sociodemographic Characteristics Form

The researcher prepared a form, including questions about the age of the parents, the level of education, the income of the family, the type of family and the child's age, gender, intellectual disability, the additional disability, and status of having sexual health education. The intelligence quotient of the children was obtained from the data they reported to the institution they received education after being evaluated by the clinicians.

Scale of Sexual Development Characteristics of Adolescents with Intellectual Disability

The Sexual Development Characteristics Scale of Adolescents with Intellectual Disability (SDCS) was developed by Gürbüz ve Eratay to determine the level of the understanding of sexual development characteristics of their children by parents of adolescents with intellectual disability during adolescence.[20] This is a five-point Likert-type scale. It consists of nine subscales. The Cronbach's alpha value of the scale was 0.85. It has no cutoff score. A median score on the total scale and subscales indicates the central level of understanding sexual development, whereas a scale score below the median value indicates a higher level of understanding sexual development. Therefore, parents with high scale scores do not understand the sexual development of their children.[20] In this study, the Cronbach alpha values was determined as 0.84 for the total scale, 0.865 for the subscale of sexual arousal, 0.84 for the subscale of information needs, 0.79 for the subscale of privacy and social trust, 0.77 for the subscale of information about bodily development, 0.87 for the subscale of sexual harassment, 0.74 for the subscale of urge for sexual satisfaction, 0.82 for the subscale of sharing sexual issues, 0.78 for the subscale of emotional change, and 0.73 for the subscale of sexual self-care.

Family Burden Assessment Scale

The Family Burden Assessment Scale for Families of Children with Intellectual Disability (FBAS), developed by Sarı and Basbakkal, was used to evaluate the burden of parents of children with intellectual disability.[21] The scale consists of 43 items and six subscales. This is a five-point Likert type scale with a cutoff score 97. A score of 97 and above indicates a high family burden, vice versa.[21] The Cronbach alpha coefficient of the scale was 0.92 and the test-retest correlation value was 0.93. In this study, the Cronbach alpha coefficient was found to be 0.88 for the total scale, 0.85 for the economic burden, 0.83 for the subscale of perceived inadequacy subscale, 0.86 for the subscale of physical burden subscale, 0.83 for the subscale of social burden subscale, 0.83 for the emotional burden, 0.75 for the subscale of need subscale.

Data analysis

Data were analyzed using the SPSS (Statistical Package for Social Sciences, IBM, Armonk, NY, USA) for Windows 25.0 program and evaluated using descriptive statistics (number, percentage, minimum and max values, median, mean, standard deviation). The suitability of the data for the normal distribution was checked using the skewness and kurtosis values, which should range from ±-1.5 to ±1.5.[22] To examine the associations between the SDCS and FBAS, a regression correlation was created using all variables. Parental family burden was determined according to the cutoff score of the Family Burden Assessment Scale. Those who scored 97 points or more on the Family Burden Assessment Scale were interpreted as experiencing a high family burden, while those who scored less than 97 points were interpreted as experiencing a low family burden. Two categorical vari-

ables (high family burden and low family burden) and predictors of family burden were subjected to analysis.

A logistic regression analysis was performed to determine the effect of sexual development characteristics (sexual arousal, information needs, privacy and social trust, information on bodily development, sexual harassment, desire for sexual satisfaction, sharing sexual issues, emotional change, and sexual self-care) of children with intellectual disabilities on family burden.

Ethical aspects

The study was approved by the Ethics Committee for Social and Human Sciences of the university in question (Protocol No. 2021-SBB-0241, dated 26 May 26, 2021). Furthermore, the research was approved by the provincial education directorate (No. 30644743, dated 02/09/2021). All study participants were required to sign an informed consent form, which was approved by the Institutional Review Board.

Table 2. Distribution of the Sexual Development Characteristics Scale of Adolescents with Intellectual Disability and the Mean Scores of the Family Burden Assessment Scale of the participants (n=815)

	Mean±SD	Min–max
The Scale of Sexual Development Characteristics of Adolescents with Intellectual Disability	127.84±9.41	98–157
Sexual arousal	23.18±4.96	10–30
Information needs	23.43±4.80	10–30
Privacy and social trust	12.41±1.67	9–15
Information about bodily development	12.44±1.65	8–15
Sexual harassment	10.28±3.03	3–15
Urge for sexual satisfaction	12.48±3.03	5–15
Sharing sexual issues	12.48±3.05	5–15
Emotional change	12.61±3.10	5–16
Sexual self–care	12.56±3.08	5–15
The Family Burden Assessment Scale	122.00±19.03	85–162
Economic burden	17.38±8.66	6–30
Perceived inadequacy	20.08±3.93	12–24
Social burden	20.27±3.95	12–24
Physical burden	22.93±2.73	11–25
Emotional burden	23.12±3.04	11–32
Need for time	18.20±3.82	7–34
	n	**%**
Family burden*		
Low	198	24.3
High	617	75.7

Results

Table 2 presents the SDCS and FBAS scores. Consequently, their SDCS total score was 127.84±9.41 and they obtained the highest score (23.43±4.8) on the subscale of information needs. Furthermore, their total FBAS score was 122.00±19.03 and they obtained the highest score (23.12±3.04) on the emotional burden. The study found that 75.70% of the families had a high family burden (Table 2).

Table 3. Correlation matrix of variables (n=815)[a]

The Family Burden Assessment Scale and subdimensions	The Scale of Sexual Development Characteristics of Adolescents with Intellectual Disability and subdimensions									
	Total score	Sexual arousal	Information needs	Privacy and social trust	Information about bodily development	Sexual harassment	Urge for sexual satisfaction	Sharing sexual issues	Emotional change	Sexual self-care
Total score	0.199*	0.238**	0.245**	-0.190*	-0.176*	0.160*	0.173*	0.176*	0.181*	0.178*
Economic burden	0.240**	0.192*	0.194*	-0.177*	-0.158*	0.101	0.144	0.147	0.148	0.147
Perceived inadequacy	0.254**	0.110	0.096	-0.116	-0.105	0.089	0.014	0.015	0.023	0.019
Social burden	0.265**	0.234**	0.119	-0.128	-0.118	0.099	0.033	0.048	0.055	0.050
Physical burden	0.191*	0.074	0.108	-0.041	-0.041	0.052	0.153*	0.142	0.143	0.146
Emotional burden	0.190*	0.224**	0.224**	-0.129	-0.126	0.164*	0.150*	0.156*	0.158*	0.153*
Need for time	0.235**	0.057	0.103	-0.040	-0.042	0.055	0.138	0.125	0.125	0.130

[a] * p≤0.05, ** p≤0.01

Table 3 shows the interrelationships of the variables. The FBAS and all its subscales were positively but negatively associated with the SDCS. As the SDCS scores increased, their general family burden (r=0.199, p=0.002), economic burden (r=0.240, p<0.001), perceived inadequacy (r=0.254, p<0.001), social burden (r=0.265, p<0.001), physical burden (r=0.191, p<0.023), emotional burden (r=0.190, p<0.003), and the need for time (r=0.235, p<0.001) also increased.

Table 4 presents the logistic regression analyses for risky sexual development factors related to family burden. The predictive model for the burden of the family explained 60% of the variance in this sample of parents (F (9.82)=16.24, p <0.001). The sexual predictors with the strongest effect on the model were previous sexual development education (β=0.74; SE=0.16, p≤0.001), followed by sexual harassmet (β=-0.56, SE=0.26, p≤0.001). Furthermore, the other sexual predictors with a significant effect on the model were sexual information needs (β=-0.46, SE=0.25, p≤0.001), sexual development characteristics (β=0.37, SE=0.17, p≤0.001), sexual self-care (β=-0.34, SE=0.14, p≤0.05) and the desire for sexual satisfaction (β=-0.16, SE=0.06, p≤0.001). In the case of the best model; sexual arousal (β=-0.01, SE=0.20, p>0.05), privacy and social trust (β=0.07, SE=0.05, p>0.05), information on bodily development (β=0.01, SE=0.17, p>0.05), sharing of sexual issues (β=-0.04, SE=0.07, p>0.05), and emotional change were not predictive of family burden (β=-0.07; t=0.07; p>0.05).

Table 4. Logistic regression model with the best fit of predictors for family burden

Effect	b	SE	OR	95% CI	
				LL	UL
Constant	0.27	0.22	1.27	0.85	1.98
Having previous sexual education[#]	0.74	0.16**	1.58	1.15	2.13
SDCS	0.37	0.17**	0.75	0.69	0.85
Sexual arousal	-0.01	0.20	0.96	0.67	1.46
Information needs	-0.46	0.25**	0.98	-0.96	0.02
Privacy and social trust	0.07	0.05	1.05	0.97	1.19
Information about bodily development	0.01	0.17	1.02	0.72	1.41
Sexual harassment	-0.56	0.26**	0.62	0.51	0.85
Urge for sexual satisfaction	-0.16	0.06**	0.86	0.76	0.97
Sharing sexual issues	-0.04	0.07	0.96	0.83	1.14
Emotional change	-0.07	0.07	0.93	0.92	1.03
Sexual self-care	-0.34	0.14*	1.40	1.07	1.84

[a] [#] yes = 0, no = 1, * – p≤0.05, ** – p≤0.001, SDCS The Sexual Development Characteristics Scale of Adolescents with Intellectual Disability, note: "Having previous sexual education, SDCS, sexual arousal, information needs, privacy and social trust, information about bodily development, sexual harassment, urge for sexual satisfaction, sharing sexual issues, emotional change, sexual self-care" is an independent variable, and family burden is a dependent variable

Discussion

Our objective was to examine the predictors of sexual development of family burden among parents of children with intellectual disability. A statistically significant relationship was found between the characteristics of sexual development of children with intellectual disability and the burden of the family. As their negative characteristics of sexual development increased, the parents' economic, physical, social and emotional burden of parents increased, causing them to feel inadequate. As the most significant risk that increases the family burden, children with intellectual disability did not receive sexual education about sexual development before. Other factors that increased the family burden were sexual harassment, sexual self-care, and the desire for sexual satisfaction.

In this study, the family burden of 75.70% was high. Perception of inadequacy and a higher level of family burden in social, physical, and emotional areas. In all studies that examine the family burden of families with children with intellectual disability, it is seen that the family burden is high.[2,23] It is stated that the rate of family burden in these families can reach up to 90%.[1] Parents experience perceived inadequacy and family burden more intensely in emotional areas. Determining the emotional factors that cause families to feel inadequate will be decisive in reducing the family burden.[24]

In this study, the total FBAS score of parents was 122±19.03 and they obtained the highest mean score (23.12±3.04) on the emotional burden. In a separate investigation conducted in Türkiye, the general FBAS score for parents of children with mild intellectual disabilities was found to be 121.91±33.50.[2] In the same study, the highest scores were observed for perception of inadequacy and emotional burden. In parents of children with intellectual disabilities, pessimism can emerge as economic, emotional, and temporal burdens increase with time. Parental problems may arise as social burdens increase, and dysfunction may emerge as physical burdens increase. This situation results in an increase in the family burden experienced by parents over time.[25] In this study, the characteristics of sexual development of children with intellectual disabilities were discussed as a factor that increases family burden.

The mean SDCS score of the parents in the study was 127.84±9.41 (98–157). According to this result, parents were inadequate in understanding their children's sexual development. Parents were more likely to feel inadequate in the areas of information needs and sexual arousal. In another study, the mean SDCS score of parents was reported as 111.33 (57 162) and information need and sexual arousal were the areas where parents felt more inadequate.[26] In their study, Krbaş and Odabaşı Aktaş reported the total SDCS score of parents as 117.73±16.78.[27] The highest scores were observed for

sexual arousal, need for information, privacy, and social trust. These findings suggest that parents may experience challenges in managing their children's sexual arousal and privacy. Furthermore, the results indicate that parents have significant information needs, particularly regarding their children's sexual development.[27] Similar to the results of this current study, several studies have reported that parents with children with intellectual disability lacked information on sexuality.[12,28] Parents can feel competent about their children's sexual health and development through education. With these educations, parents were able to deal with their children's problems related to sexual development and effectively solve their children's problems.[16]

As another significant risk that increased family burden, most of parents of children with intellectual disability (81.97%) did not receive education about their children's sexual development before. In a parallel investigation, 45.7% of mothers indicated that they perceived themselves to be inadequately equipped to provide sexual education to their disabled adolescent children, and 97.6% stated that they had not received any information on this subject.[26] The findings of our study, as well as similar studies in the literature, indicate that a significant proportion of parents perceive themselves as lacking the necessary skills to effectively provide their children with sexual education.[26,27] This can result in an increase in parental stress and a greater family burden.[29] Parents of children with intellectual disability may have low awareness of their children's sexual development. They may not know what to discuss, when to talk, or how to direct conversations with their children.[8,14] Parents may be worried about not being able to provide education at a level that their child with intellectual disability can understand due to his intellectual disability. Parents may lack information on these issues and may feel helpless.[18,28]

Two important of the sexual predictors of family burden were sexual harassment and sexual satisfaction in this study. Similarly, in another study, it was reported that the average score of the scale was high in the subdimensions of sexual arousal (20.97±5.71), sexual harassment (12.22±3.13), sexual satisfaction (15.79±3.85), and sharing sexual topics (12.74±2.67).[26] These high rates indicate that some sexual behaviors are performed problematically in children with intellectual disabilities.[29] Children with intellectual disability may frequently engage in touching, hugging, and kissing behaviors that will not be accepted by their social environment and cause discomfort to individuals.[15] Exhibiting these behaviors in public places can cause stigma in families. Such behaviors may be perceived as sexual harassment and may cause embarrassment to their parents in the social environment.[12] The underlying reason why mothers of children with intellectual disability feel distressed about their children's social environment is that they worry about their children getting hurt.[18,28] Children with intellectual disability may be abused due to lack of attention, inability to react at the right time and place, insufficient understanding and comprehension skills and limited abilities, and fulfilling what they are requested without questioning.[30] Children with intellectual disability who receive sexual health and communication education exhibit less problematic behavior.[31] In a study examining the effectiveness of the sexual education program given to both adolescents with intellectual disabilities and their mothers, it is stated that the program is effective in children's gaining social skills, learning how to behave in dating, friendship, and family/parent relationship.[32] Children can organize their social relations more easily by exhibiting less problematic behavior with such educations.[9,33] Also, by providing privacy education to children, these behaviors will not cause stigma in families.[30] With the sexual health and abuse educations prepared for children with intellectual disability, children's level of privacy increases, they can learn to distinguish private and public areas, where to practice behaviors such as masturbation/walking around naked, and the concept of strangers.[34]

Study limitations and strengths

The study included all special education centers located in the city center. The diverse sociodemographic characteristics of the children attending these centers, who come from various districts and villages with transport services, reinforce the study's findings. While the study has several strengths, it also has some limitations. The research's limited scope, conducted solely in one city center in Türkiye, restricts the generalizability and representativeness of the results.

Conclusion

This present study provided a need for information about the sexual development of children with intellectual disability and their sexual harassment behaviors, sexual self-care needs and urge for sexual satisfaction increase family burden. Providing education on sexual development in children with intellectual disability to both these children and their parents can facilitate their management of challenges in adolescence period. This education should be given by a multidisciplinary professional team, including nurses. Therefore, families of children with intellectual disability can cope more easily with situations that cause stress and anxiety about their children's sexual development process, and their family burdens can be reduced.

Declarations

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No financial resources are associated with the work in this article.

Authors' contributions

Conceptualization, A.K, F.D., E.G.Ş., and İ.B.U. and A.K, F.D., E.G.Ş., and İ.B.U.; Methodology, A.K, F.D., E.G.Ş., and İ.B.U.; Software, A.K, F.D., E.G.Ş., and İ.B.U.; Validation, A.K, F.D., E.G.Ş., and İ.B.U.; Formal Analysis, A.K, F.D., E.G.Ş., and İ.B.U.; Investigation, A.K, F.D., E.G.Ş., and İ.B.U.; Resources, A.K, F.D., E.G.Ş., and İ.B.U.; Data Curation, A.K, F.D., E.G.Ş., and İ.B.U.; Writing – Original Draft Preparation, A.K, F.D., E.G.Ş., and İ.B.U.; Writing – Review & Editing, A.K, F.D., E.G.Ş., and İ.B.U.; Visualization, A.K, F.D., E.G.Ş., and İ.B.U.; Supervision, A.K, F.D., E.G.Ş., and İ.B.U.; Project Administration, A.K, F.D., E.G.Ş., and İ.B.U.; Funding Acquisition, A.K, F.D., E.G.Ş., and İ.B.U.

Conflicts of interest

No potential conflict of interest was reported.

Data availability

The data used in the study and the details of the method can be requested from the corresponding author.

Ethics approval

The study was approved by the Social and Human Sciences Ethics Committee of the university in question (Protocol No. 2021-SBB-0241, dated May 26, 2021).

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ORIGINAL PAPER

The management and clinical outcomes of electrothermal burn injury patients over a ten-year period

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ABSTRACT

Introduction and aim. This study investigates electrothermal burns in children, including their incidence, causes, associated systemic changes, and treatments. Electrothermal burns are unique and can be caused by factors such as moisture, leading to heat conduction from the contact site. The study aims to determine the frequency of these burns in children, identify their causes, and evaluate various treatment methods. The outcomes of interest include wound healing, scarring, and long-term complications. The results of this study will help develop better treatment strategies and reduce the incidence of such injuries.

Material and methods. The Grodno Regional Children's Clinical Hospital, Belarus treated 666 children for burn injuries between 2014 and 2023, 35 of them diagnosed with electrothermal skin burns.

Results. According to our analysis, electrothermal burns make up approximately 5.3% of all burn injuries. Upon reviewing hospitalization records, it was observed that the number of hospital visits related to this type of injury ranged from 3 to 5 annually, except for 2022, when there were nine recorded cases.

Conclusion. Our study shows that electrothermal burns are usually caused by household appliances. There is a gender imbalance in those affected. Early detection and appropriate medical intervention are crucial in the management of these burns.

Keywords. biochemical blood test, dermoplasty, electrocardiography, electrothermal burn, treatment

Introduction

Electrothermal burns occur as a result of heat generated outside the skin due to electrical activity. Burns resulting from contact with high tension current, due to the leaping of an electric arc from the conductor to the skin, are the most commonly observed.[1] Direct and indirect mechanisms come into play, with direct damages being caused by contact with electrical energy and the electric arc, and indirect injuries being secondary mechanical trauma that can be linked to falls or burns caused by electrical arcs or flames that ignite combustible materials.[2] The harmful effects of electric energy include thermal action at the body level and electrolytic action at the cell level, which can lead to serious injuries such as electrothermal burns resulting in tissue damage and life-threatening complications.[3] Extensive full-thickness burns and soft tissue injuries are seldom observed after accidents with electricity used for home appliances (<1000 V); however, in high-voltage (>1000 V) accidents, there is a well-recognized relationship between extensive burns and necrosis of soft tissue coagulation necrosis.[4] The characteristics of electrothermal injuries

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to display an alveolar soot arrangement and skin metallization.[5]

Cardiac changes from electrical injuries often show up as rhythm or conduction problems like sinus tachycardia, ventricular and atrial ectopy, bundle branch block, heart block, atrial fibrillation, supraventricular tachycardia, and ventricular fibrillation.[6] The myocardium can be damaged by high- and low-voltage current, direct electrothermal conversion, electroporation, or contusion from a lightning strike.[7]

Respiratory complications can result from inhibition of respiratory drive of the CNS, paralysis, and tetany of respiratory muscles, leading to acidosis due to poor tissue perfusion and lactic acid production.[8,9] The literature often underreports neurological symptoms in patients with electrical injury, such as seizures, motor weakness, decreased sensation, left hemiparesis, and loss of consciousness.[10,11]

Electric current can cause significant damage to blood vessels, leading to heat generation, coagulation necrosis, and occlusion of small vessels, especially those supplying muscles.[12] It is important to take into account that electrothermal heating leads to muscle damage and is typically observed only when exposed to high voltage with long contact and current flow.[13] On arteriography, this can be visualized as arterial pruning proximal to occlusion, indicating an area of irreversible muscle injury. This may mimic the appearance of progressive muscle necrosis.[14] In severe cases of high-voltage injuries, muscle necrosis can spread to sites far away from the observed skin injury, causing compartment syndromes due to vascular ischemia and muscle edema.

Furthermore, the damaged muscle releases a massive amount of myoglobin which may result in myoglobinuric renal failure.[12] The risk of myoglobinuria, which is caused by muscle damage, is acute renal failure. This requires prompt treatment with crystalloid loading to a target urine output of 2 $mLkg^{-1}h^{-1}$.

Anemia is the most frequent complication observed in electrothermal burn cases, which can be diagnosed through a complete blood count (CBC).[15] Additional diagnosis can be aided through urine myoglobin and creatinine kinase (CK) testing.[16] Elevations in lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) are significant in terms of low-to-high voltage difference.[17] To detect the possibility of life-threatening arrhythmias, it is recommended to monitor electrocardiography (ECG) during transport.[6]

Additionally, treatment with sodium bicarbonate, mannitol, and furosemide facilitates myoglobin excretion and protects against renal tubular injury.[18] Electrothermal injury treatment is complex and difficult and should be customized based on the patient's particularities. Traditional treatment of electrothermal trauma included infusion therapy and the use of drugs to improve blood rheology, glucocorticoids, protease inhibitors, drugs to improve cardiac and respiratory activity, analgesics, neuroleptics, hepato-protectors, antioxidants, and antibacterial therapy. If necessary, patients also received transfusions of blood, plasma, and albumin.

The treatment of wounds is a crucial aspect of medical care that requires a careful and methodical approach. Local wound treatment involves the use of wet-drying and ointment dressings, which are chosen based on the specific phase of the wound process. For instance, gauze wet-drying dressings with antiseptic solutions such as iodopovidone, iodopyrine, and betadine are used for drying necrotic tissues, while multicomponent ointments with water-soluble bases may be applied in other situations. There is conclusive evidence supporting the effectiveness of early decompression incisions, neurectomies, and surgical debridement in promoting wound healing. These interventions facilitate the removal of necrotic tissue, relieve skin tension, improve blood flow, and eliminate edema. Additionally, the use of early fasciotomy on the first day after injury and early necrectomy has been shown to significantly reduce the frequency of crippling operations such as amputation and exarticulation of limbs. This approach also enables auto-dermoplasty to be conducted one week earlier, compared to traditional tactics. Furthermore, it enhances the engraving ability of autografts and reduces the duration of inpatient treatment. According to radiographic densitometric studies, the process of osteonecrosis after electrothermal trauma is completed within two weeks after the injury. As a result, osteo-necrectomy can be initiated one to three weeks earlier, including simultaneous radical osteo-necrectomy over the entire surface of osteonecrosis. These findings have significant implications on electrothermal trauma and its management and represent a valuable addition to the existing body of knowledge.[19,20]

Aim

The research aims to bridge the existing gap in literature concerning electrothermal burn trauma. It intends to delve into the innovative treatment approaches and the analysis of post-treatment care.

Material and methods

We conducted a thorough retrospective review of the pediatric population with electrothermal injuries who received inpatient treatment at the Grodno Regional Children's Clinical Hospital, Belarus. During the period spanning from 2014 to 2023, a total of 666 pediatric burn patients were admitted to the hospital for treatment, of which 35 presented with electrothermal skin burns. Of these 35 patients, 23 were male and 12 were female.

The statistical analysis has been conducted. The authors generated quantitative data on various subjects, which they then presented visually using curve charts,

bar charts, and pie charts. These visual representations served to illustrate the data clearly and concisely.

The present study has specific criteria for inclusion and exclusion of patients. The inclusion criteria consist of patients who have been subjected to electrothermal burns and are within the pediatric age group, residing in the Grodno region. Conversely, the exclusion criteria involve patients who have been subjected to burns that occurred by factors other than electrothermal burns, those who are above 18 years of age, and those who belong to regions other than Grodno. These criteria have established the certainty that the study is focused and to maintain the integrity of the study's findings. The study was approved by the institutional ethics and misconduct wing (approval number 2410).

Results

During our retrospective analysis of patient cases, we identified that out of the 666 patients who underwent inpatient treatment, a total of 35 patients were reported to have experienced electrothermal skin burns. We have included a few examples of such instances in Figure 1.

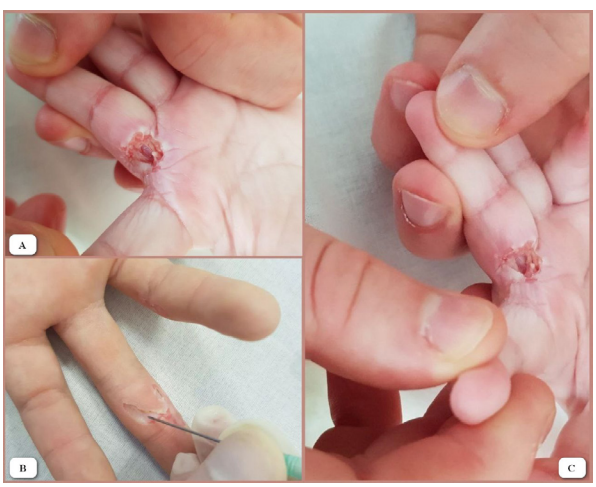

Fig. 1. A and C: III-degree burn, B: diagnostic sensitivity test (needle test) on III -degree burn

The tabulated data presenting the demographic information for the study is in Table 1.

Table 1. The demographic data of the study

Characteristics	n
Patients	35
Mean age (years)	9
Gender	
Male	23
Female	12

In our research, we included a parameter of hospitalizing a patient with electrothermal skin burns, which is shown in Figure 2. Our findings indicate that in the year 2014, no patient experienced electrothermal skin burns. However, among the 35 patients who experienced electrothermal skin burns, the highest number of patients, 9 (25.7%), was recorded in 2022, followed by 5 (14.3%) patients in 2018 and 2020, 4 (11.4%) patients in 2023, and 3 (8.6%) patients in 2016, 2017, and 2021. 2 patients in 2019 and one patient (2.8%) in 2015 experienced electrothermal skin burns.

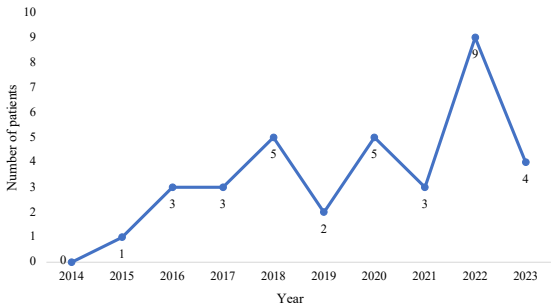

Fig. 2. Hospitalization of patients with electrothermal skin burns over nine years

The incidence of electrothermal burns varies depending on the age and gender of the affected individuals. According to our findings, children between the ages of one and three years are the most susceptible to electrothermal burns, and the incidence rate of electrothermal burns is higher in males as compared to females. The second most common age group affected by electrothermal burns is children between the ages of four and eight years, as evidenced by statistical data presented in Table 2.

Table 2. The distribution of children with electrothermal burns by age and gender

Age	Boys (n)	Girls (n)	Total (n)
Before 1	5	0	5
1–3	6	6	12
4–8	7	2	9
9–14	3	2	5
14–18	2	2	4
Total	23	12	35

According to Figure 3, electrothermal burns exhibit a gender-based distribution, with males experiencing such burns at about 65.7%, which is twice as frequent as females, who experience them at about 34.3%.

When examining the geographic distribution of children seeking inpatient care, it was observed that the regional pediatrics hospital in Grodno was chosen by 30 patients who were residents of the city itself. Conversely, the district hospital of the Grodno region was sought out by 5 patients residing on the outskirts of Grodno, as in Figure 4.

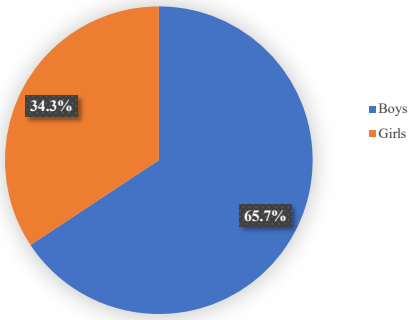

Fig. 3. Electrothermal burn according to gender distribution

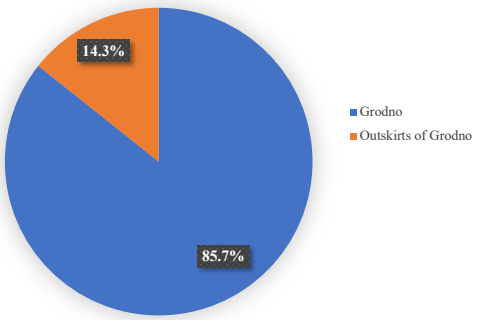

Fig. 4. The distribution based on the region of residence

The study sought to investigate the duration between electrothermal burns and the time patients sought medical attention. In Figure 5, a majority of the patients (65.7%) opted to seek medical assistance within an hour of the incident. Approximately 25.7% of the patients sought medical assistance within 1–3 hours, while only 5.7% sought help after a day. The remaining 2.9% of patients waited for up to 7 days before seeking medical attention. The results indicate that the majority of patients were proactive in seeking medical attention shortly after the incident, which is encouraging as prompt medical attention is critical in managing the associated complications of electrothermal burns.

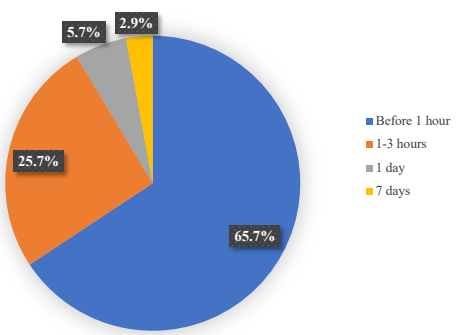

Fig. 5. The distribution depending on when a person seeks medical attention

Electrothermal injury caused by electrical appliances is a significant concern, particularly in children. Among young children, inserting metal objects, such as hairpins, metal nails, and metal rods, into a 220V socket was identified as the fundamental cause of low-voltage injury, accounting for 48.6% of cases. In contrast, 42.9% of children experienced injury due to bare electric wires, 5.7% due to contact with a light bulb base, and 2.8% due to exposure to a 380V transformer substation, as presented in Figure 6. These outcomes highlight the need for increased awareness particularly among young children, to reduce the incidence of electrothermal injury caused by electrical appliances.

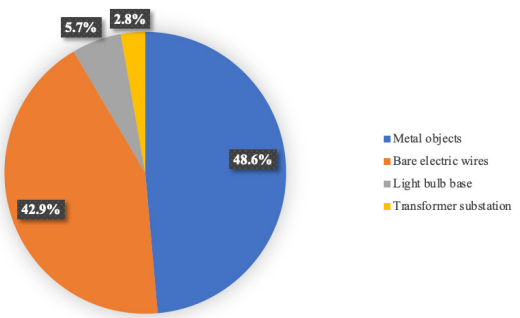

Fig. 6. The distribution depending on the etiology of the injury

The data presented in Figure 7 illustrates the seasonal distribution of electrothermal burns. A total of 35 children were injured during the study period, and the majority of the cases (34.3%) occurred during the summer. In contrast, 25.7% and 14.3% of the injuries were observed in winter, autumn, and spring, respectively. These findings suggest that the summer months pose a higher risk for electrothermal burn injuries among children. It is recommended that guardians take extra precautions during this season to minimize the risk of burns.

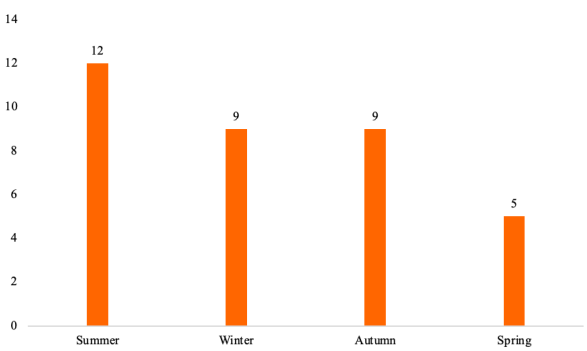

Fig. 7. The distribution of injuries by season

An analysis was conducted to evaluate the occurrence of injuries based on the day of the week. The findings indicate that the highest number of injuries were

reported on Sunday, with 9 children (25.7%) experiencing an injury. Friday followed with 6 children (17.2%), while Saturday recorded 5 cases (14.3%). Monday, Wednesday, and Thursday had 4 cases each (11.4%), while Tuesday had the lowest number of injuries with only 3 cases (8.6%). These results are in Figure 8.

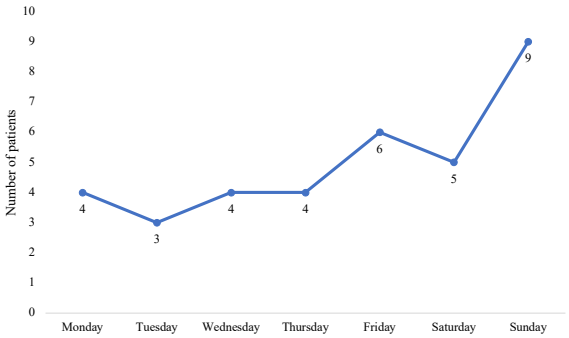

Fig. 8. The distribution varying depending on the day of the week

Upon admission, the medical care provided adhered to the clinical protocols. These protocols outlines the basic requirements for providing medical care to patients with burn injuries and their complications in both outpatient and inpatient settings. Among the admitted children, 22 (62.8%) were hospitalized in the emergency surgical department from the emergency room, while 10 (28.6%) received treatment in pediatric emergency medicine. Additionally, 3 (8.6%) children were admitted to the anesthesiology and resuscitation department, as in Figure 9.

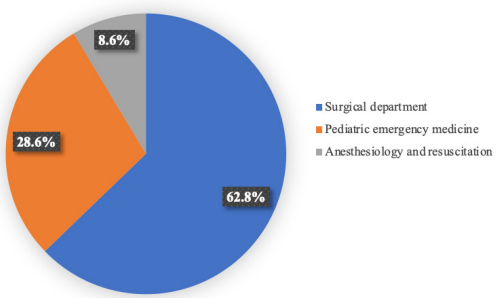

Fig. 9. The distribution of children by hospital department upon admission

A full blood count (FBC) was conducted on all patients on admission. Mild anemia was observed in 7 children (20%), leukocytosis in 11 children (31.4%), and an increase in erythrocyte sedimentation rate in 3 children (8.6%). These findings suggest the presence of an underlying inflammatory process, which may warrant further investigation.

Upon admission, a biochemical blood test was conducted on 18 patients to determine the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH). The results indicated that AST levels were elevated in five of the children, while two children had elevated ALT levels. Four children had elevated CK levels, and one child had elevated LDH levels.

Eight patients underwent a coagulation profile test upon admission. The test results revealed that one child had an elevated level of fibrinogen, while another patient showed an increase in international normalized ratio (INR) to 1. These findings suggest the possibility of coagulation disorders. It is essential to monitor the patient's health status closely and take appropriate measures to manage their conditions.

Electrocardiography (ECG) was conducted on all patients. The analysis of the ECGs revealed certain changes in the cardiac parameters. Specifically, a decrease in voltage was noted in four children (11.4%), while three children (8.6%) exhibited a shortening of the PQ interval. Two children (5.7%) showed an atrioventricular block, while another two children (5.7%) showed a partial block in the right bundle branch. Further, one child (2.9%) had tachycardia, while another child (2.9%) showed bradycardia and atrial rhythm, as in Figure 10.

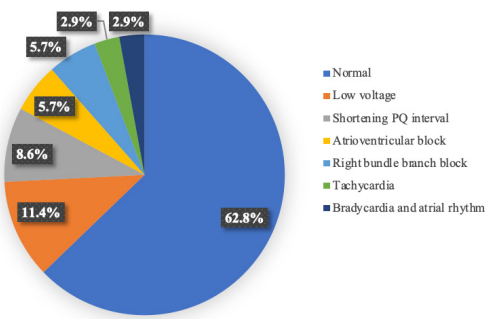

Fig. 10. The distribution of changes in ECG following electrothermal burn injury

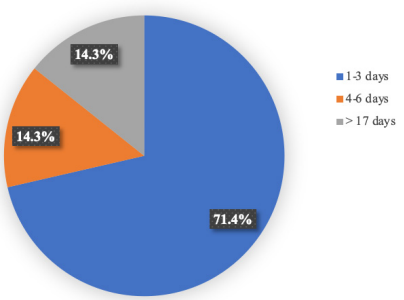

Fig. 11. The distribution according to length of stay after receiving an electrothermal burn injury

Based on the distribution of hospital stays, the majority of children, specifically 71.4%, remained hospitalized for 1–3 days. A smaller proportion of patients, comprising 14.3% of the total, were admitted for 4–6

days while the remaining 14.3% of patients were hospitalized for a duration exceeding 17 days, as shown in Figure 11.

On admission, an evaluation was conducted to determine the localization, burn area, and depth of the wound. The location of the electrothermal burns is presented in Table 3.

Table 3. Localization of electrothermal burns in children

One anatomical region			Many anatomical regions		
n=25 (71.4%)			n=10 (28.6%)		
Anatomical region	n	%	Anatomical region	n	%
Right hand	16	64	Both hands	9	90
Left hand	7	28	Left hand, leg	1	10
Left shin	1	4			
Head	1	4			

Among the 35 patients under review, it was observed that the majority of the lesions (71.4%) were localized in a single anatomical region, primarily among 25 children. The right hand was affected in 16 children (64%), while the left hand was affected in 7 children (28%). One child (4%) experienced the lesion on the left shin, while another child (4%) had it on the head. Additionally, there were 10 children (28.6%) who exhibited lesions involving multiple anatomical regions. Among these, both hands were affected in 9 children (90%), while the left hand and leg were affected in 1 child (10%).

Typically, electrothermal burn wounds do not exhibit significant damage over a wide area. However, it is common to observe skin lesions in the appearance of distinct marks, as summarized in Table 4.

Table 4. Distribution of electrothermal burns depending on the affected area

(%) of affected area	<0.05	0.1–0.2	0.3–0.8	1
Number of patients	20	9	5	1

Burns are classified into different degrees based on their severity, with superficial burns being classified as first-degree burns, partial-thickness burns as second and third-degree burns, and full-thickness burns as fourth-degree burns. All pediatric patients underwent primary surgical intervention for their wounds, and the depth and extent of their burns were thoroughly evaluated upon admission. The observed scars exhibited a mosaic pattern, affecting the superficial and deep layers of the skin. Three children were diagnosed with IV-degree electrothermal burns.

During the initial day of hospitalization, we implemented wet-to-dry dressings with antiseptics for local treatment. Subsequently, we utilized 1% silver sulfadiazine or 2% silver sulfathiazole cream, as well as ointment forms based on povidone-iodine and chloramphenicol for further treatment.

Two pediatric patients underwent surgical procedures, one of which involved one-stage plastic skin grafting, while the other received delayed dermoplasty involving Italian dermatoplasty and auto-dermoplasty utilizing a free split skin flap measuring 0.4 mm thick. In another case, a single child underwent partial resection of the distal phalanx, followed by stump plastic surgery using local tissues, as in Figure 12.

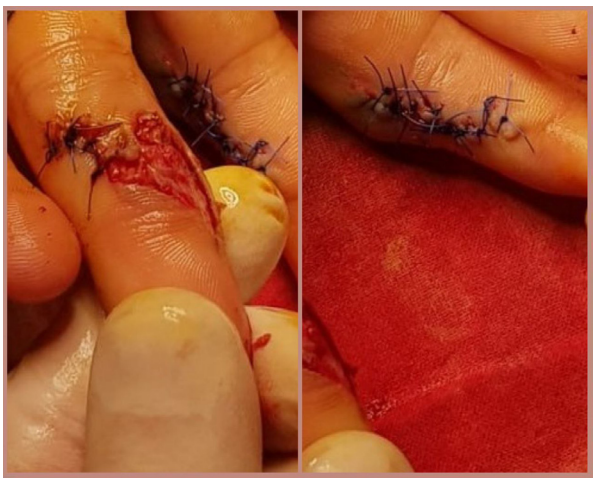

Fig. 12. Primary surgical intervention

Additionally, primary sutures on the wound were ligated to only one child, as outlined in Table 5.

Table 5. Methods of surgical treatment

Surgical treatment				
n=4 (100%)				
Anatomical region	Type of surgery	Day of surgical treatment	n	%
Right hand	Early necrectomy	6	2	50
	One-stage plastic surgery with skin flap	6	1	25
	Delayed Italian dermatoplasty and auto-dermoplasty with a free split skin flap	13	1	25
	Primary sutures on the wound	1	1	25
Left hand	Partial resection of the distal phalanx with stump plastic surgery using local tissues	25	1	25

As part of their treatment plan, nine children were prescribed a rigorous regimen of antibacterial therapy, with the addition of a comprehensive course of physiotherapy and physical therapy. This complex therapy approach was designed to address their specific needs and promote optimal healing and recovery.

Following their discharge from the hospital, all children underwent a thorough examination conducted by a pediatric surgeon. This examination took place one month after the complete epithelialization of the wounds as shown in Figure 13, and was followed by a course of conservative treatment aimed at preventing the development of pathological scars. Subsequently, the

children were taken for follow-up, following established protocols.

Upon admission, all pediatric patients underwent culturing at sites of tissue damage exhibiting no microbial growth. Only one patient experienced infectious complications, while rapid marginal epithelization occurred in the others. For more extensive injuries, necrectomy followed by plastic surgery was performed, effectively preventing the development of infectious complications. After a year of treatment, it was observed that a significant majority of the children (97.1%) showed positive outcomes and were excluded from further observation. However, in the case of a single child who suffered an electrothermal burn caused by a transformer substation, the surgeon observed the formation of a flexion contracture. Additionally, the child exhibited psychiatric complications such as hyperkinetic behavior disorder.

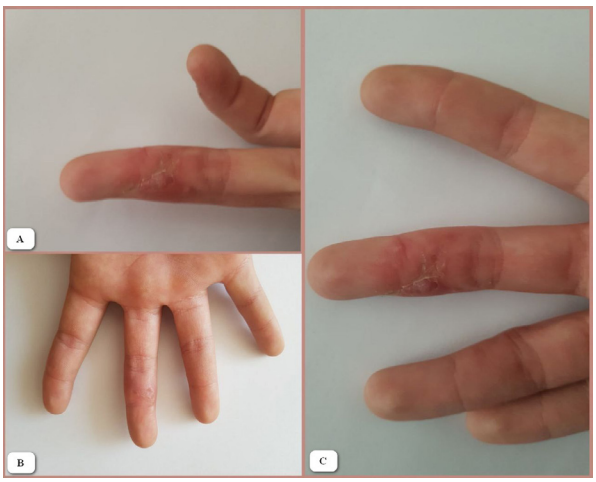

Fig. 13. A and C: epithelialization of the transplanted skin flap after a month, B: epithelialization of the transplanted skin flap after 12 months

Discussion

Electrothermal burns are a serious concern as they can result from low-voltage sources that are capable of producing large currents. When a metal object comes into contact with a live terminal and a grounding, it can short-circuit the battery and rapidly heat the metal object.[21] Children have been reported to have had electrothermal burns from various objects such as metal objects, bare electric wires, light bulb bases, and transformer substations. Electrothermal injury can occur due to different reasons such as capacitive coupling, direct application, direct coupling, insulation failure, etc.[22] The severity of the injury depends on several factors such as voltage, amperage, type of current (alternating or direct), tissue resistance, tissue susceptibility, duration of contact, the path of electrical flow through the body, and extent of grounding.[23] It is worth noting that most patients with electrothermal injuries are young and healthy.[24] Gender and season distribution analysis shows that boys are at higher risk of electrothermal burns during the summer season compared to girls.

According to the results of an ECG, changes in low voltage were observed in 11.4% of children, while 8.6% of children showed a shortening of the PQ interval. Atrioventricular block and right bundle branch block were detected in 5.7% of children, and tachycardia, bradycardia, and atrial rhythm were observed in the remaining 2.9% of children. It has been found that electrothermal conversion and electroporation can cause damage to the myocardium, with the direct impact of electric current being a primary cause of such damage. Furthermore, elevated levels of myocardial damage parameters, such as CK-myocardial band isoenzyme and troponin levels, could indicate cardiac complications. However, an ECG remains a crucial diagnostic tool for identifying myocardial damage as a complication of electrothermal injury.[24] Cardiac events usually occur immediately after the accident, resulting in the proarrhythmic effect of electric shock. Electromechanical disturbances, such as sinus tachycardia, ventricular premature beats, atrial fibrillation, ventricular tachycardia, ventricular fibrillation, and conduction abnormalities, such as sinus bradycardia or bundle-branch blocks, or varying degrees of atrioventricular blocks, are all potential consequences of electrothermal injury.[25]

During the FBC analysis, the patients exhibited anemia, leukocytosis, and an elevation in ESR levels. Anemia and leukocytosis are the most frequent laboratory findings among these patients.[26] Furthermore, patients' coagulation profile tests revealed increased levels of fibrinogen and INR. A biochemical blood test revealed elevated levels of AST, ALT, CK, and LDH. The severity of the injury may be correlated with the LDH level.[27] Within an hour of the electrothermal burn incident, most children sought medical attention, while parents of 25.7% of children decided to seek medical attention within 1-3 hours, and the remaining children sought medical attention within 1–7 days.

According to the clinical protocols of the Ministry of Health of the Republic of Belarus, children with burns were treated in different departments based on the extent and depth of the injury. 62.8% of children required treatment in the surgical department, 28.6% received treatment in pediatric emergency medicine, and 8.6% were treated in anesthesiology and resuscitation. It is imperative to consider varying monitoring and treatment concepts for patients, especially after sustaining an injury.[28] The condition can be managed through conservative treatment, primary intervention, and epithelialization.[29] In our study, all children underwent primary surgical treatment of wounds upon admission, which helped to determine the burn's depth

and area. Wet-to-dry dressings with antiseptics were performed on the first day, followed by a prescription of creams like 1% silver sulfadiazine or 2% silver sulfathiazole cream, and ointment forms based on povidone-iodine and chloramphenicol. Additionally, complex therapies such as physiotherapy, physical therapy, and antibacterial therapy were prescribed to a few children.

Some children had undergone early necrectomy using different surgical techniques, including one-stage skin grafting, delayed Italian dermatoplasty, and auto-dermoplasty with a free split skin flap measuring 0.4 mm thick. Primary suture ligated on the wound in one child. Furthermore, partial resection of the distal phalanx with stump plastic surgery using local tissue was performed in a single child. In the hospital, most children had a shorter stay, ranging from 1–3 days, while 14.3% had more extended stays of more than 17 days. Follow-up care after a month showed complete epithelization of the wound, and conservative treatment was prescribed to prevent the development of pathological scars. After a year, 34 children had fully recovered with no complications, except for one child, who had developed a flexion contracture and exhibited hyperkinetic behavior disorder. It is crucial to note that neurological complications, including loss of consciousness, memory problems, hypoxic encephalopathy, intracerebral hemorrhage, and stroke, were observed in previous studies.[9]

Conclusion

According to our research, electrothermal burns are observed in 5.3% of hospitalized children with burn injuries. It has been observed that patients under the age of three are the most commonly affected group, accounting for 48.6% of cases. Furthermore, boys are twice as likely as girls to suffer from such burns, with the majority of cases occurring during the summer season and on weekends. These findings highlight the need for enhanced awareness and preventive measures to mitigate the risk of electrothermal burn injuries among pediatric patients. Parents should exercise increased vigilance with children under the age of 4, as this age group is particularly vulnerable to sustaining electrothermal burns from common household appliances.

Injuries caused by electrical shock are primarily attributed to contact with household instruments, with the majority of cases (97.2%) involving incidents such as inserting metallic objects into a 220 V socket and contact with bare conductors. In the episode of an electrical injury, most children (65.7%) seek medical attention within an hour of the incident. The most common site of injury is the hands (91.4%), with a sizeable proportion of cases (11.4%) requiring surgical intervention.

Declarations

Funding

This research received no funding.

Author contributions

Conceptualization, A.I.V.H. and G.R.P.; Methodology, G.R.P. and L.R.S.D.L.; Software, An.V.H. and Y.V.A.; Validation, A.I.V.H., G.R.P., L.R.S.D.L., An.V.H. and Y.V.A.; Formal Analysis, G.R.P., An.V.H. and Y.V.A.; Investigation, A.I.V.H., G.R.P., L.R.S.D.L., An.V.H. and Y.V.A.; Resources, A.I.V.H., An.V.H. and Y.V.A.; Data Curation, A.I.V.H., G.R.P. and L.R.S.D.L.; Writing – Original Draft Preparation, G.R.P. and L.R.S.D.L.; Writing – Review & Editing, A.I.V.H., An.V.H. and Y.V.A..; Visualization, A.I.V.H.; Supervision, A.I.V.H., An.V.H. and Y.V.A.; Project Administration, A.I.V.H., G.R.P., and L.R.S.D.L.; Funding Acquisition, A.I.V.H.

Conflicts of interest

None declared.

Data availability

All data generated or analyzed during this study are included in this manuscript.

Ethics approval

It was approved by institutional ethics and misconduct wing (approval number 2410).

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ORIGINAL PAPER

In vitro and in vivo models of immunomodulatory activity of a hydroalcoholic fraction of *Turnera ulmifolia* Linn

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ABSTRACT

Introduction and aim. The present study explores the immunomodulatory effects of the hydroalcoholic extract of *Turnera ulmifolia* through in vitro and in vivo models.

Material and methods. The study examined cytotoxicity, cytokine production, and nitric oxide (NO) levels using RAW 264.7 murine macrophage cells, while *in vivo* assessments were performed using BALB/c mice.

Results. *In vitro, T. ulmifolia* extract significantly increased cytokine levels and NO production in unstimulated cells while effectively inhibiting overproduction in LPS-stimulated cells, suggesting immunomodulatory and anti-inflammatory activities. In *vivo* experiments demonstrated that *T. ulmifolia* extract enhanced the immune response by improving macrophage phagocytic activity, increasing delayed-type hypersensitivity, increasing serum hemolysin levels, and enhancing thymus and spleen indices. These results highlight the potential of the *T. ulmifolia* extract as an immunomodulatory agent, regulating cytokine secretion and enhancing immune responses without causing cytotoxicity.

Conclusion. The findings indicate promising therapeutic applications for *T. ulmifolia* extract in modulating immune function and inflammation.

Keywords. hydroalcoholic, immunomodulatory activity, in vitro, in vivo, *Turnera ulmifolia*

Introduction

Turnera ulmifolia Linn., sometimes called ramgoat dashalong, is a flowering plant classified under the *Passifloraceae* family. *T. ulmifolia,* a plant widely spread across the Americas, including South and Central America and the Caribbean, is renowned for its therapeutic powers and decorative value. The plant often exhibits a prostrate growth habit, reaching heights of up to one meter, with conspicuous yellow flowers and leaves with toothed edges.[1] This species thrives in many environments, including open forests, places that have been disrupted, and beside highways, often seen in tropical and subtropical countries. The dashalong ramgoat plant has a long-standing history of being used in traditional herbal treatments in its indigenous habitat. Multiple components of the plant, including leaves, stems, and roots, are used to create decoctions, infusions, and poultices to treat a diverse array of diseases.[2,3] *T. ulmifolia* is highly regarded in traditional Caribbean medicine for its alleged ability to reduce inflammation, relieve pain, increase urine production, and relax muscles. It is often used to alleviate symptoms of diseases such as lung infections, gastrointestinal problems, menstrual cramps, and urinary tract infections. Moreover, it is widely believed that the plant has aphrodisiac characteristics and is sometimes used to increase desire and sexual performance.[4,5]

T. ulmifolia is planted for decorative reasons due to its beautiful leaves, vivid yellow blooms, and thera-

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peutic applications. Commonly cultivated in gardens, parks and landscapes, this plant is an ornamental addition that enhances the aesthetic appeal and visual appeal of outdoor areas. Furthermore, the plant offers nectar and a home for pollinators such as bees and butterflies, thus improving local biodiversity and ecological well-being.[6,7] Although *v T. ulmifolia* has been highly valued in traditional medicine for many years, there is a lack of scientific studies on its pharmacological qualities and possible therapeutic uses. Additional research is required to clarify the bioactive substances found in the plant, their modes of operation, and their effectiveness and safety characteristics. Furthermore, initiatives aimed at preserving the natural populations of *T. ulmifolia* and advocating for sustainable farming methods can guarantee its accessibility for future generations while benefiting local people who depend on its medicinal and economic advantages.[8-10] To explore the pharmacological potential of *T. ulmifolia,* we extracted the plant using a hydroalcoholic solvent, and the active fraction was subjected to *in vivo* and *in vitro* immunomodulatory activity.

T. ulmifolia, commonly known as ramgoat dashalong, has been widely used in traditional medicine for its various therapeutic benefits, such as anti-inflammatory, analgesic, and aphrodisiac properties. Despite its historical and widespread use, the pharmacological potential of *T. ulmifolia* has not been thoroughly explored in modern scientific research. There remains a significant gap in understanding its immunomodulatory effects, particularly its potential to regulate the immune system and combat inflammation without cytotoxic side effects. Given the growing interest in herbal medicines for immune modulation, this study aims to address the lack of rigorous scientific investigation of the plant's bioactive compounds. By focusing on both *in vitro* and *in vivo* models, this research seeks to uncover the immunomodulatory activities of *T. ulmifolia* extract, contributing valuable insights to both the fields of immunopharmacology and research on natural products.

Aim

This study is unique in that it systematically evaluates the immunomodulatory effects of *T. ulmifolia* through a comprehensive approach that includes both *in vitro* and *in vivo* models. This research utilizes advanced methodologies to assess cytokine production, nitric oxide (NO) levels, and macrophage function, providing novel information on the plant's biphasic effect on immune responses. Additionally, this work distinguishes itself by demonstrating the ability to enhance immune responses in unstimulated cells while simultaneously inhibiting excessive immune reactions in stimulated conditions, suggesting dual immunomodulatory and anti-inflammatory effects. These findings significantly advance our understanding of the pharmacological properties of *T. ulmifolia,* positioning it as a potential therapeutic agent for immune-related conditions.

Material and methods

Reagents

Glowderma Labs Pvt. Ltd. (Mumbai, India) supplied levamisole hydrochloride. In contrast, Infobio Lifesciences (Delhi, India) supplied Mouse TNF-α, IL-1β, IL-6, and IFN-γ ELISA kits from Krishgen Biosystems, Mumbai, India (catalog numbers: TNF-α: KET7012, IL-1β: KET7013, IL-6: KET7014, IFN-γ: KET7015). The remaining chemicals utilized in the study were acquired from HiMedia Laboratories in Mumbai, India.

Preparation of T. ulmifolia extracts

The protocol for the preparation of *T. ulmifolia* extracts involves the extraction of plant or biological samples using a mixture of ethanol and water in a 70:30 ratio. This extract is then subjected to column fractionation, which is based on the polarity of the solvent system. Various solvents are used sequentially and the sample is placed on top of a silica gel column (60-120 mesh size) to ensure even distribution. A cotton bolus is placed above and below the sample to maintain uniformity. Different solvents, including ethyl acetate, benzene-ethyl acetate mixtures, chloroform, and acetone, are used, each with specific run times until the complete separation of the components is achieved. The fractions obtained are subsequently analyzed using thin-layer chromatography (TLC) to determine the number and type of compounds present in each fraction, and further analysis performed on the most promising fraction for compound identification.

Cell culture

Aaranya Biosciences Private Limited supplied the RAW 264.7 murine macrophage cell line. Cells were cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. They were kept at 37°C in a humidified incubator with an atmosphere of 5% CO_2 and 95% air.

Cell viability assay

The MTT assay, used to assess cell viability, was obtained from HiMedia Laboratories, Mumbai, India. RAW 264.7 cells were seeded at a density of 4×10^5 cells/mL into 96-well plates and allowed to incubate overnight. The following day, different concentrations of *T. ulmifolia* extract (ranging from 0–150 µg/mL, diluted in 0.1% DMSO) were added to the cells and incubated for 24 hours. After incubation, 50 µL of MTT reagent was added to each well, and the cells were further incubated for 4 hours. The MTT reagent was then removed and 100 µL of DMSO was added to each well to dissolve

the formazan crystals formed by viable cells. The optical density (OD) was measured at 570 nm using a microplate reader to determine cell viability.

Cytokine assays

RAW 264.7 cells were seeded in 96-well plates at a density of 4×10^5 cells/mL. The cells were then treated for 24 hours with 1 μg/mL of lipopolysaccharide (LPS) or with 10, 25, or 150 μg/mL of trifluoroacetic acid (diluted in 0.1% DMSO). After treatment, cytokine analysis was performed on the cell-free supernatants. Concentrations of TNF-α, IL-1β, IL-6, and IFN-γ in the supernatants were measured using ELISA kits, following the manufacturer's instructions.

Nitrite measurement

NO production of NO was determined by measuring the accumulation of nitrite in the cell culture medium using the Griess procedure. RAW 264.7 cells were seeded in 96-well plates and treated with *T. ulmifolia* extract at concentrations of 10, 25, 100, and 150 μg/mL (dissolved in 0.1% DMSO). Cells were either exposed to 1 μg/mL of LPS or left untreated for 24 hours. After incubation, cell supernatants were collected, and NO generation was evaluated using the Griess reagent. Equal volumes of the samples and Griess reagent were mixed and incubated at room temperature for 15 minutes. Absorbance was measured at 540 nm using a microplate reader. The nitrite concentration was calculated on a standard sodium nitrite dilution curve.

Animals

BALB/c mice weighing 18–22 g were purchased from the National Institute of Nutrition (NIN) in Hyderabad, India. The animals were housed in isolation cages with unrestricted access to food and water. Before the experiments, the mice were allowed at least one week to acclimate to the experimental environment. All experimental procedures involving animals were conducted strictly in accordance with the guidelines established by the Ethical Committee for the Experimental Use of Animals.

In vivo immunomodulatory effect of T. ulmifolia extract

Carbon clearance test to measure macrophage phagocytic activity in mice

Six groups of mice (n=6 per group) were randomly assigned as follows: NC (normal control) group, DC (disease control) group, TU-200 group (treated with 200 mg/kg of *T. ulmifolia* extract), TU-400 group (treated with 400 mg/kg of *T. ulmifolia* extract), and a standard group. Mice in the TU-200 and TU-400 groups were administered the extract orally (p.o.) using sodium carboxymethyl cellulose (CMC) as a vehicle for seven days. The standard group received levamisole (LEV) at a dose of 30 mg/kg intraperitoneally (i.p.). Mice in the NC and DC groups were given sodium CMC p.o. On days 2, 4, and 6 of the experiment, mice in all groups except the Standard group were administered cyclophosphamide (CTX) at a dose of 40 mg/kg ip to induce immunosuppression. One hour after the final dose, India ink (diluted to 10 μL/kg) was injected into the caudal vein of each mouse. Blood samples (20 μL) were collected from the tail vein at two minutes and ten minutes after injection. The collected blood samples were immediately mixed with 2 mLof 1 mg/mL sodium carbonate (Na_2CO_3) solution to lyse the red blood cells and stabilize the ink particles. The absorbance of the samples was measured at 650 nm using a microplate reader. The phagocytic index was calculated using the following formula:

$$\text{Phagocytic index}=(t_{10} - t_2)/(\log OD_2 - \log OD_{10})$$

Where:

t_{10}=10 minutes post-injection

t_2=2 minutes after injection

OD_2=absorbance at 2 minutes

OD_{10}=absorbance at 10 minutes

After the final blood collection, the mice were sacrificed and the spleen and liver were carefully removed and weighed. Organ weights were expressed as a ratio of body weight to the combined weight of the spleen and liver, providing additional information on the mice's immune function.

Assessing mice for delayed-type hypersensitivity

The footpad edema test assessed mice's delayed-type hypersensitivity (DTH) response. The experimental design was maintained, with the animals divided into appropriate groups. On the second day of drug administration, each mouse was sensitized via an intraperitoneal (i.p.) injection of 0.2 mL of 2% sheep red blood cell (SRBC) suspension. This sensitization initiated the immune response in preparation for the challenge. On the seventh day of drug administration, a challenge was performed by injecting 20 μL of a 20% SRBC suspension subcutaneously into the right posterior footpad of each mouse. This subcutaneous injection was performed to induce the DTH response in the immune-sensitized mice. After 24 hours, the thickness of the right (injected) and left (non-injected) rear foot pads was measured using a vernier caliper. The differential thickness of the footpads was recorded before and after the injection of SRBC to assess the degree of inflammation. The degree of DTH was determined by calculating the difference in thickness between the right (injected) and left (non-injected) footpads before and after the SRBC challenge. This differential measurement indicated the inflammatory response, with more significant swelling indicating a stronger DTH response. This method is designed to assess the cellular immune response in mice and can be used to evaluate the immunomodulatory effects of various substances. The test can be accurately replicated and

results reliably reproduced by following these detailed steps.

Determination of serum hemolysin level in mice

The serum hemolysin assay was performed to assess the humoral immune response in mice. The experimental design remained consistent and the mice were grouped as appropriate. On the third day of treatment, each mouse received 0.2 mL of a 2% sheep red blood cell (SRBC) suspension via intraperitoneal (i.p.) injection to stimulate the immune system and induce antibody production. Exactly 24 hours after SRBC injection, blood samples were taken from the brachial plexus of mice. The blood collected was immediately processed to obtain serum by centrifugation at 3000 rpm for 10 minutes. The serum was then diluted 100-fold in physiological saline for further analysis. A hemolysin reaction mixture was prepared by combining 1 mL of the diluted mouse serum, 0.5 mL of a 10% sheep red blood cell (SRBC) suspension (serving as the antigen), and 0.5 mL of a 10% complement solution. The mixture was then incubated to assess the hemolytic activity of the serum, which indicates the presence of hemolysin antibodies that interact with SRBC in the presence of the complement. The reaction mixture was incubated in a 37°C water bath for 30 minutes to allow the hemolysin (antibodies) in the serum to lyse the SRBC in the presence of complement. After 30 minutes of incubation, the reaction was stopped by placing the tubes in an ice bath. The reaction mixture was centrifuged at 3000 rpm for 10 minutes to separate the lysed cells from the supernatant. The supernatant absorbance was then measured at 540 nm using a microplate reader, indicating the hemolysin activity in the serum. The absorbance values reflect the level of hemolysin antibodies produced by the mice, with a higher absorbance indicating greater hemolytic activity and a stronger humoral immune response.

Immune organ index determination

After seven days of drug administration, the experimental design remained unchanged and the mice were weighed to record their body weights. Subsequently, mice were humanely euthanized via cervical dislocation. After euthanasia, the thymus and spleen were carefully removed from each mouse. Care was taken to remove any extraneous tissues attached to the organs. The thymus and spleen were then weighed separately using a precision balance. Organ weights were recorded in milligrams (mg). Organ weights were normalized to the body weight of each mouse to calculate thymus and spleen indices. These were expressed in milligrams of organ weight per gram of body weight (mg/g). This index provides insight into the effect of the drug on the immune organs of mice.

Statistical analysis

Statistical analysis was performed for the investigation of the immunomodulatory activity of the *T. ulmifolia* extract using standard methods to ensure the accuracy and validity of the findings. Data are expressed as the mean±standard deviation (SD) of independent experiments. The differences between groups were evaluated using one-way analysis of variance (ANOVA) followed by post hoc tests such as Tukey's multiple comparison test. Significance was considered when p-values were less than 0.05 ($p<0.05$), indicating a statistically significant difference. Furthermore, for comparisons involving multiple groups, values such as $p<0.01$ and $p<0.001$ were reported to indicate a higher significance level (SSPS, IBM, Armonk, NY, USA).

Ethical approval

The study was carried out according to ethical standards and ethical approval was obtained from Flair Labs, located at Plot No. B 510, Palsana, Surat-394315, Gujarat, India. The study was approved under the registration number 1250/PO/RcBi/S/23/CPCSEA.

Results

In vitro studies

Effect of lipopolysaccharide extract on cell viability

The extract of T. ulmifolia did not induce significant cytotoxicity in the concentration range of 0–100 µg/mL in RAW 264.7 macrophage cells, whether in the presence or absence of LPS stimulation (Fig.1). This is evident from the minimal variation in cell viability values, all of which remained close to or above 0.8, with most reaching or exceeding 1.0. In particular, the highest concentration of *T. ulmifolia* extract (100 µg/mL) led to a slight reduction in cell viability without LPS. However, this was not statistically significant, suggesting that the extract was non-toxic at the tested doses. The ability to maintain or slightly enhance cell viability, particularly at 10 and 25 µg/mL, suggests that the extract of *T. ulmifolia* may have an immunostimulatory effect without inducing cellular damage. Furthermore, in the presence of LPS, which is known to activate macrophages and cause an inflammatory response, cell viability remained relatively stable. This indicates that the *T. ulmifolia* extract may modulate the immune response without exerting cytotoxic effects.

Effect of T. ulmifolia extract on cytokine release in RAW 264.7 macrophages that are unstimulated or stimulated by LPS

This work investigated how the extract of *T. ulmifolia* affected the cytokine release in RAW 264.7 macrophages. The findings demonstrated that relative to the control group, *T. ulmifolia* extract at doses of 10, 25, 100, and 150 µg/mL markedly improved the production

of TNF-α, IL-1β, IL-6, and IFN-γ ($p<0.05$ or $p<0.01$ or $p<0.001$) (Fig 2). Significantly, this induction showed immunomodulatory activity in innate immunity as it was carried out without any cytotoxic effects and produced a favorable modulation of RAW 264.7 macrophage function. To assess the anti-inflammatory effects of the *T. ulmifolia* extract in a laboratory environment, we carried out measurements on how much inflammatory cytokine was present in RAW 264.7 cell supernatants after exposure to LPS stimulation.

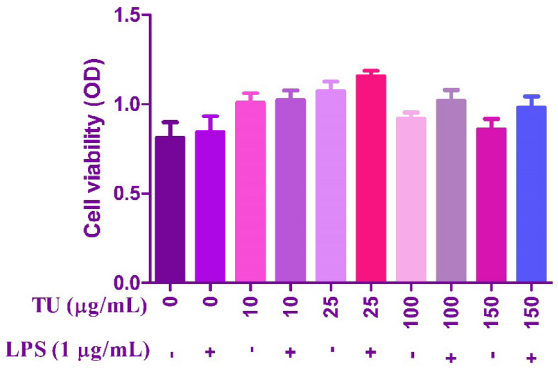

Fig. 1. Effect of *T. ulmifolia* extract on RAW 264.7 viability

LPS stimulation markedly ($p<0.01$) boosted cytokine production compared to the control group. Concentrations of 10, 25, 100, and 150 µg/mL of *T. ulmifolia* extract markedly decreased cytokine synthesis (Fig. 3). A dose-dependent pattern of this inhibition ($p<0.05$ or $p<0.01$) suggested anti-inflammatory action. The results imply that the extract of *T. ulmifolia* may affect RAW 264.7 macrophages' cytokine release, impacting the immune system and lowering inflammation.

Effects of T. ulmifolia extract on NO production in RAW 264.7 macrophages with and without LPS stimulation

Our investigation found that the extract of *T. ulmifolia* has a concentration range that could promote NO generation in unstimulated RAW 264.7 macrophages and be non-cytotoxic. Comparing the concentrations of the *T. ulmifolia* extract at 10, 25, 100, and 150 µg/mL to the control group, the former significantly increased NO generation ($p<0.05$) (Fig. 4). Proportional to dose, the extract of *T. ulmifolia* at doses of 25, 100, and 150 µg/mL markedly reduced NO overproduction of NO as against the LPS-stimulated group ($p<0.05$, $p<0.01$, and $p<0.01$) (Fig. 5). The results show that the extract of *T. ulmifolia* affects RAW 264.7 macrophages' release of the inflamma-

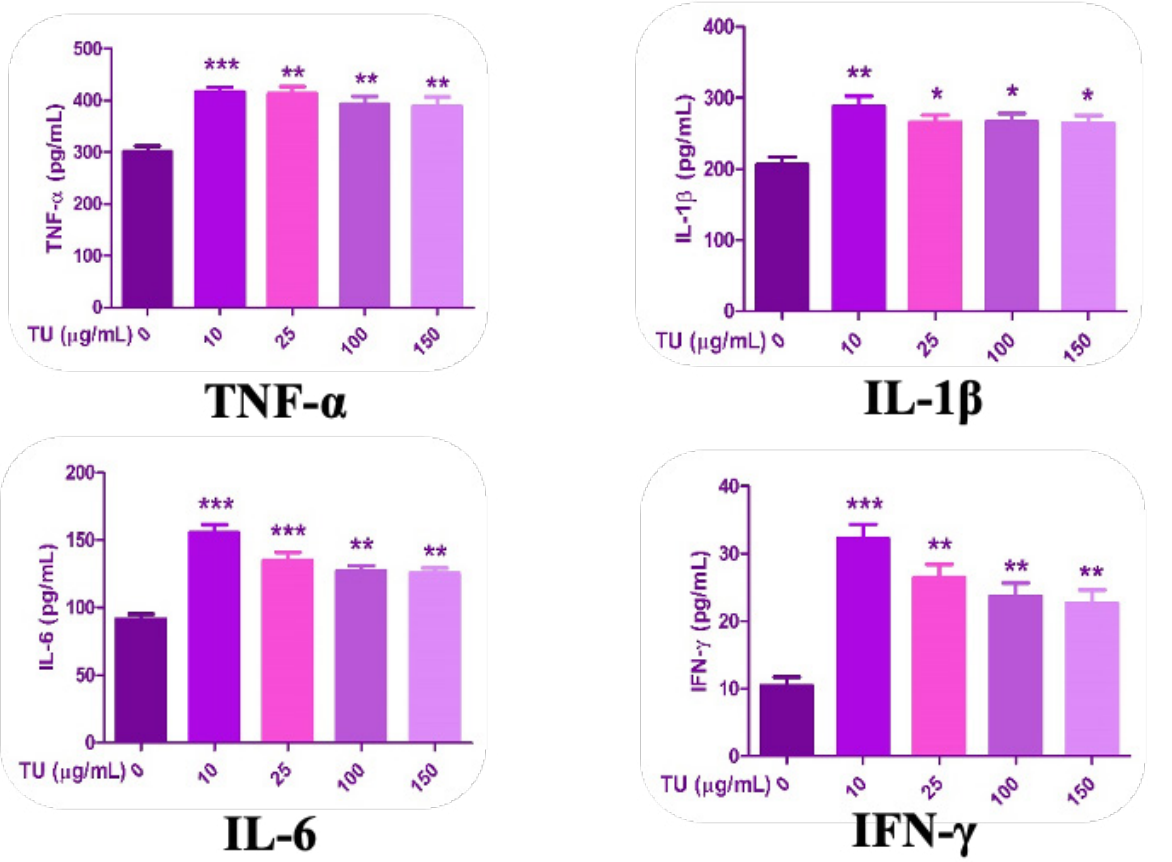

Fig. 2. Effect of the *T. ulmifolia* extract on cytokine release in unstimulated RAW 264.7 macrophages, *** – $p<0.001$, ** – $p<0.01$, * – $p<0.05$, with respect to the control

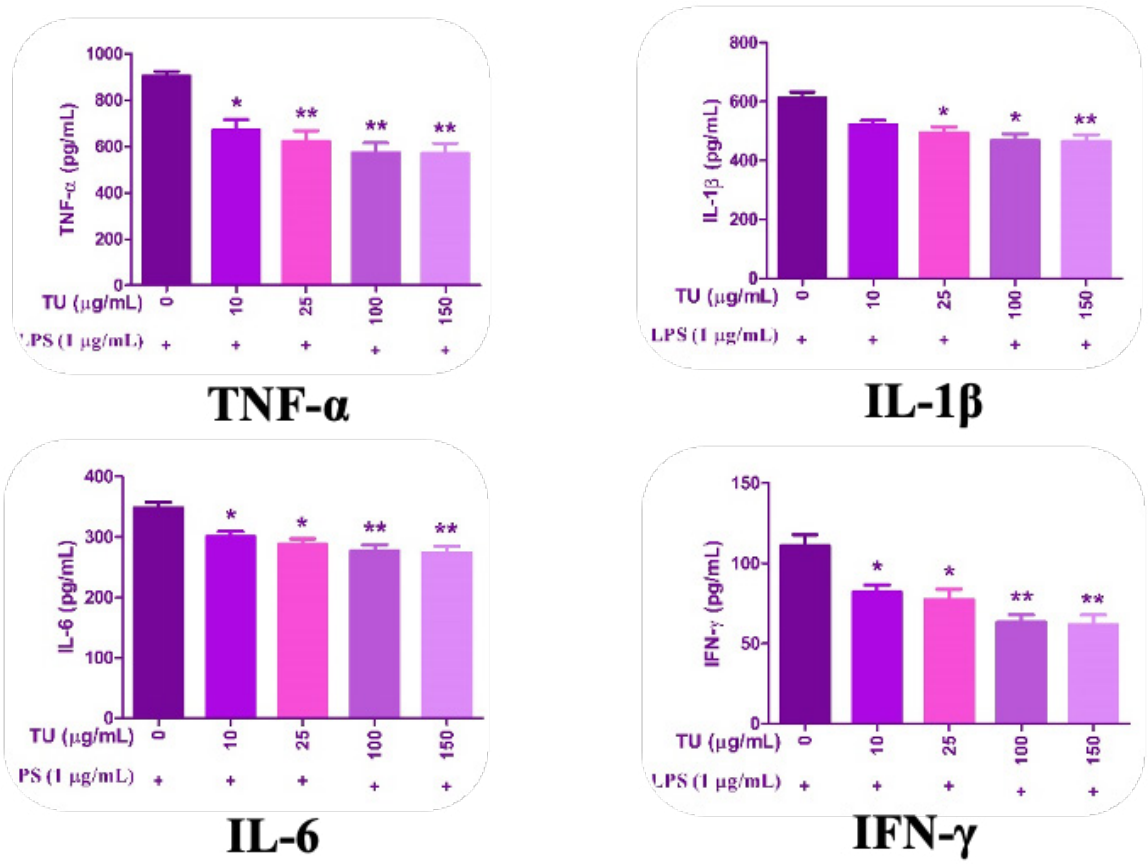

Fig. 3. Impact of the *T. ulmifolia* extract on the production of cytokines by RAW 264.7 macrophages stimulated by LPS, ** – p<0.01, * – p<0.05, with respect to the control

tory mediator NO by RAW 264.7 macrophages in both directions, therefore having immunomodulatory effects.

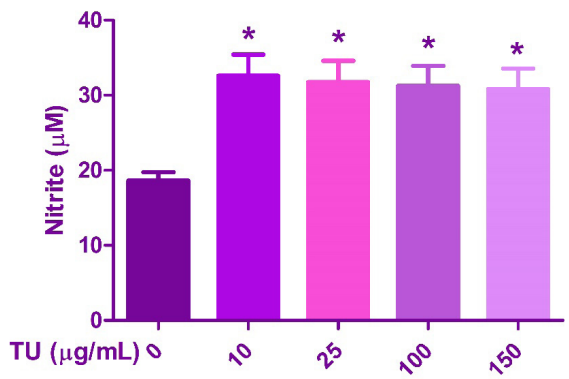

Fig. 4. Effects of *T. ulmifolia* extract on NO production in non-stimulated RAW 264.7 macrophages, * – p<0.05, with respect to control

Immunomodulatory effects of the T. ulmifolia extract in vivo

Effects of T. ulmifolia extract on mice's macrophage phagocytic activity

The phagocytic index was significantly lower in the DC group (2.5±0.12) compared to the normal control group (NC) group (3.6±0.17), reflecting immune suppression in the DC group. However, treatment with *T. ulmifolia* extract restored phagocytic activity, with the TU-200 group showing a phagocytic index comparable to the NC group (3.6±0.17) and the TU-400 group showing a slight decrease (3.4±0.078). This suggests that *T. ulmifolia* extract enhances the innate immune response by promoting macrophage phagocytic function, particularly at the dose of 200 mg/kg (Fig. 6).

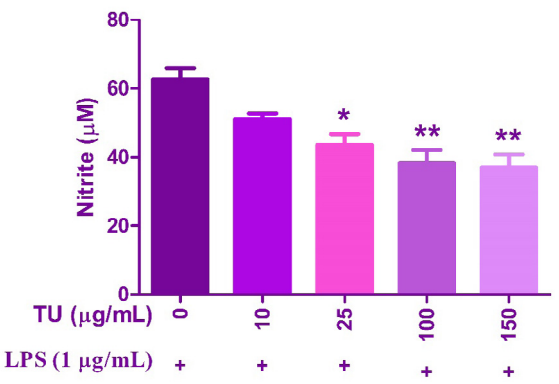

Fig. 5. Effects of the *T. ulmifolia* extract on NO production in RAW 264.7 macrophages stimulated by LPS, ** – p<0.01, * – p<0.05, with respect to the control

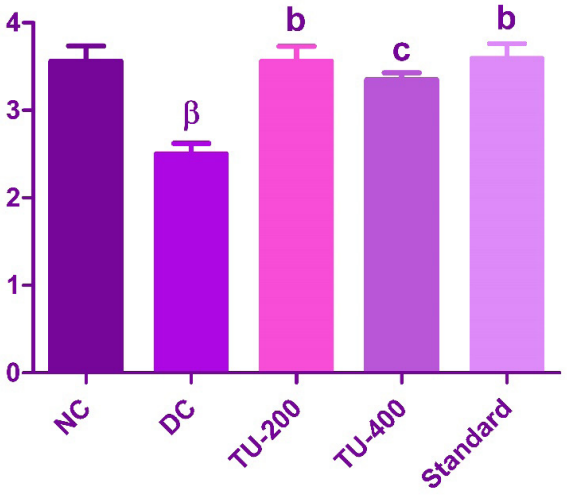

Fig. 6. Effects of *T. ulmifolia* extract on macrophage phagocytic function, $^{\beta}$ – p<0.01, when compared to the NC group, ^{b} – p<0.01, ^{c} – p<0.05, when compared to the DC group

Impact of T. ulmifolia extract on mice with DTH

Footpad thickness, a measure of the DTH response, was significantly reduced in the DC group (0.73±0.12 mm) compared to the NC group (1.5±0.07 mm), indicating an altered immune response. Both the TU-200 (1.3±0.10 mm) and TU-400 (1.2±0.092 mm) groups showed a significant increase in footpad thickness compared to the DC group, demonstrating that the extract of *T. ulmifolia* helps restore cell-mediated immunity in immunosuppressed mice. However, the response was slightly lower than in the NC group, indicating a partial but significant recovery of immune function (Fig. 7).

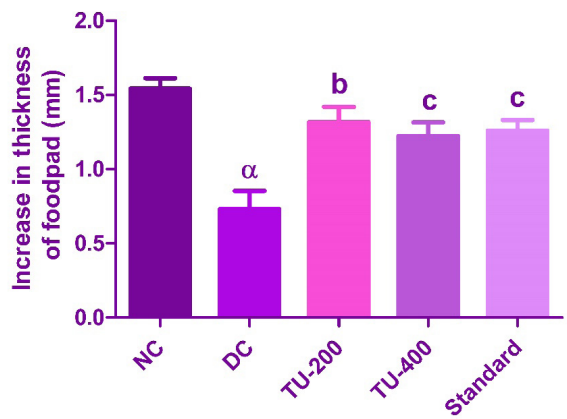

Fig. 7. Effects of *T. ulmifolia* extract on delayed-type hypersensitivity, $^{\alpha}$ – p<0.001, when compared to the NC group, ^{b} – p<0.01, ^{c} – p<0.05, when compared to the DC group

Effects of T. ulmifolia extract on serum hemolysin level in mice

Serum hemolysis levels were markedly lower in the DC group (0.16±0.024 OD value) compared to the NC group (0.31±0.035 OD value), showing reduced humoral immune function. Treatment with extracts of *T. ulmifolia* significantly increased serum hemolysis levels, with TU-200 (0.30±0.021 OD value) and TU-400 (0.28±0.021 OD value) approaching the levels of the NC group. This indicates that *T. ulmifolia* extract effectively enhances the humoral immune response, probably by increasing antibody production (Fig. 8) .

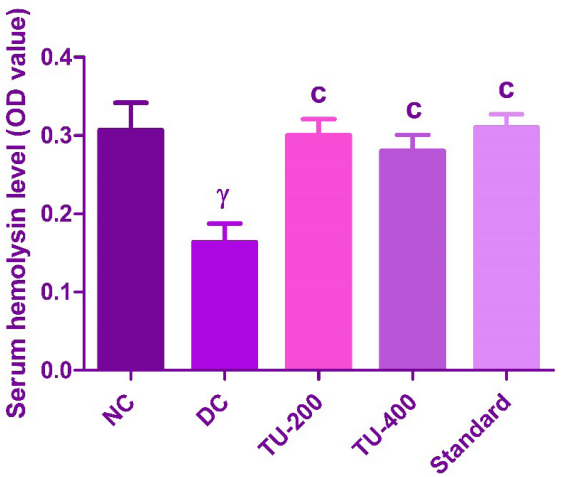

Fig. 8. Effects of *T. ulmifolia* extract on serum hemolysin level, $^{\gamma}$ – p<0.01 when compared to the NC group, ^{c} – p<0.05 when compared to the DC group

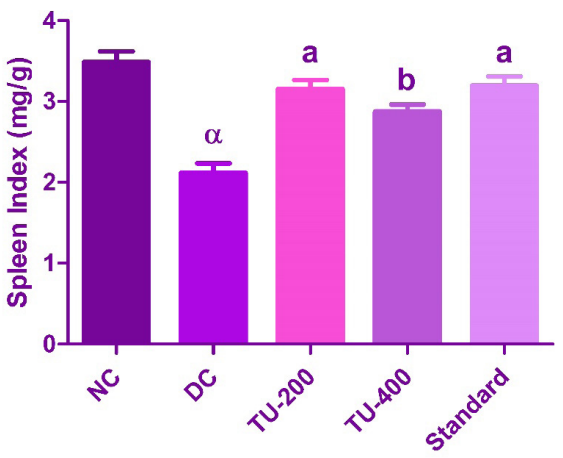

Fig. 9. Effects of *T. ulmifolia* extract on spleen index, $^{\alpha}$ – p<0.001, when compared to the NC group, ^{a} – p<0.001, ^{b} – p<0.01, when compared to the DC group

Effects of T. ulmifolia extract on immune organ index in mice

The thymus and spleen indices (Fig. 9 and Fig. 10), which serve as indicators of immune organ health, were

significantly lower in the DC group (1.2±0.091 mg/g for thymus, 2.1±0.12 mg/g for spleen) compared to the NC group (2.7±0.17 mg/g for thymus, 3.5±0.14 mg/g for spleen), reflecting immunosuppression. Both thymus and spleen indices increased significantly in the TU-200 and TU-400 groups, and the TU-200 group showing values closer to those of the NC group.

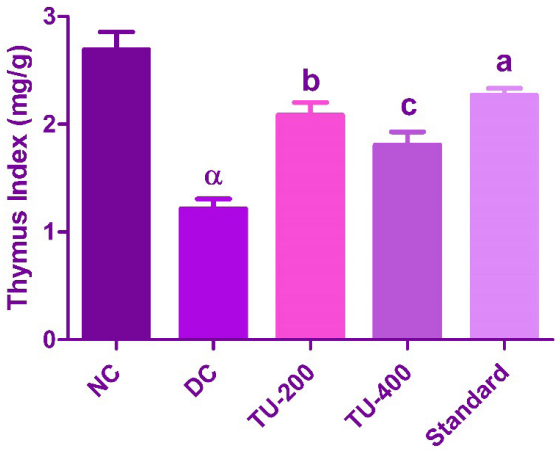

Fig. 10. Effects of *T. ulmifolia* extract on the thymus index, [α] – p<0.001 when compared to the NC group, [a] – p<0.001, [b] – p<0.01, [c] – p<0.05, when compared to the DC group

Discussion

The RAW 264.7 cell line is a valuable tool for assessing *in vitro* the immunomodulatory effects of numerous compounds. Among the immunomodulatory agents secreted by these macrophages are cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and leukocyte adhesion molecules. Together, these chemicals promote the development of T and B lymphocytes, trigger the phagocytosis process in macrophages, and kill germs as part of secondary immune responses.[11] Cytokines play an essential role in modulating immune responses.[12] TNF-α is a tumor necrosis factor and a vital host regulatory molecule.[13] Macrophages release TNF-α and IL-1β, categorized as "early response cytokines", in reaction to inflammatory stimuli. These cytokines help raise endothelial cells' adhesion molecule levels, facilitating the migration and movement of phagocytes to tissue damage sites.[14] In contrast, IL-6 is essential for the host's immunological response, rapidly synthesizing proteins, and maintenance of homeostasis.[15,16]

Inflammatory cytokine overproduction can have detrimental effects, such as systemic exposure that may be fatal and collateral to normal cells. However, it is vital to remember that tissue healing and host survival from infections depend on the release of inflammatory cytokines.[17,18]

Although a volatile gas, NO is essential for several physiological functions, including inflammation, neurotransmission, and immunological responses.[19] It shows a dual biological role in affecting the activity of resident cells, tumor cells, and immune cells in many tissues and organs at suitable concentrations or, at low levels, acting as neurotransmitters.[20] On the other hand, excessive or uncontrollably released NO can cause damage to inflammatory tissue and host cell death. Therefore, controlling NO production may help reduce inflammatory and immunological disorders.[21]

In phagocytosis, specialized cells, called phagocytes, take in foreign substances and destroy them, including bacteria, tumor cells, inorganic fragments, and tissue waste. It is critical to the defense mechanisms. Phagocytes, which include neutrophils, monocytes, and macrophages, are among the first to the invasion of respond to a pathogenic organism.[22] When the epithelial barrier is penetrated, macrophages, ancient and evolutionary conserved cells in multicellular creatures, act as the first line of defense. A reflection of the phagocytic activity of the mononuclear phagocytic system, the macrophage phagocytic index is regarded as a diagnostic for identifying nonspecific immunity.[23] T-lymphocytes can develop into sensitized lymphocytes in response to antigen presentation, which can cause aberrant reactive inflammation to arise locally. This allergic inflammation exhibits necrosis and cell degradation and has a delayed start.[24] The presence of serum hemolysin antibodies can be used to estimate the degree of humoral immunity accurately. In reaction to different antigens, B-lymphocytes release this antibody.[25] This suggests that the *T. ulmifolia* extract helps restore immune organ health, particularly at the dose of 200 mg /kg, supporting the overall immune response.[26]

The results of this study reveal the significant immunomodulatory activity of the hydroalcoholic extract of *T. ulmifolia* in both *in vitro* and *in vivo* models. In vitro experiments using RAW 264.7 murine macrophage cells demonstrated that the extract could enhance the production of key cytokines (TNF-α, IL-1β, IL-6, and IFN-γ) and NO without causing cytotoxic effects. The increase in cytokine production indicates that *T. ulmifolia* extract has an immunostimulatory effect on macrophages, which are critical players in the innate immune response. However, it is essential to note that the extract also exhibited anti-inflammatory activity by inhibiting the overproduction of cytokines and NO in LPS-stimulated cells. This dual immunomodulatory and anti-inflammatory effect is significant because an excessive inflammatory response can lead to tissue damage and other adverse effects. At the same time, cytokines are necessary for effective immune function.[27] In *vivo* studies further support the immunomodulatory potential. The extract enhanced macrophage phagocytic activity, as observed in the carbon-clearance test in mice. This enhancement of the innate immune response was par-

ticularly evident at the 200 mg/kg dose, suggesting an optimal concentration for the efficacy of the extract. Additionally, the extract positively influenced cell-mediated immunity, as shown by the increased DTH response in immunosuppressed mice. The improvement in the DTH response points to the role in restoring cell-mediated immunity, an essential aspect of the immune system's ability to recognize and respond to foreign antigens.[28] The study also found that *T. ulmifolia* increased serum hemolysin levels, boosting the humoral immune response. The production of hemolysin antibodies by B-lymphocytes is a vital part of the body's defense mechanism against pathogens, and the ability to elevate these levels highlights its potential as an immunostimulant. Furthermore, the increase in thymus and spleen indices in treated mice suggests that the extract may positively impact the health and functionality of these critical immune organs.[29,30] Overall, the findings of both *in vitro* and *in vivo* models suggest that the extract of *T. ulmifolia* has significant immunomodulatory effects, enhancing immune responses and providing anti-inflammatory benefits. The biphasic effect of stimulating cytokine production in unstimulated cells and inhibiting excessive cytokine production in activated cells indicates that the extract can modulate immune responses in a balanced manner. These properties make *T. ulmifolia* extract a promising candidate for developing therapeutic agents targeting immune-related conditions, where fine-tuning of the immune system is crucial. More research is needed to isolate the active compounds responsible for these effects and to elucidate the underlying mechanisms of action.

Conclusion

The findings of this study demonstrated that the *T. ulmifolia* extract has significant immunomodulatory effects, both *in vitro* and *in vivo*. *In vitro* studies revealed that the extract improved cytokine production and nitric oxide levels in unstimulated RAW 264.7 macrophage cells while inhibiting the overproduction of these immune mediators in LPS-stimulated cells, thus exhibiting immunostimulatory and anti-inflammatory properties. Furthermore, in vivo studies in BALB/c mice showed that the *T. ulmifolia* extract improved macrophage phagocytic function, increased DTH response, and elevated serum hemolysin levels, further indicating a strong immunostimulatory effect. The extract also improved the thymus and spleen indices, suggesting its ability to enhance overall immune responses. These results highlight the potential of *T. ulmifolia* extract as an effective immunomodulatory agent with promising applications in the management of immune-related disorders.

Declarations

Funding

The authors received no external funding.

Author contributions

Conceptualization, V.S. and G.B.S.R.; Methodology, V.S.; Software, V.S.; Validation, V.S. and G.B.S.R.; Formal Analysis, V.S.; Investigation, V.S.; Resources, G.B.S.R.; Data Curation, V.S.; Writing – Original Draft Preparation, V.S.; Writing – Review & Editing, G.B.S.R.; Visualization, V.S.; Supervision, G.B.S.R.; Project Administration, G.B.S.R.; Funding Acquisition, G.B.S.R.

Conflicts of interest

The authors declare that they have no potential conflicts of interest regarding the research, authorship, and publication of this article.

Data availability

All data generated or analyzed during this study are included in the published article. Any additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

Ethical approval for the study was obtained from Flair Labs, located at PLOT NO B 510, PALSANA, Surat-394315, Gujarat, India, under the registration number 1250/PO/RcBi/S/23/CPCSEA.

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ORIGINAL PAPER

Outcome prediction criteria for multiple trauma patients with combined cranio-thoracic injuries

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ABSTRACT

Introduction and aim. Blunt chest trauma and traumatic brain injury are considered two of the most significant injury entities with a high potential for complications. In the early post-traumatic period, trauma care frequently encounters limitations in diagnostic capabilities within trauma centers. The objective of this study was to develop simple signs to predict outcomes at three time points during the early post-traumatic period for patients with multiple blunt trauma with combined cranio-thoracic injuries.

Material and methods. This retrospective cohort study was conducted on 51 polytraumatized male patients. Examinations of the patients were performed on the 1st–2nd, 3rd–4th,and 5th–6th day after trauma. Mortality was set as the primary outcome. Receiver operating characteristic curve analysis was used to investigate the predictive capacity of the estimated markers for each time period.

Results. The most significant differences between survivors and non-survivors on the 1st to 2nd day after trauma were observed in terms of SpO_2/FiO_2 index, hemoglobin and red blood cell count. On the 3rd–4th day – SpO_2/FiO_2 index. The oxygen content, SpO_2/FiO_2 index and hemoglobin exhibited the greatest disparity between patients groups on the 5th–6th day.

Conclusion. A set of criteria can be employed to monitor the clinical course of multiple trauma patients with combined cranio-thoracic injuries. The predictive value of special markers varies depending on the time period. Each of the investigated time periods is characterized by its own specific predictive signs. The predictive capacity of the estimated markers varies depending on the time period under consideration. It is not an accurate approach to employ the same predictive markers throughout the entire posttraumatic period.

Keywords. blunt injury, clinical decision rules, critical care, hospital mortality, multiple trauma

Introduction

Blunt chest trauma and traumatic brain injury (TBI) are considered two of the most significant single injury entities with a high potential for complications.[1–3] It is clear that the integrity of the respiratory system is crucial in providing optimal respiratory care to patients with moderate to severe TBI.[4] Chest trauma is a significant contributor to mortality and the development of complications in multiple trauma cases. It is an independent predictor of mortality in trauma patients, regardless of the severity of

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the trauma.[5] There is a lack of evidence on the impact of chest trauma on functional outcome after TBI, but what evidence does exist suggests chest trauma is a leading determinant of adverse outcome after multiple injuries.[1,4,6]

It is imperative that all traumas, including those with multiple cranio-thoracic injury, be managed in a systematic way, with the involvement of a highly experienced, interdisciplinary trauma team with expertise in critical care, anesthesia and surgical disciplines.[7] This is essential for ensuring high-quality management and low morbidity and mortality rates.[8]

The lack of a universal scoring system for predicting mortality in multiple trauma patients, particularly in the context of TBI and severe thoracic trauma, is a consequence of the challenging applicability of some scales, as well as resource limitations and the lack of predictive outcome significance.[9]

The pathophysiology of multiple organ dysfunction syndrome (MODS) after polytrauma represents a complex network of interactions between the immune, endocrine, neural, and other systems.[10–12] There are multiple interactions between the mechanisms responsible for harmful effects and involved in the progression of MODS, on the one hand, and compensatory reactions directed to maintain homeostasis during the early posttraumatic period, on the other hand.[13–15] In this setting, the same laboratory or clinical markers cannot predict the prediction of the outcome during different time periods.

Another issue related to trauma care in the early post-traumatic period is the limited diagnostic capabilities of the initial, secondary, and tertiary trauma centers. In low- and middle-income countries, such as Ukraine, the diagnostic capabilities of these centers are particularly limited. The optimal prognostic tool should therefore be based on simple predictors that can be easily calculated by different healthcare professionals in different settings, allowing for the correct interpretation by those with varying levels of expertise.[16]

Therefore, the issue becomes that of developing simple and dynamic outcome predictive signs to prepare more effective management strategies for such patients by the trauma team at the initial clinical presentation and the early post-traumatic period.

Aim

The objective of this study was to develop simple signs to predict outcomes at three time points during the early posttraumatic period for patients with multiple blunt trauma with combined cranio-thoracic injuries.

Material and methods

Study design

Data for this retrospective cohort study were extracted from the previous single center prospective observational cohort study that was conducted in the Department of Anaesthesiology and Intensive Care for Patients with Combined Trauma of the Kharkiv City Clinical Hospital of Emergency Aid named by prof. O.I. Meshchaninov.[17] A total of 51 male patients with combined TBI and severe thoracic trauma, as defined by the inclusion criteria, were included in the study cohort. All subjects gave their informed consent for inclusion before participating in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Kharkiv National Medical University (N8/2016, October 5, 2016).

Patient selection and data collection

The inclusion criteria were the following: age ≥18 years, injury severity score (ISS) ≥16, two or more injured body regions, presence of TBI and abbreviated injury scale (AIS) thorax 3 after blunt trauma. The presence of a concomitant chronic disease in the subcompensation or decompensation phase, as well as penetrating injuries, was identified as exclusion criteria. A diagnostic evaluation was carried out for all patients according to existing protocols, and their treatment was conducted in accordance with the Advanced Trauma Life Support Program. Laboratory tests and clinical examination were performed on the 1st–2nd (11–33 hours), 3rd–4th (48–75 hours) and 5th–6th (97–122 hours) days after trauma during their management in the ICU.

In-hospital mortality was defined as the endpoint of the study. The survival to non-survival ratio of polytraumatized patients was 24 to 27 on the 1st–2nd day, 24 to 20 – on the 3rd–4th and 24 to 18 – on the 5th–6th day after trauma.

The severity of polytrauma was evaluated using the AIS, ISS, revised trauma score (RTS), and trauma and injury severity score (TRISS) scales.[18–20] The Kigali modification of the Berlin criteria has been used for the diagnosis of ARDS.[21]

Statistical analysis

All data are presented as the median with 95% contingency interval for ordinal variables, the mean± standard deviation for continuous variables, and the number with percentage for categorical variables. Fisher's exact test, the chi-square test for trends and the Mann-Whitney U test were employed to compare demographic and laboratory data using GraphPadPrism 5.03 (GraphPad Software, California, USA). The receiver operating characteristic (ROC) curves were constructed for variables that showed statistically significant differences, and the cutoff values were calculated according to Youden's index.[22] All p-values were calculated using a two-sided test. A value of $p<0.05$ was considered to indicate statistical significance.

Table 1 presents a summary of the main demographic characteristics of the patient groups.

Table 1. Characteristics of the survival and non-survival groups of multiple trauma patients with combined cranio-thoracic injuries

		Survivors n=24	Non-survivors n=27	p
Age, years		39.5 (35.92–44.16)	40 (34.85–45.59)	0.9813
ISS score		27.5 (24.85–32.31)	34 (31.09–39.87)	0.0324
RTS score		7.84 (6.607–7.672)	6.084 (5.219–6.413)	0.0005
TRISS score		0.9442 (0.8085–0.9603)	0.7179 (0.5479–0.7647)	0.0004
Admission time, hours		1.127±1.275	1.933±4.184	0.8366
GCS at admission		14 (11.36–14.06)	12 (8.337–11.66)	0.0059
Systolic BP on admission, mmHg		107.3±24.98	94.44±39.06	0.0676
Number of patients with concomitant alcohol exposure		11 (45.8%)	13 (54.2%)	0.999
Controlled mechanical ventilation >48 h		7 (26.9%)	19 (73.1%)	0.005
ARDS		5 (20.8%)	21 (77.8%)	<0.0001
Injury severity, n (%)				
AIS skin	0	9 (37.5%)	12 (44.4%)	
	1	14 (58.3%)	13 (48.2%)	0.8224
	2	1 (4.2%)	2 (7.4%)	
AIS head	1	10 (41.6%)	7 (25.9%)	
	2	1 (4.2%)	1 (3.8%)	
	3	9 (37.5%)	7 (25.9%)	0.0518
	4	3 (12.5%)	6 (22.2%)	
	5	1 (4.2%)	6 (22.2%)	
AIS facial	0	18 (75%)	22 (81.5%)	
	1	5 (20.8%)	3 (11.1%)	
	2	0	1 (3.7%)	0.8504
	3	1 (4.2%)	1 (3.7%)	
AIS thorax	3	5 (20.8%)	3 (11.1%)	0.4505
	4	19 (79.2%)	24 (88.9%)	
AIS abdomen	0	10 (41.7%)	10 (37%)	
	1	6 (25%)	6 (22.2%)	
	2	0	2 (7.4%)	0.6731
	3	5 (20.8%)	4 (14.8%)	
	4	3 (12.5%)	5 (18.5%)	
AIS extremities	0	8 (33.3%)	8 (29.7%)	
	1	3 (12.5%)	2 (7.4%)	
	2	6 (25%)	7 (25.9%)	0.4984
	3	7 (29.2%)	9 (33.3%)	
	4	0	1 (3.7%)	
Mechanism of injury, n (%)				
Car driver		11 (45.8%)	3 (11.1%)	
Bicycle accident		3 (12.5%)	2 (7.4%)	
Car passenger		1 (4.2%)	3 (11.1%)	
Pedestrian		5 (20.8%)	5 (18.5%)	
Falls from height		4 (16.7%)	11 (40.8%)	0.0015
Assault		0	1 (3.7%)	
Crushed by the heavy object		0	1 (3.7%)	
Injury by manufacture machines		0	1 (3.7%)	

Results

The 51 patients were admitted to the ICU after primary surgery, depending on the injuries suffered. The first examination with laboratory tests (on the 1st–2nd day) was performed the next morning after resuscitation measures.

There were no statistically significant differences between survivors and non-survivors with respect to age, time of admission, number of patients with concomitant alcohol exposure, systolic blood pressure at admission, or the severity of injury to the involved body regions (Table 1). The most common cause of trauma among the survival cohort occurred in car drivers. While falling from height was the main etiological factor among non-survivors. In five victims of this etiological group, polytrauma included trauma to the spine: 3 at the thoraco-lumbar level; 1 at the lumbar level and 1 – at the thoracic level. In the survivor group, a patient had lumbar spine trauma as a result of a pedestrian accident. The greatest discrepancy was observed among the severity scales was observed between patient populations with respect to the TRISS model.

Table 2. Dynamic physiological data in the blunt multiple trauma patients with combined cranio-thoracic injuries*

	Groups	The 1st–2nd day	The 3rd–4th day	The 5th–6th day
SpO_2,% (mean±SD)	S	95.08±0.9834	93.25±1.3	94.29±0.8437
	NS	96.15±0.7115 p=0.4476	95.55±0.6746 p=0.4415	92.61±2.666 p=0.7958
SpO_2/FiO_2 (mean±SD)	S	367.6±18.27	386.8±17.1	387.4±18.5
	NS	234±8.63 p<0.0001	235.8±16.98 p<0.0001	227±18.37 p<0.0001
Hemoglobin, g/L (mean±SD)	S	115.2±3.504	100.4±3.941	107.3±2.828
	NS	98.39±4.476 p=0.0063	90.15±4.004 p=0.1108	93.59±2.929 p=0.0018
Hematocrit,% (mean±SD)	S	36.29±1.189	30.24±1.219	31.88±0.8797
	NS	31.06±1.474 p=0.0127	28.73±1.117 p=0.4165	29.72±0.921 p=0.1298
Red blood cell count, $\times 10^{12}$/L (mean±SD)	S	3.915±0.1048	3.455±0.1175	3.724±0.08523
	NS	3.376±0.1396 p=0.002	3.254±0.1404 p=0.4098	3.397±0.09409 p=0.0322
Oxygen content, mL/L (mean±SD)	S	142.5±4.852	121.9±5.278	131.2±3.214
	NS	122.9±5.682 p=0.0206	111.9±4.945 p=0.2583	112.7±4.885 p=0.0037

* SD standard deviation, S – group of survived patients, NS group of patients who did not survive

The dynamics of the investigated variables are not consistent over time or between survivors and non-survivors (Table 2). Laboratory markers and polytrauma severity scales were selected to perform the ROC-analysis based on the degree of difference between groupings of patients for each time period. Figure 1 shows the ROC-curves, exhibiting the highest values of the Area under the ROC curve (AUROC). The TRISS model demonstrated the highest value of AUROC among polytrauma severity scales (0.7824 (0.6520 to 0.9128); p=0.0006). Furthermore, the AUROC for the GCS score on admission to the trauma center was 0.722 (0.5799 to 0.8640) with p=0.0072. According to the statistics of the contingency table, the TRISS model with a cut-off value of <0.8339 (odds 11.88 (2.927 to 37.97), p=0.0002) is the most influential among polytrauma predictive models

in determining the probability of mortality for patients with multiple trauma with combined cranio-thoracic injuries. The GCS score at admission, with a cut-off value of <13, was found to have an odds ratio of 4.091 (1.208 to 12.49), with a p-value of 0.0246.

The most pertinent predictive marker of unfavorable outcome for patients with multiple trauma with combined cranio-thoracic injuries during the investigated polytrauma period was the SpO_2/FiO_2 index. However, its AUROCs and cutoff values were different for each time period: <308 for the 1st–2nd day (AUROC 0.9074 (0.8259 to 0.9889); p<0.0001; odds 78 (9.333 to 835.9), p<0.0001), <325 for the 3rd-4th day (AUROC 0.9046 (0.8171 to 0.9921); p<0.0001; odds 46.14 (5.509 to 502.9), p<0.0001) and <356.7 for the 5th–6th day of the early posttraumatic period (AUROC 0.8854 (0.7851 to 0.9857); p<0.0001; Odds 36 (4.381 to 395.1), p<0.0001).

Furthermore, hemoglobin concentration and red blood cell count demonstrated a significant predictive influence on survival on the 1st–2nd day 1 after admission to the ICU. The AUROC for hemoglobin concentration was 0.7207 (0.5813 to 0.8601), p=0.007 with a cutoff value of <104 (odds ratio 4.75 (1.349 to 15.28), p=0.0206). For the count of RBC on the 1st-2nd day the AUROC was 0.7477 (0.6115 to 0.8838), p=0.0025 with a cutoff value of $<3.845 \times 10^{12}$/L (odds 7.333 (1.984 to 25.09), p=0.0018).

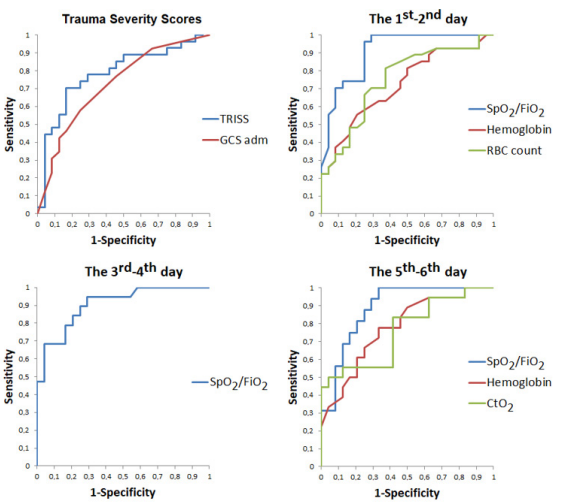

Fig. 1. The ROC curves of the predictive markers during early posttraumatic period in case of multiple trauma with craniothoracic injuries, GCS adm – GCS at the time of admission to the hospital, CtO_2 – oxygen content of the blood

The highest value of AUROC was estimated for hemoglobin concentration on day following trauma (0.7766 (0.6354 to 0.9178); p=0.0024), with a cut-off value of <101.5 g/L (odds ratio 7 (1.835 to 23.36), p=0.0058). The probability of survival can also be evaluated according to the level of CtO_2 on day after trauma, with an AUROC degree of 0.7593 (0.6089 to 0.9096), p=0.0044 and cut-off value <109 ml/L with odds ratio 23 (3.233 to 260.5), p=0.0008.

It is also pertinent to consider the impact of pulmonary complications on survival in patients with multiple trauma with severe craniothoracic injuries. The prolonged use of CMV in this category of polytrauma patients has a negative impact on the outcome, with an odds ratio of 5.768 (1.719 to 18.60), p=0.005. Furthermore, the appearance of ARDS symptoms during the first 5-6 days after trauma is a more severe predictor of mortality, with a odds ratio of 13.30 (3.305 to 54.16), p<0.0001. The AUROCs are presented in Figure 1 for comparison.

Discussion

Multiple body regions and organs are often damaged in high-impact trauma. Severe extracranial injuries to the chest, abdomen and extremities occur in about one third to one-half of TBI cases.[6]

Automobile accidents represent the most common cases of blunt injuries, as evidenced by the present study (Table 1) and other studies conducted by other authors.[4,23,24] In this study, the most prevalent mechanisms of injury were falls from height, followed by vehicular collisions and pedestrian accidents. In addition, there were statistically significant differences between the groups in terms of the the etiology of trauma. Among the patients in the survival cohort, the driver of a car was the most common cause, while among nonsurvivors, falling from height was the main etiology factor (Table 1). One possible explanation for this finding is that the study included patients with only multiple trauma and combined cranio-thoracic injuries.

Among polytrauma severity scales, the TRISS scale exhibits the highest predictive power, which is not surprising given that it incorporates both the RTS and the ISS scales into its equation, thus improving mortality prediction accuracy.

Our findings suggest that the dynamics of SpO_2/FiO_2 can serve as an indicator of disease progression during the early posttraumatic phase of patients with blunt multiple trauma with combined cranio-thoracic injuries. Furthermore, it was shown that the necessity for prolonged mechanical ventilation and the appearance of signs of ARDS signs during the early posttraumatic period are reliable indicators of a poor prognosis in the case of multiple traumas involving combined cranial and thoracic injuries. It is established that people who have multiple trauma who also have injuries to the thorax tend to have longer periods of mechanical ventilation (2 vs. 8 days) and longer stays in intensive care (4 vs. 11 days) than patients who have suffered polytrauma but without injuries to the thorax.[11]

Another notable observation is the hemoglobin concentration cutoff value of <104 g/L on the first 2nd day following trauma. The observed value exceeds the threshold specified in polytrauma transfusion guidelines.[25] A review of the existing literature suggests that red blood cell transfusions in patients with TBI with hemoglobin levels above 100 g/L are not warranted.[26] However, in the present study a different subset of patients with combined cranio-thoracic injuries was included. This finding suggests that these patients may benefit from a liberal transfusion strategy. More randomized controlled studies are required to confirm this value of hemoglobin concentration as a potential target in the context of a standardized management approach for this population of patients with polytrauma.

In contrast to expectations, the cutoff value of the CtO_2 acquires high predictive importance only on the 5th-6th day after trauma. The aforementioned outcome indicates that prolongation of combined hypoxia (arising from both anemia and reduction in hemoglobin saturation) during the initial posttraumatic phase of multiple trauma patients with combined cranio-thoracic injuries is more detrimental than the degree of this hypoxia itself.

It is of paramount importance to improve the accuracy of medical diagnosis, treatment, and monitoring to optimize the outcomes of multitrauma patients, particularly those with combined cranio-thoracic injuries. This is of particular significance in contemporary, multitiered medical trauma services, where the quantity of data received about the traumatic victim significantly impacts the precision of decisions made by critical care physicians.[27] The full potential of risk prediction models for use in ICUs has yet to be realized. These models have the potential to support decision-making and diagnosis in critical care.[28]

The additional criteria enable trauma team members to provide more objective guidance for decisions on the survival of multiple trauma patients with combined craniothoracic trauma. This is based on simple clinical and laboratory data obtained during the early post-traumatic period. The judicious application of current concepts and management protocols may be expected to ensure the best outcomes.[3] Furthermore, it allows for the comparison of patients from different trauma centers, classified according to the level of trauma care provided. This can be beneficial in facilitating prompt and precise decisions regarding interhospital transfers. Comparison of estimated to observed mortality rates can be used as an evaluation criterion and as a means of monitoring the quality of intensive care unit work.

Study limitations

As with other retrospective studies, this study is subject to certain limitations. This is a single-center study and the results require validation in other trauma centers and regions. A further limitation is that the groups are similar in age, which precludes the possibility of analyzing how this parameter contributes to the prediction process. Previous studies have indicated that age is a relevant factor in the context of mortality risk in polytrauma.[5] However, other studies have found that this contribution is not statistically significant.[29–31] Additionally, the current study is based on a relatively small sample of participants. Another limitation of the study is that the prediction criteria were calculated for each time period on the basis of varying numbers of participants and an increasing ratio of survivors to nonsurvivors. However, the results of the discrimination statistics indicate that the proposed criteria can accurately define patients with a high risk of negative outcome. Despite these limitations, our results appear to be significant, agree with other clinical and experimental studies, and we hope that our scoring method can improve the management of patients with blunt multiple trauma with combined cranio-thoracic injuries during the early post-traumatic period.

Conclusion

A series of proposed predictive markers was developed with the intention of assisting in the estimation of individual mortality risk in patients presenting with blunt multiple trauma with combined cranio-thoracic injuries. These markers were developed based on the results of routine diagnostic tests performed daily in the ICU during the first five to six days following the traumatic event. A simple set of criteria can be employed to monitor the clinical course of polytraumatized patients and to identify those at high risk for negative outcomes, thus improving patient care during the early intensive focused phase of treatment. The predictive value of clinical and laboratory markers varies depending on the time period of the initial phase of intensive care following traumatic injury. Each of the investigated time periods is characterized by its own specific predictive signs. The predictive capacity of the estimated markers varies depending on the time period under consideration. It appears that employing the same predictive markers throughout the early posttraumatic period for patients with multiple trauma with cranio-thoracic injuries is not an accurate approach.

Declarations

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Author contributions

Conceptualization, M.S.; Methodology, M.S.; Software, M.S.; Validation, M.S. and O.B.; Formal Analysis, M.S.; Investigation, M.S. and O.B.; Resources, M.S. and O.B.;

Data Curation, M.S.; Writing – Original Draft Preparation, M.S.; Writing – Review & Editing, M.S. and O.B.; Visualization, M.S.; Supervision, O.B.; Project Administration, M.S.

Conflicts of interest

The authors declare no competing interests.

Data availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All subjects gave their informed consent for inclusion before participating in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Kharkiv National Medical University (N8/2016, October 5, 2016).

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ORIGINAL PAPER

Hepatoprotective effect of *Costus afer* (Lin) on toxic metal mixture treated rats mediated by regulation of oxidative stress markers, inflammatory cytokines and bio-metal chelation

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ABSTRACT

Introduction and aim. Cadmium, lead, and mercury have been considered to exhibit their hepatotoxic effects by oxidative induction damage and the generation of reactive oxygen species (ROS). The current work evaluated the protective activity of aqueous leaf extracts of *Costus afer* (ALECA) on liver damage arising from exposure to toxic metal mixture (TMM): 1.61 mg/kg cadmium chloride ($CdCl_2$), 20 mg/kg lead chloride ($PbCl_2$), and 0.40 mg/kg mercury chloride ($HgCl_2$).

Material and methods. Five groups of weight-matched Sprague Dawley rats were treated for 90 days. Metal mixtures and deionized water were used to treat the 2 groups of rats whereas the other 3 groups were treated with various doses of the ALECA through oral gavage with TMM. Hepatic function parameters, oxidative biomarkers, inflammatory cytokines, morphological changes, and metal levels in the liver were monitored.

Results. Treatment with TMM resulted in significant increases in alanine transaminase, aspartate transaminase, alkaline phosphatase, bilirubin, interleukin 6, malondialdehyde, but decreased albumin, total protein, interleukin 10, superoxide dismutase, catalase, and glutathione levels. TMM also caused some morphological changes and increased the concentrations of heavy metals (Pb, Cd, and Hg) in the liver.

Conclusion. ALECA showed beneficial effects against TMM-induced hepatotoxicity via metal chelation, anti-inflammatory, and antioxidant mechanism. ALECA may be beneficial in the management of liver toxicity.

Keyword. *C. afer,* environment, exposure, heavy metal mixture, hepatotoxicity

Introduction

Trace metals occur from both natural and human activities. The continued use of metals in various industries like agriculture, medicine, technology, etc. has posed both public and eco-health concerns.[1] Metals are systemic toxic agents known to cause organ damage even at low exposure levels. Lead (Pb), mercury (Hg) and cadmium (Cd) can be hepatotoxic.[2] These elements are

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ranked among metals of enormous concern to the public health (WHO).[3] Pb is a toxic metal found naturally in the Earth's crust.[4] The negative consequences of exposure to Pb exposure have been shown in some organs such as the brain, liver, kidney, heart, and systems, for example, reproductive, musculoskeletal, developmental and immunological systems.[5] Inorganic Hg induces neurotoxic, hematotoxic, cardiovascular, hepatotoxic, genotoxic and nephrotoxic effects.[6-12] Acute exposure to Cd affects the lungs by causing irritation, while chronic exposure leads to accumulation in the kidneys, liver and other organs resulting in dysfunction of the organs.[13-14]

Uptake and elimination of extraneous compounds are vital functions of the liver.[15] Hepatotoxicity implies hepatocyte impairment arising from the overload of chemicals and exogenous compounds that include heavy metals.[16] Owing to the dominance of these metals in the ecosystem, the long-term effect of a combination of lead, cadmium, and arsenic have been studied and liver toxicity reported by Bhattacharjee et al.[17] Treatment of rats with lead and cadmium have also resulted in liver damage.[18] Until now, the focus on the unfavorable health effects of metals is largely based on human case studies of chronic high exposure seen in the metal industry or in densely polluted environments. Occupational and environmental exposure to metals is common. Therefore, more studies on the consequences of chronic environmental trace metals and their mixtures in animal models that can be extrapolated to humans.[19] To mimic real-life situations, it is imperative to evaluate the toxicities of metal mixtures.

Chelation with meso-2,3-dimercaptosuccinic acid (Succimer or DMSA) and D, L-2,3-dimercapto-1-propanesulfonic acid (Dimaval or DMPS); the 2,3-dimercaptopropanol (British Anti Lewisite, BAL or Dimercaprol), Ethylenediamine-tetracetic acid (EDTA) and D-penicillamine remain the mainstay of the treatment of metal poisoning.[20,21] Despite their contribution in the treatment of metal poisoning, they also have some drawbacks, including toxicity issues, availability, and affordability concerns. These disadvantages constitute the need to provide effective and safe pretreatment therapy. Understanding the relevance of natural antidotes, considered 'generally considered safe' GRAS and given their affordability and availability, as chemopreventive agents in metal poisoning has gained traction recently.

The different pharmacological properties of *C. afer* have led to the study of its bioactive compounds that may show promise as drugs. Protection of the liver from noxious trace metal mixtures is considered worthwhile given its importance. The anatomical proximity of the liver to the intestines predisposes the liver to toxic attack. Hepatotoxicity refers to liver damage resulting from various chemicals and xenobiotics, including heavy metals and their metabolites.[22] Due to the ubiquity of metals in the environment, Bhattacharjee et al. evaluated the effects of long-term exposure at a low dose to a mixture of Cd, As, and Pb and concluded that chronic exposure to of heavy metal mixture at a very low environmentally relevant dose produced hepatotoxic effects in albino rats.[17] Hepatoxicity has also been reported by Yuan et al. to be among the toxicities resulting from the mixture of Pb and Cd on Sprague Dawley rats.[18] Hepatotoxicity is usually characterized by increased membrane permeability and changes in enzyme levels. Other liver damage, reported substantial decrease in serum ALP and AST at 400 mg/kg of *C. afer.*[23] The stem extract of *C. afer* was protective in alcohol-mediated liver damage in rats, suggesting a possible benefit in alcohol-mediated liver cirrhosis.[23-24] *C. afer* is a herbaceous, perennial and unbranched rhizomatous herb that belongs to the Zingiberaceae family. It grows up to 4 m high and has been proven in many studies to have strong therapeutic effects.[25] It is commonly found in shady or moist forests or riverbanks of tropical West Africa, including Cameroun, Ghana, and Nigeria.[26] It is commonly known as bush cane or ginger lily. Among Igbos in Nigeria, it is commonly called 'Okpoto', 'Okpete Ohia' or "Okpete".[27] The Hausas call it 'Kakizuwa', Yoruba call it "Tete-egun" and the Efik calls it 'Mbritem' 27 *C. afer* has been evidenced to have many therapeutic effects in humans and animals. Reports on the phytochemical analysis of *C. afer* revealed that the plant is rich in steroidal saponins, flavonoids, alkaloids, tannins, terpenoids, sapogenins, oxalates, furans, furan derivatives, and starches without any form of toxicity.[27] These phytochemicals are rich in antioxidants. Pharmacological activities associated with *C. afer* include antioxidant property, hepatoprotective, nephroprotective, antidiabetic, and antinociceptive role.[28-30]

Previous studies have shown that ALECA may be organoprotective (kidney and testis) in lead-mediated kidney and testes damage through antioxidant mechanisms.[23,31,32] As much as various studies have reported exposure of single heavy metal studies, there is insufficient information on the heavy metal mixtures that represent the real situation of these toxicants in various environmental matrices. Classical and synthetic metal chelators, which have become the mainstay of antidotal management of metal intoxication together with the numerous side effects, are scarce and expensive especially in developing countries.[33]

Aim

This study focuses on investigating the hepatoprotective action of ALECA in male albino rats exposed to lead, cadmium, and mercury.

Material and methods

Harvesting of C. afer

Samples of *C. afer* leaves were collected, in the month of July 2021, from a farmland in the University of Port Harcourt, Rives state, Nigeria, in an area free of air pollution

due to vehicular traffic. Mr. A. O. Ozioko, of the Botany Department, University of Nigeria, Nsukka, helped verify the plant for its authenticity prior to its use.

Preparation of ALECA

The leaves of *C. afer* were washed to remove sand particles, pulverized, and stored. Two hundred and fifty grams of the pulverized leaf samples were macerated in 500 ml of deionized water for 24 hours amidst continuous agitations after the method of Ezejiofor and Orisakwe.[29] The mixture was shaken and filtered using Whatman No 1 filter paper to obtain the extract with a yield of 0.11g/ml which was stored in a refrigerator at 4°C.[34] The process was repeated after every four days of treatment to obtain fresh extract throughout the 90 days treatment period.[34]

Determination of the ALECA dosage

A total of 12 male albino rats of approximately 8 weeks old with 100-200 g weights separated into four equivalent groups that received *C. afer* 1000, 2000, 4000 and 5000 mg/kg bw respectively were observed for 24 hours for any change in physical characteristics or death. This administration was carried out by oral gavage and at the end of the treatment no death or change in physical features was recorded. A previous study from our lab showed that subchronic administration of ALECA was not toxic.[29]

To determine the dose used for this study, given the safety of ALECA, 3000 mg/kg bw ALECA was chosen and then 25% of 3000 mg/kg bw as the low dose, 50% of 3000 mg/kg bw as the medium dose and 75% of the 3000 mg/kg bw as high dose.

Phytochemical screening of the plant material

The phytochemical constituents of *C. afer* were tested to confirm the existence of tannins, alkaloids, saponins, flavonoids, and phenolic compounds using the standard procedures of Trease and Evans.[35]

Animal care handling

The study used 25 male albino rats that were about 8 weeks old with 100-200 g weights procured from the Animal House of the Faculty of Pharmacy, University of Port Harcourt Rivers state, Nigeria. The study used the animal husbandry procedure established in previous studies by Ezejiofor and Orisakwe and Anyanwu et al.[29,36] Rats were kept under standard laboratory conditions with ambient temperature (25±2°C), relative humidity (55-64%) and 12-hour light-dark condition cycles. The rats were acclimatized for 2 weeks and fed standard rat chow (Sander Nigeria Ltd) with water *ad libitum*. The protocol for the study was allowed by the University of Port Harcourt and was assigned the reference number UPH/CEREMAD/REC/04.

Design of the experiment

Twenty-five rats consisting of five weight matched male albino rats per group of each were used for this study. The first group was as control which received only deionized water whereas the second group received only TMM ($PbCl_2$, 20 mg/kg; $CdCl_2$, 1.61 mg/kg; $HgCl_2$, 0.40 mg/kg) (Sigma Aldrich WGK Germany).[34,37] Groups 3, 4 and 5 received a toxic metal mixture TMM and ALECA at 750, 1500 and 2250 mg/kg respectively, according to a previous study from our laboratory.[34] These treatments were done five times in a week to mimic occupational exposure for 90 consecutive days by oral gavage.

Necropsy

On the 91st day, the animals were sacrificed under ether anesthesia. The liver was harvested, washed in ice-cold saline, blotted with Whatman No.1 filter papers, and weighed afterward to get the absolute weight. Normal saline and formalin were used for storing the samples for biochemical, histopathological, oxidative markers, and inflammatory analysis. These samples were collected according to the procedures recorded by Anyanwu et al.[34,36]

Preparation of liver homogenate

Two grams of liver sample was homogenized in cold phosphate buffer (5 mM, pH 7.4). The supernatant was collected after centrifugation for inflammatory and antioxidant analysis.

Metal digestion (acid digestion method) and analysis

About 6 ml and 2 mL of nitric acid and perchloric acid, respectively, were used for the acid digestion of the liver after isolating the weighed organ. The samples were left for 30 min after acidification before being heated at 105°C until digestion was complete. Whatman filter paper Number (1) (pore size 11 μm) of was used for filtration to obtain clearer samples. The solution was later made up to 15 ml (final volume) with deionized water. All glassware was thoroughly washed and rinsed before use. Calibration curves for Pb, Cd, and Hg as previously described by Anyanwu et al.[34,36] The solar thermo-elemental flame atomic absorption spectrometer (Model SG 71906) was used to determine the levels of Pb, Cd and Hg at a detection limit of <0.001 mg/kg. Standard operating parameters were set and the hollow cathode lamps for Pb, Cd and Hg (Model SG 71906) were employed as radiation source and fuel was air acetylene. All samples and standards were run in duplicate.

Hepatic biomarkers

Estimation of alanine aminotransferase (ALT) and aspartate transaminase (AST)

The ALT and AST activities of the liver samples were tested using a Randox kit.[38]

Estimation of alkaline phosphatase (ALP)
The ALP function was determined with the aid of standard diagnostic kits (Randox Laboratories Ltd, UK) using the colorimetric endpoint.[39]

Estimation of total and direct bilirubin
This was done using standard diagnostic kits in a colorimetric process (Randox Laboratories Ltd, UK).[40]

Estimation of total protein and albumin
A hepatocellular injury is indicated by a decrease in total protein.[41] The level of total protein in serum was estimated using standard diagnostic kits (Randox Laboratories Ltd, UK). Similar to total protein, a reduction in albumin level also signifies liver injury.[42] With the standard diagnostic kits (Randox Laboratories Ltd, UK), the albumin level in the serum was determined.

Antioxidant analysis
Estimation of catalase (CAT) activity
CAT activity was assayed by adapting the method of Clairborne.[43]

Estimation of liver glutathione (GSH) level
The GSH level was estimated after the method of Sedlak and Lindsay.[44]

Estimation of superoxide dismutase (SOD) activity
Following the method of Misra and Fridovich, SOD was determined.[45]

Lipid peroxidation marker (MDA) activity
Following the method of Ohkawa and Ohishi, the MDA was evaluated.[46]

Evaluation of Inflammatory cytokines [interlukin-6 (IL-6) and interlukin-10 (IL-10)]
Enzyme-linked immunosorbent assay (ELISA) was employed. The ELISA kit (Bioassay Technology Laboratory, Shanghai, China) with a sensitivity of 0.052 ng/L and 1.51 pg/mL, respectively, as described in Anyanwu et al.[34]

Histopathological examination
The tissues were soaked in 10% formaldehyde, sectioned, and treated with hematoxylin and eosin (H&E). The H&E treated tissues were finally examined with a microscope at 200x magnification following the procedures outlined in Anyanwu et al.[34]

Statistical analysis
Analysis of variance (ANOVA) was applied to the sequence of observations for the purpose of comparative analysis at 5% significance. Multiple comparisons were performed with Duncan's multiple comparison method. Principal component analysis (PCA) was employed to select the principal factors (or independent variables) for the development of the multiple regression equations. In this study, the use of PCA was carried out using XLSTAT (Microsoft, Redmond, Washington, USA).[36,47] The annotation on the bar graphs indicates whether there is a significant difference between two groups. Groups that have different alphabets signify that there is a significant difference in the mean concentration, while groups with the same alphabet signify that there is no significant difference in the mean concentration.

Results

Phytoconstituents of ALECA
The phytoconstituents of ALECA are shown in Table 1. Flavonoids were the main phytoconstituents in ALECA (25±0.13 mg/100 g) which accounted for approximately 70% of the total chemical constituents. Alkaloids, tannins, and saponins were also found in ALECA.

Table 1. Quantitative phytochemical screening (mg/100 g) of ALECA*

Chemical constituents	Content
Alkaloids	4.3±0.1
Saponins	2.9±0.08
Tannins	2.72±0.11
Flavonoids	25±0.13

* values are expressed as mean±SD, n=5

Effect of ALECA on body weight, absolute and relative weight of liver
Treatment with TMM did not decrease the body weight of the rats. There was an increase in body weight in all the groups. Rats treated with TMM only had a considerable increase in liver weight ($p<0.05$) compared to normal control rats (received only deionized water). Rats that received both ALECA and TMM had decreased liver weight compared to the TMM only treated rats (Table 2).

Table 2. Effect of *C. afer* on body weight, absolute and relative weight of liver of male albino rats treated with TMM*

Treatment	Absolute (g)	Relative (%)	Body weight (g)
Deionized H_2O (only)	5.08 ± 0.95^{a}	2.45 ± 0.41^{a}	I=118.2±7.46; F=207.1±42.61 % diff.=139.1
Metal mixture (only)	9.4 ± 1.35^{c}	3.62 ± 0.47^{c}	I=194.2±10.74; F=260±10 % diff.=62.7
Metal mixture + 750 mg/kg	$8.48\pm1.13^{b,c}$	$3.52\pm0.42^{b,c}$	I=174.2±8.19; F=248.3±25.63 % diff.=78.7
Metal mixture + 1500 mg/kg	$8.36\pm0.52^{b,c}$	3.15 ± 0.17^{bc}	I=158.1±4.77; F=247.1±33.02 % diff.=104.1
Metal mixture + 2250 mg/kg	7.86 ± 0.22^{b}	3.06 ± 0.08^{b}	I=150.6±3.03; F=228.6±30.37 % diff.=44.1

* values are expressed as mean±SD, n=5., data with different superscripts (a, b, c) are significantly different from each other ($p<0.05$), data with the same superscripts are not significantly different, initial weight, F – final weight, % diff. – % difference

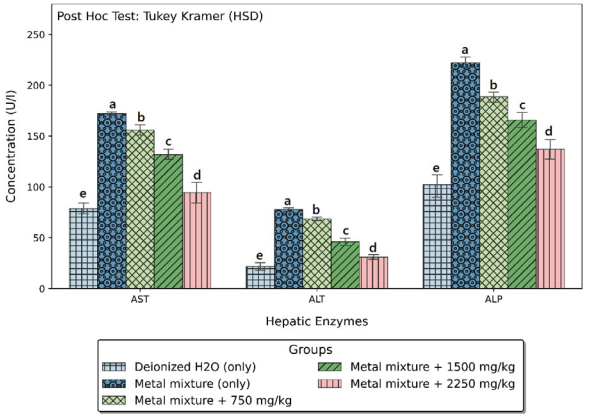

Fig. 1. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different

Effect of C. afer extract on serum liver enzymes, bilirubin and proteins

Liver function were done to evaluate the likely protective role of *C. afer* treatment from exposure to the metal mixture. Treatment with TMM caused a significant elevation in AST, ALT, and ALP levels relative to the control Figure 1. There was a significant increase in bilirubin (total and direct) in the TMM treated only compared with control Figure 3. In Figures 1 and 3, there were significant reductions in liver enzyme concentrations (ALT, AST and ALP) and bilirubin (total and direct) in rats that received both ALECA and TMM. Figure 2 showed a significant decrease in total protein and albumin when rats were treated with TMM only compared to the control group. Treatment with ALECA caused a significant increase in total protein and albumin when the ALECA concentration exceeded 750 mg/kg. AST, ALT and ALP, total bilirubin, direct bilirubin, total protein and albumin levels in rats treated with only TMM were significantly different (172 µg/L, 77.8 µg/L, 222.2 µg/L, 32.6 mg/dL, 15.2 mg/dL, 43.8 g/L, 25.4 g/L, p<0.05) respectively, from the groups that received both ALECA and TMM.

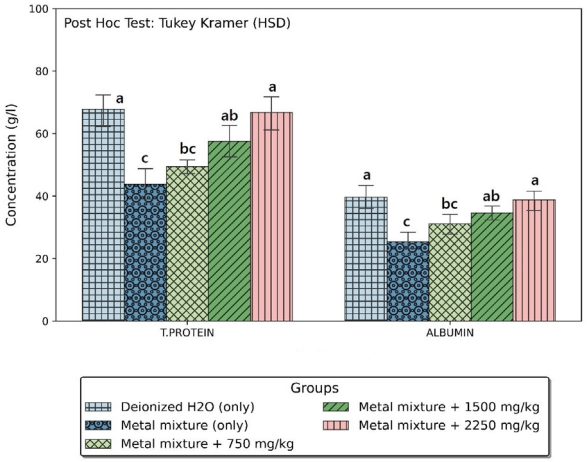

Fig. 2. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different, total protein

Effect of C. afer on anti-inflammatory cytokines and pro-inflammatory cytokines on the liver

An evaluation of the inflammatory status after TMM treatment by evaluating the pro- and anti-inflammatory cytokines in the liver. Cotreatment with *C. afer* significantly decreased the levels of pro- and increased (p<0.05) the anti-inflammatory cytokines (IL-6 and IL-10) in liver tissue respectively in comparison to the TMM-only treated group (Fig. 4), suggesting anti-inflammatory activity of ALECA. Pro and anti-inflammatory cytokine levels (IL-6 and IL-10, respectively) of rats administered with TMM only were significantly different (61.8 pg/mg of tissue and 14.7 pg/mg tissue, p<0.05) from the inflammatory cytokines seen in rats cotreated with *C. afer*.

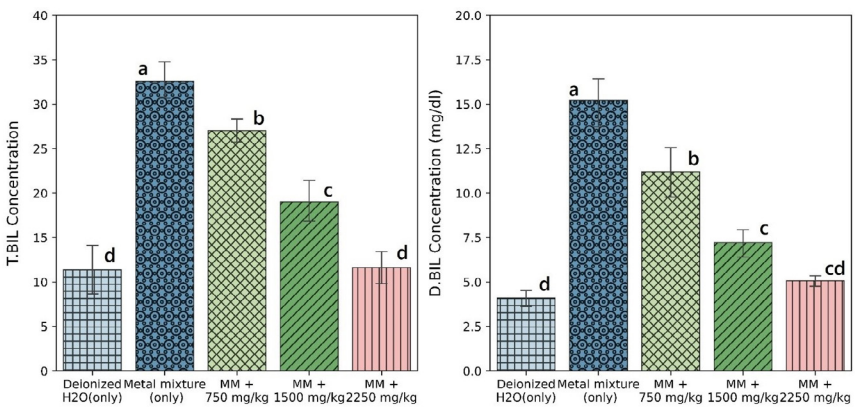

Fig. 3. Effect of ALECA on serum liver enzymes of male albino rats treated with TMM, values with different superscripts (a, b, c, d) are significantly different from each other (p<0.05), while those with the same superscripts are not significantly different, TBIL total bilirubin, DBIL – direct bilirubin

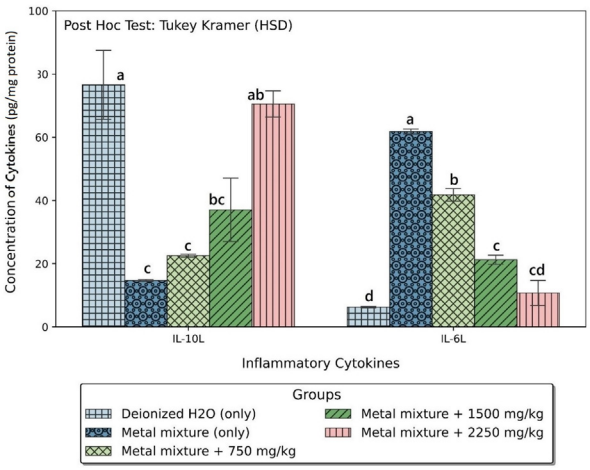

Fig. 4. Effect of *C. afer* on anti-inflammatory cytokines (interlukin-10 (IL-10) and pro-inflammatory cytokines interlukin-6 (IL-6) on the liver of male albino rats treated with TMM, values with different superscripts (a, b, c) are significantly different from each other ($p<0.05$), while those with the same superscripts are not significantly different.

Effects of C. afer on markers of liver oxidative stress

The oxidative status in the hepatocyte was evaluated after treatment with the metal mixture using the lipid peroxidation marker, MDA level. The 90-day treatment that was done with the following metal mixture at the following dosage $PbCl_2$ – 20 mg/kg, CdCl2 1.61 mg/kg, $HgCl_2$ – 0.40 mg/kg body weight induced oxidative reaction in the liver. The liver MDA level increased significantly ($p<0.05$) in rats treated with TMM only compared to those of the control group (Table 4). A significant decrease in MDA level was observed in the rats treated with *C. afer* and the metal mixture when compared to those treated with only TMM. Treatment with TMM resulted in a significant decrease ($p<0.05$) in GSH, SOD, and CAT levels compared to the control. Rats that received ALECA plus TMM had higher levels of GSH, SOD, and CAT compared to rats that received only TMM.

Table 3. Effects of *C. afer* on liver oxidative stress markers of male albino rats treated with a metal mixture

Treatment	CAT (U/mg)	SOD (U/mg)	GSH (µmol/g)	MDA (nmol/g)
Distilled H_2O (only)	2.87±0.95^{b}	0.35±0.06^{c}	1.36±0.28^{b}	0.39±0.08^{b}
Metal mixture (only)	2.36±1.85^{b}	0.24±0.06^{c}	1.82±0.2^{c}	0.64±0.1^{a}
Metal mixture + 750 mg/kg ALECA	5.64±3.39^{a,b}	0.33±0.06^{c}	1.35±0.24^{b,c}	0.45±0.110^{b}
Metal mixture + 1500 mg/kg ALECA	6.08±1.84^{a,b}	0.54±0.08^{b}	1.04±0.07^{b}	0.24±0.04^{c}
Metal mixture + 2250 mg/kg ALECA	9.18±1.9^{a}	0.82±0.12^{a}	0.81±0.18^{a}	0.12±0.03^{c}

* values with different superscripts (a, b, c, d) are significantly different from each other ($p<0.05$), while those with the same superscripts are not significantly different.

Heavy metal concentration on the liver of the rat samples

The concentration of trace metals (Pb, Cd, and Hg) in the liver was markedly elevated ($p<0.05$) in the liver of rats treated with the TMM compared to the control (Table 5). Treatment of rats with ALECA plus TMM resulted in a significant reduction in trace metal levels (Pb, Cd, and Hg) compared to rats that received only TMM. Furthermore, the TMM group only had the highest level of metals (Pb=90.992±13.284, Cd=0.78±0.133 and Hg=0.305±0.0439) in comparison to the control group. Pearson's rank correlation analyzes indicate the inter-trace metal relationship between metals in liver of rats showed strong positive correlation ($r>0.90$) between metals such as: Pb and Cd, Pb and Hg, and Cd and Hg. All correlations were significant at $p<0.01$ (Fig. 5).

Table 4. Metal levels (mg/kg) in the liver

Treatment	Cadmium (Cd)	Mercury (Hg)	Lead (Pb)
Deionized H_2O (only)	<0.001^{a}	<0.001^{a}	0.392±0.552^{a}
TMM (only)	0.782±0.133^{b}	0.305±0.039^{b}	90.992±13.284^{c}
TMM + 750 mg/kg ALECA	0.143±0.046^{a}	0.051±0.02^{a}	31.007±6.017^{b}
TMM + 1500 mg/kg ALECA	0.033±0.024^{a}	0.009±0.001^{a}	8.857±4.849^{a}
TMM + 2250 mg/kg ALECA	0.003±0.002^{a}	0.001±0.001^{a}	3.500±1.141^{a}

* values are expressed as mean±SD, values in the same column with different superscripts are significantly different from each other ($p<0.05$) and those with the same superscripts in the same column are not significantly different.

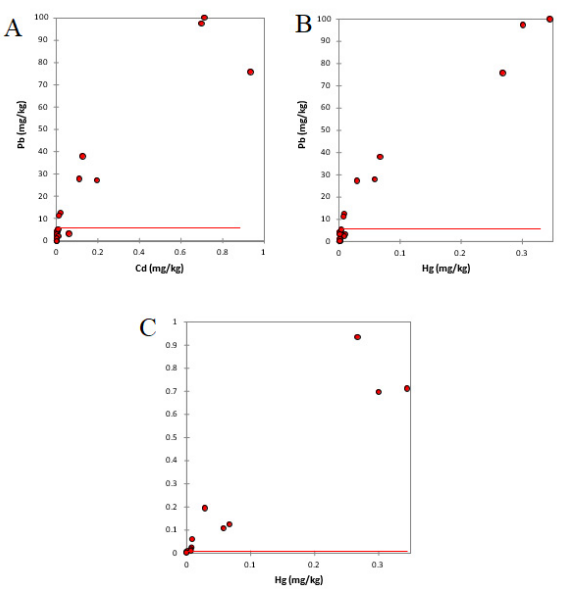

Fig. 5. The correlation among metals in the liver of rats showed a strong positive correlation ($r>0.90$) between metals such as (a) Cd and Pb (b) Hg and Pb (c) Hg and Cd during the study. All correlations were significant at $p<0.01$

Histopathology of the liver

The liver sections of six different groups were sectioned and presented in (Fig. 6 (G1), (G2), (G3), (G4) and (G5)

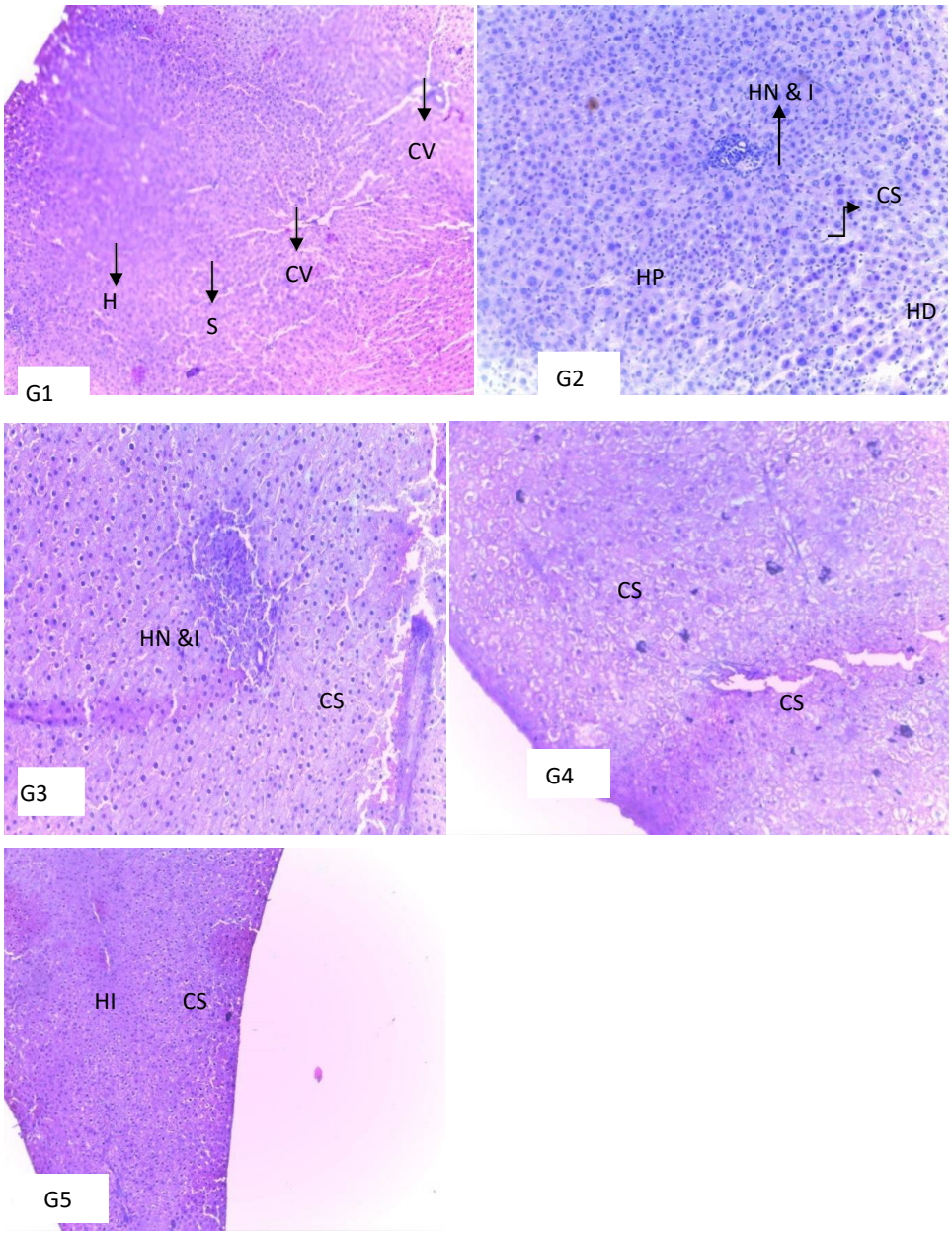

Fig. 6. Histopathology of the liver: G1 (H_2O), G2 (TMM), G3 (TMM + 750 mg/kg of ALECA), G4 (MM + 1500 mg/kg of ALECA), G5 (TMM + 2250 mg/kg ALECA). Staining was performed with H&E magnification X 200 in all panels, cytoplasmic swelling, HN & I – hepatocyte necrosis and inflammation of HN and I hepatocytes, cytoplasmic vacuolation CV, fatty change, HP – hepatocyte pleomorphism, HD hepatocyte dysplasia, S – sinusoids, H – hepatocytes

representing groups treated with deionized H20, metal mixture (MM), (MM 362 + 750 mg/kg ALECA MM + 1500 mg/kg ALECA, and MM + 2250 mg/kg ALECA. The rats treated with TMM only showed cytoplasmic swelling; hepatocyte necrosis and inflammation); cytoplasmic vacuolation; fatty change; hepatocyte pleomorphism and dysplasia of hepatocytes. These histological changes were reduced by ALECA. Fig. 7 is a synoptic capture or graphic illustration of the antioxidant and anti-inflammatory mechanism suggested of ALECA in metal mixture induced hepatotoxicity in rats.

Discussion

According to Jarup, most health challenges can be attributed to Pb, Cd, and Hg.[48] These metals have also been associated with infertility, neurotoxicity, osteoporosis, and various organ failures in humans.[18,49-50] The liver helps in metabolism and excretion and therefore its susceptibility to the adverse effects of foreign compounds.[15] Given the ubiquity of metals, some researchers have investigated the effects of chronic exposure of low doses of a mixture of cadmium and mercury.[51,52] They posited that long-term exposure to trace metal mixture even at low affected the liver of rats adversely.[51,52]

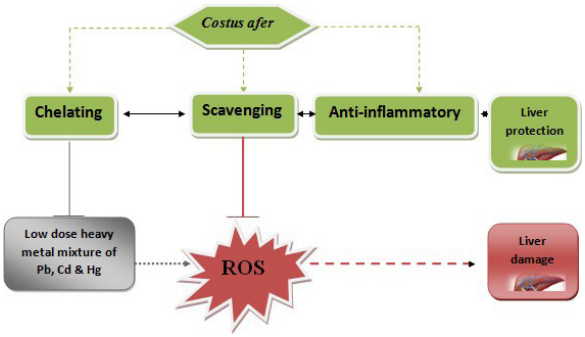

Fig. 7. Summary of the suggested mechanism

Majority of the actions of ALECA can be attributed to the inherent antioxidant properties of *C. afer*. Alkaloids, saponins, flavonoids, tannins, and phenols are the phytoconstituents of ALECA and may be responsible for its biological activity. Flavonoids are water-soluble polyphenolic compounds with 15 atoms that are antioxidant in nature.[29,53] Natural phenolic compounds have antioxidant and anti-inflammatory effects. Furthermore, tannins are water-soluble polyphenolic compounds within plants of secondary metabolism and are usually of two classes: hydrolyzable and condensed tannins.[29,54] Their solutions are acidic with a characteristic astringent taste; they are also known to have strong antimicrobial activities in addition to other physiological effects that help to enhance blood clotting, as well as antihypertensive, antihyperlipidemic, and immunomodulatory.[55] Saponins are glycosides that are usually foamy in nature and have good medicinal value due to their therapeutic action. Desai et al. reported that they help protect plants from pathogenic attacks, giving rise to their antimicrobial activity[56] Alkaloids are nitrogenous compounds that are used as supplements, ingredients, supplements and for medical and pharmaceutical purposes.[57,58]

A significant increase ($p<0.05$) was observed in weights of liver treated with the TMM (only) in comparison to the control rats. However, a significant reduction in weight (absolute and relative) was observed in the samples administered with ALECA (750, 1500 and 2250 mg/kg) respectively in a dose-dependent manner in corroboration with the study by Bhattacharjee et al. that reported a marked elevation in the weight of the liver in rats treated with a trace metal mixture of Pb, Cd and As. Kluwe, Simmoni et al., and Orisakwe et al. further highlighted that an increase or decrease in organ weight after treatment with a chemical substance suggests toxicity.[59-61] Hence, this indicates that the liver could be a target organ for TMM toxicity. The increased liver weight observed in the present work confirms the work of Ahmad et al.[62] This current study indicates that ALECA may be hepatoprotective.

The significant rise ($p<0.05$) seen in rats that received TMM with respect to ALT, AST, ALP, total and direct bilirubin levels that formed the markers of injury to the hepatocyte could have arisen from increased membrane permeability and cell loss due to membrane oxidation[63-64] Liver cell death (hepatocellular necrosis) or membrane lesions increase serum levels of AST and ALT serum levels, flow into the bloodstream from the liver, in line with increased enzyme levels of metal mixture intoxicated rats. These observations indicate cell loss and loss of functional cellular membrane integrity in the liver in accordance with the studies by Zhang et al. and Ramachandran and Jaeschke.[64-65]

The increased level of bilirubin is another marker of abnormal liver function66 The liver is a chemical laboratory of the body involved in oxidation and metabolic conversion of fatty acids, the production of cholesterol and phospholipids and the elimination of specific classes of serum lipoprotein.[67] The apparent decrease in total protein and albumin levels in rats treated with TMM only could be due to inhibition of protein synthesis and metabolism that gave rise to hepatotoxicity.[68,69]

Many previous studies described trace metals as immune suppressors that provoke elevated levels of TNF-α, Il-1β and IL-6 (pro-inflammatory cytokines). The elevated level of IL-6 in this study may be due to increased production of reactive oxygen species.[70,71] Oxidative stress could be associated with an excessive production of pro-inflammatory cytokines.[70] The concomitant administration of ALECA and TMM resulted in dose-dependent reduction in levels of IL-6 and an increase in IL-10. Inflammation is caused by several factors, such as a group of secreted polypeptides called cytokines that control host responses to lesions; some are anti-inflammatory cytokines, while others function as pro-inflammatory cytokines72-73 An elevation in the pro-inflammatory cytokine (IL-6) and a decrease in the anti-inflammatory cytokine (IL-10) were observed in rats exposed to TMM only. These effects were alleviated upon administration of ALECA in a dose-dependent manner suggestive of anti-inflammatory potency of ALECA.

Elevated levels of antioxidant enzymes were observed in rats that received ALECA plus TMM compared to those that received only TMM, which may suggest that ALECA has antioxidant and hepatoprotective effects. This observation is in corroboration with the work of Ezejiofor and Orisakwe.[29] SOD is the first defense against the conversion of superoxide radical anion to the production of free radicals involved in hydrogen peroxide, while CAT serves as the second antioxidant protection mechanism by reducing hydrogen peroxide to oxygen and water.[74] Thus, ALECA increased antioxidant enzyme levels, which agrees with the previous study protective effects of *C. afer* in the liver by Ezejiofor and Orisakwe.[29]

Although the toxicity of the exact mechanism of the metal mixture is unclear, the observations in this study indicate that treatment with TMM elicits ROS generation and alters cellular antioxidant capacity. This

could result in an imbalance between free radical species and the resistance against cellular damage.[75] Therefore, supplementation of antioxidant molecules would be exogenously beneficial in the protection of cell antioxidants to neutralize heavy metal poisoning. ALECA contains antioxidant phytochemicals such as phenolics and flavonoids.[29] This study has shown the protective effect of ALECA on TMM-induced toxicity in rats. MDA, a marker of lipid peroxidation, showed a substantial increase ($p<0.05$) in the liver of rats that received TMM compared to the groups that received ALECA plus TMM. MDA is a marker used to measure the level of oxidative stress in an organism.[76] A similar finding revealed an elevation in lipid peroxidation in the liver after metal poisoning.[29] This suggests that peroxidative injury may be involved in the development of complications of severe heavy metal toxicity.

The significant decrease in Pb, Cd, and Hg following administration of ALECA is prominent. The chelation of these trace metals by some phytoconstituents of ALECA may be a plausible mode of action notwithstanding the fact that the actual mechanism remains largely unknown. Only recently have some researchers opined that mopping of free radicals and metal chelation are essential characteristics in management of oxidative stress.[77-79] There is a need for further studies in this regard to ascertain the actual route of metal elimination. Pearson's rank correlations of Pb, Cd, and Hg in the liver show strong significant relationships ($r>0.90$) between Cd and Pb ($r=0.940$, $p<0.05$, $n=18$), Hg and Pb ($r=0.982$, $p<0.05$, $n=18$), Hg and Cd ($r=0.960$, $p<0.05$, $n=18$) in the liver. This strong association indicates a close physiological connection.[80] The strongest association was observed between liver Hg and liver Pb. This relationship between these trace metals could be due to the similarity of their oxidative states, which makes them exhibit similar chemical properties.

Certain histological changes, such as necrosis and inflammation and severe dysplasia of the hepatocytes, were observed in rats that received trace metal mixture compared to the rats that received only deionized water. Hepatic necrosis, inflammation, and cytoplasmic swelling are common symptoms of liver damage.[81] The deleterious histomorphological features in the TMM treated rats are consistent with the oxido-inflammatory effects of metals in the liver. The protective effect of *C. afer* was observed in the rats co-treated with ALECA at doses of 750, 1500, and 2250 mg/kg. Whereas rats which received low dose ALECA plus trace metal mixture showed hepatocyte necrosis and cytoplasmic swelling, higher doses of ALECA plus trace metal mixture showed mild cytoplasmic swelling and mild hepatocyte inflammation. The dose-dependent histomorphological protection of ALECA against trace metal-mixture-mediated damage may confer on ALECA a beneficial role. Perhaps some of the limitations of the present study which will be addressed in follow-up studies are the use of crude extract of *C. afer* and an in-depth mechanistic consideration of the beneficial effect of *C. afer* in heavy metal-mediated hepatotoxicity.

Conclusion

The suggested mechanism of action is shown in Fig. 10. Exposure to trace metal mixtures may be of significant health effect arising from oxidative damage, inflammation, and distortion of the liver in rat model. The observed attenuation of the destructive effects of TMM by the effects tended to be attenuated by ALECA, which could provide some hope as an alternative remedy and circumvent the major drawbacks of notable chelators: increase in toxicity, unavailability, and prohibitive cost. *C. afer* may have promise in the management of chronic hepatotoxicity arising from metal exposure.

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Declarations

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Author contributions

Conceptualization, O.E.O.; Methodology, B.O.A. and A.N.E.; Validation, B.O.A., A.N.E. and O.E.O.; Formal Analysis, B.O.A., A.N.E. and O.E.O.; Investigation, B.O.A., A.N.E. and O.E.O.; Data Curation, B.O.A., A.N.E. and O.E.O.; Writing – Original Draft Preparation, B.O.A. and O.E.O.; Writing – Review & Editing, D.N.A. and O.E.O.; Supervision, A.N.E. and O.E.O.

Conflicts of interest

Authors declare no conflict of interest.

Data availability

All data have been provided.

Ethics approval

The protocol for the study was permitted by the University of Port Harcourt Research Ethics Committee and was assigned the reference number UPH/CEREMAD/REC/04.

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ORIGINAL PAPER

Comparison of falls and non-fall admissions to the emergency department in older adults and evaluation of the Barthel index and the Falls Efficacy Scale International scores

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ABSTRACT

Introduction and aim. The aim of this study was to describe the characteristics of patients aged 65 years and older who experienced falls and to examine the distribution of the Barthel index (BI) and Falls Efficacy Scale International (FES-I) scores.

Material and methods. Participants over 65 years of age who were admitted to the emergency department (ED) between 31.07.2019 and 31.01.2020 and who met the inclusion criteria were included in the study. Characteristics, BI, and FES-I scores of patients admitted to the emergency department for falls and nontraumatic reasons were compared.

Results. The study was carried out with 259 participants, 133 in the fall group and 126 in the control group. The mean age was 79±8.3 years in the fall group and 76.3±7.9 years in the control group (p=0.011). In the fall group, 61 (46.2%) were male and 71 (53.8%) were females. The most common trauma after a fall was soft tissue trauma. The FES-I and BI scores were found to be at higher risk in the fall group aged 75-84 years compared to the control group and there was a statistically significant difference between them (p=0.009; p=0.030, respectively).

Conclusion. FES-I and BI did not show significant differences between fall and control groups in all age groups. In the 75-84 age group, both scales showed higher values in the fall group. We believe these scales can be used as follow-up tools in screening and preventing fall risks, especially in this age group.

Keywords. Barthel Index, emergency department, fall, Falls Efficacy Scale International, older adult

Introduction

Trauma, which is a serious cause of morbidity and mortality in the elderly, is a health problem that should be prevented. Falls are the most common cause of trauma in the elderly and 1/3 of the population aged 75 years and older falls at least once a year. Worldwide, more than 50% of injury-related hospitalizations are seen in patients aged 65 years and older and 10–15% of all emergency department (ED) admissions are due to mild or severe injuries.[1]

The Barthel index (BI) is a scale based on measuring the individual's performance in activities of daily living and monitoring activities of daily living.[2] Its predictive value is in predicting the effects caused by neuromuscular and musculoskeletal diseases, the duration of hospitalization, and care needed. It is easy to use but limited in application due to unreliable results in patients with malignancy, dementia, and pelvic trauma.[3,4]

The Falls Efficacy Scale International (FES-I) was developed as a solution to the problems of the fear of falling, self-efficacy and balance confidence. FES-I is a test that shows almost perfect measurement characteristics in people with and without moderate cognitive impairment and has been validated for use in older

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adults.[5] The aim of both scores is to assess the patient's fall risk and implement early precautions. BI has mainly been used to monitor rehabilitation in cerebrovascular disease and predict mortality in various conditions.[6-11] Its role in fall prediction is less explored. Toots et al. identified high BI scores and environmental factors as fall risks, with greater morbidity observed in women with high BI values, especially those aged 70-90, where BI was a better predictor of falls in women than men.[12] In a study by Figueiredo et al., FES-I demonstrated high internal consistency when used with socially isolated older adults in the community.[13]

Aim

The aim of this study was to explore and compare the characteristics of patients over 65 years old who present to the ED with fall complaints versus those presenting with other complaints. Specifically, we sought to determine if there are significant differences between the fall group and a control group within the same age bracket. Our focus was on comparing the BI and the FES-I scores between these two groups. While the BI has been compared with various other scales in literature, the FES-I is less frequently utilized. We chose to use the FES-I to evaluate whether it provides a better prediction of fall risk compared to the BI. To avoid confusion, we focused on external risk factors, as both the Barthel Index and the Falls Efficacy Scale International are based on physical capacity and ability. Both scores aim to assess the patient's fall risk and implement early precautions. By including patients presenting with other complaints as the control group, we were able to better evaluate the effectiveness of these indices in assessing external factors. Additionally, the study aimed to identify risk factors associated with falls, including patient-related factors such as visual and auditory impairments and frequent falls, as well as environmental factors like indoor stairs and living alone. By investigating these factors, we aimed to understand their impact on fall risk. Furthermore, we documented the diagnoses of the patients included in the study to identify the most common conditions encountered. This study was conducted with these objectives in mind to better understand fall risk and inform preventive strategies.

Material and methods

Study design

The study was a descriptive cross-sectional study. Patients over the age of 65 who were admitted to Ege University Faculty of Medicine Hospital ED between July 31, 2019, and January 31, 2020, and met the study criteria were included. The Ethics committee approval for the study was obtained from Ege University Clinical Research Ethics Committee (Date: 31.07.2019, No: 19-7T/7). Informed consent was obtained from all patients via an informed consent form.

Study population

In the study, participants were divided into two groups: the fall group and the control group. The fall group consisted of patients aged 65 and older who presented to the ED with complaints of falling due to causes such as slipping, hitting an object or another person, or being pushed or shoved by a person. The height of the fall was not used as a criterion. However, falls due to non-mechanical causes (e.g., syncope, cerebrovascular events, etc.) were not included in the study. The control group consisted of patients aged 65 and older who were presented to the ED only for non-traumatic reasons. The inclusion criteria for the fall group were defined as being 65 years of age or older, presenting to the ED with a fall as described above, and agreeing to participate in the study. For the control group, the criteria were defined as being 65 years of age or older, presenting to the ED for non-traumatic reasons, and agreeing to participate in the study.

Exclusion criteria were defined as unconsciousness or not suitable for evaluation, sequelae of cerebrovascular events, trauma with mechanisms other than falls for the fall group, and ED visits due to falls within the last 1 year.

Sample size calculation

The minimum number of patients required to find a significant difference between BI and FES-I scores in trauma and non-trauma patient groups over 65 years of age was calculated as 210 in total, 105 patients in each group, using Gpower, with 95% power, type 1 error level 0.05, and a medium effect size of 0.5 under the independent sample t test.

Study procedure

Patients were evaluated by the primary responsible emergency physician. There was no intervention in the routine diagnosis, treatment and examination practices. A history was taken by the physician and then BI and FES-I scales were administered. These were recorded in the case report form.

Data and outcome measures

The date of admission, time of admission, age, gender, living arrangements, presence of stairs, visual and hearing problems, history of frequent falls, comorbidities, presenting complaints and diagnoses were taken into consideration. Information about the mechanism of trauma, physical examination findings, and hospital diagnosis were recorded. BI and FES-I scores of the patients were calculated and fall probability was determined.

BI and FES-I

BI is a 10-item scale. These are feeding, washing, self-care, dressing and undressing, bowel control, bladder

control, toilet care, transfer from wheelchair to bed and vice versa, mobility (walking on a smooth surface, using a wheelchair), going up and down stairs. In the scale consisting of a total of 100 points, 0–20 is categorized as fully dependent, 21–61 as highly dependent, 62–90 as moderately dependent, 91–99 as mildly dependent and 100 as fully independent.

The FES-I consists of a total of 16 parameters and each parameter is scored from 1 to 4. I am not worried at all scored 1 point, a little worried is scored 2 points, quite worried is scored 3 points and very worried is scored 4 points. These are cleaning the house, getting dressed, cooking easy meals, bathing, shopping, sitting up and down in a chair, walking up and down stairs, walking around the house, facing an object above the head or on the floor, answering a landline phone before the caller gives up, walking on wet or icy slippery ground, visiting a friend or relative, walking in a crowded place, walking on uneven ground such as stony ground, walking up and down hills, going out of the house for community activities. The total score is scored from 16 to 64. A score of 16–19 is considered as low risk, 20–27 as medium risk and 28–64 as high risk.

Statistical analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS, IBM, Armonk, NY, USA) Windows 22. The conformity of the data to normal distribution was evaluated by Kolmogorov-Smirnov test. Normally distributed numerical data were presented as mean±standard deviation and non-normally distributed numerical data were presented as median and interquartile range (IQR 25–75%). Categorical variables were presented as number (n) and frequency (%). Categorical variables were compared by Chi-square or Fisher Exact test and continuous variables were compared by Student t test or Mann Whitney U test. Statistical significance level was accepted as $p<0.05$.

Results

A total of 259 participants were included in the study. We excluded 1 patient who was unconscious or not eligible for evaluation, 1 patient with sequelae of cerebrovascular events and 1 patient who presented to the ED due to a fall in the last 1 year. The study was conducted with 259 participants, 133 in the fall group and 126 in the control group. The total number of female participants was 131 (51%), while the total number of male participants was 126 (49%).

The mean age was 79±8.3 years in the fall group and 76.3±7.9 years in the control group (p=0.011). In the fall group, the number of male participants aged 65–74 years was 22 (48.9%), and the number of female participants was 23 (51.1%); the number of male participants aged 75-84 years was 25 (49%), and the number of female participants was 26 (51%); the number of male participants aged 85 and over was 14 (38.9%), and the number of female participants was 22 (61.1%) (Table 1).

The number of people living alone at home was 7 (5.3%) in the fall group and 13 (10.4%) in the control group. There was no significant difference between the groups in terms of living alone at home (p=0.127). The number of stairs in the house was 76 (57.6%) in the fall group and 61 (48.8%) in the control group (p=0.159) (Table 1).

Table 1. Comparison of demographic data, fall risk factors and comorbidities between groups

n (%)	Fall group n=132	Control group n=125	p
Male gender	61 (46.7)	65 (52)	0.354
Female gender	71 (53.7)	60 (48)	
Age group			
65–74	45 (34.1)	59 (47.2)	0.100
75–84	51 (38.6)	38 (30.4)	
≥85	36 (27.3)	28 (22.4)	
Risk factors			
Frequent history of falls	32 (24.2)	20 (16)	0.100
Use of hearing aid	7 (5.3)	8 (6.4)	0.708
Use of glasses	35 (26.5)	45 (36)	0.101
Living alone at home	7 (5.3)	13 (10.4)	0.127
Presence of stairs inside the home	76 (57.6)	61 (48.8)	0.159
Comorbidities			
Hypertension	77 (58.3)	69 (55.2)	0.612
Diabetes mellitus	36 (27.2)	39 (31.2)	0.489
Coronary artery disease	29 (21.9)	43 (34.4)	0.027
Congestive heart failure	15 (11.3)	27 (21.26)	0.027
Malignancy	9 (6.8)	25 (20)	0.002
Dementia	14 (10.6)	8 (6.4)	0.228
Chronic kidney disease	4 (3.03)	18 (14.4)	0.001
Chronic obstructive pulmonary disease	4 (3.03)	16 (12.8)	0.004
Asthma	7 (5.3)	2 (1.6)	0.073
Venous thromboembolism	6 (4.5)	3 (2.4)	0.502
Heart valve replacement	2 (1.5)	5 (4)	0.260
Hypothyroidism	2 (1.5)	0 (0)	0.359
Epilepsy	1 (0.75)	1 (0.8)	0.073

The most common comorbidities in the fall group were hypertension (HT) in 77 participants (58.3%), diabetes mellitus (DM) in 36 participants (27.2%) and coronary artery disease (CAD) in 29 participants (21.9%). CAD was statistically significantly more frequent in the control group (p=0.027). In the fall group, the number of participants with congestive heart failure (CHF) was 15 (11.4%), chronic renal failure was 4 (3%), chronic obstructive pulmonary disease (COPD) was 4 (3%) and malignancy was 9 (6.8%). In the control group, the number of participants with CHF was 27 (21.6%), with CKD was 18 (14.4%), with COPD was 16 (12.8%) and with malignancy was 25 (20%). The frequency of CHF, CKD, COPD and malignancy was significantly higher in the control group (p=0.027, p=0.001, p=0.004, p=0.002, respectively). The number of participants without comor-

bidities was 32 (24.2%) in the fall group and 18 (14.4%) in the control group (p=0.046) (Table 1).

When the most common outcome diagnoses of the participants with falls were classified, soft tissue trauma was seen in 62 (45.9%), lower extremity trauma in 41 (30.3%), and upper extremity trauma in 14 (10.3%) participants. This was followed by head trauma, vertebral trauma, thoracic trauma, maxillofacial trauma and cervical trauma. The most common association was the association of lower extremity trauma with head trauma, which was seen in 4 participants (3.1%) (Table 2, (Fig. 1).

Table 2. Distribution of injury regions after falls

	Fall group (n=132)	
	n	%
Soft tissue	62	45.9
Upper extremity	14	10.3
Lower extremity	41	30.3
Head	8	5.9
Cervical	3	2.2
Maxillofacial	5	3.7
Thoracic	7	5.18
Vertebral	8	5.9

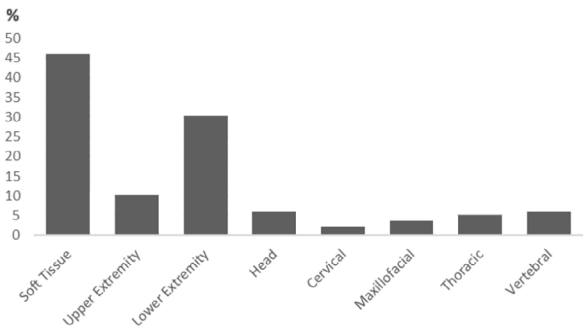

Fig. 1. Graphic of injury regions distribution

According to FES-I, there were 32 (24.2%) low-risk, 13 (9.8%) moderate-risk, 87 (66%) high-risk participants in the fall group and 20 (16%) low-risk, 18 (14.4%) moderate-risk, 87 (69.6%) high-risk participants in the control group (p=0.184). According to BI, there were 6 (4.5%) fully dependent, 37 (28%) severely dependent, 40 (30.3%) moderately dependent, 19 (14.4%) mildly dependent, 30 (22.7%) completely independent participants in the fall group; 15 (12%) fully dependent, 31 (24.8%) severely dependent, 37 (29.6%) moderately dependent, 17 (13.6%) mildly dependent, 25 (20%) completely independent participants in the control group (p=0.300) (Table 3). Participants aged 75–84 years who underwent FES-I and BI were found to have higher fall scores in the fall group compared to the control group and a statistically significant difference was found between them (p=0.009, p=0.03, respectively) (Table 3).

Table 3. Comparison of FES-I and BI between groups*

	Fall group n=132			Control group n=125			p
Age group	65–74	75–84	>85	65–74	75–84	>85	
FES-I							
Low risk	23 (71.9)	6 (18.8)	3 (8.4)	12 (60)	8 (40)	0 (0)	0.184[a]
Moderate risk	6 (46.2)	6 (46.2)	1 (7.7)	13 (72.2)	2 (11.1)	3 (16.7)	0.009[b]
High risk	16 (18.4)	39 (44.8	32 (36.8)	34 (39.1)	28 (32.2)	25 (28.7)	
BI							
Completely dependent	1 (16.7)	1 (16.7)	4 (67.7)	5 (33.3)	5 (33.3)	5 (33.3)	
Severely dependent	4 (10.8)	11 (29.47)	22 (59.5)	10 (32.3)	12 (38.7)	9 (29)	0.300[a]
Moderately dependent	12 (30)	20 (50)	8 (20)	16 (43.2)	12 (32.4)	9 (24.3)	0.030[b]
Mildly dependent	7 (36.8)	11 (57.9)	1 (5.3)	8 (47.1)	7 (41.2)	2 (11.8)	
Independent	21 (70)	8 (26.7)	1 (3.3)	20 (80)	2 (8)	3 (12)	

* FES-I – Falls Efficacy Scale International, BI – Barthel Index, p[a] – the p-value for the Pearson Chi-Square test between the fall and control groups, p[b]– The p-value for the Pearson Chi-Square test between the fall and control groups within the 75–84 age group

Discussion

In this study, aiming to define characteristics of older adult patients presenting with falls and compare BI and FES-I scale properties in this group with other admissions, FES-I and BI comparison showed no significant difference between fall and control groups across all age groups; however, in the 75-84 age group, both scales exhibited higher values in the fall group.

In the study conducted by Galet et al. the distribution of age groups among falls in older adults was found to be 24.93% between the ages of 65–74 years, 35.59% between the ages of 75–84 years, and 39.48% aged 85 years and older.[14] In our study, 45 (33.3%) aged 65–74 years, 51 (37.7%) aged 75–84 years and 36 (26.6%) aged 85 years and older were found. We are of the opinion that the relatively younger population in the country where we conducted the study is related to the lower number of admissions aged 85 years and older. Similar to our study, in the systematic review conducted by Hopewell et al., the mean age of the elderly who fell was 77.7±8.4 years.[15]

There are studies showing that the female gender is more risky for falls than the male gender.[16-18] In a study by Henfy et al. it was shown that 60.9% of patients aged 65 years and older who presented with falls were women.[19] In the study conducted by Gökçek et al. in our country, it was found that 60.8% of patients admitted to the ED with falls were women.[20] Studies in the literature showing that falls are more common in women have explained these results by the fact that women present to the hospital more frequently and that female patients are more prone to traumas related to age-related osteoporotic process.[19,20] However, some studies suggest no significant relationship between gender and fall admissions. It is theorized that this may be due to similar rates of activity restriction among both sexes with aging, leading to the disappearance of differences between them at older ages.[21]

In a study by Gökçek et al. 91.6% of older adults who presented with falls had any comorbidity and the most common comorbidity was found to be HT.[20] In the same study, it was shown that the risk of falls increased with increasing number of chronic diseases.[20] In the study by Mitchell et al. it was observed that individuals with falls had multiple chronic diseases.[22] In our study, comorbidities in the control group were: CAD, CHF, malignancy, CKD and COPD were more common in the control group without falls. We believe that the fact that the control group in our study consisted of patients admitted to the ED for reasons other than falls was effective on this result. The finding that certain comorbidities were higher in the control group who presented to the ED for other reasons is an expected result for our study. This result can be interpreted as no prominent comorbidity in the fall group among all ED admissions of older adults.

According to Berry et al. the most common injury sites in older adults in falls are the head and thorax with a total rate of 54.7%.[23] Choi et al. reported that lower extremity injuries (32.1%), upper extremity injuries (23.1%), shoulder, neck, back and/or hip injuries (23.9%) were the most common injuries after falls in older adults, followed by head injuries (15.3%).[24] In the study group in which data were collected in Choi et al. and Berry et al. study, the trauma group was handled more broadly and high-energy trauma was also included.[23,24] We believe that limiting the mechanism of trauma to fall and not including other mechanisms of trauma in our study explains these results.

BI has been primarily studied in monitoring rehabilitation response in cerebrovascular disease patients and predicting mortality across various conditions.[5,14-18] Few studies focus on fall prediction. Toots et al. found high BI scores and presence of ≥2 environmental factors to be fall risk factors.[12] They also noted greater morbidity in women with high BI values, particularly among those aged 70-90, where BI was a better fall predictor in women than men.

In Figueiredo et al.'s study, FES-I was given to socially isolated older adults living in the community, showing high internal consistency.[13] FES-I scores were notably higher in females, those with lower education levels, and individuals reporting fear of falling. In Sparrow et al.'s study on a demanding balance program for Parkinson's disease patients, those compelled to undergo the regimen experienced a decrease in fall rates, as evidenced by FES-I. The FES-I score significantly decreased following the exercise program.[25] In our study, no significant difference was found between the fall and control groups in terms of BI scores. Our study sample consisted of patients with high comorbidity, multiple risk factors and high BI scores. We think that our sample selection was effective on our results. However, in our study, BI was significantly higher in the fall group aged 75–84 years. We believe that this result is due to the fact that patients aged 65–74 years are more independent and patients over 85 years are more dependent. Therefore, we believe BI may be more meaningful in predicting fall risk, especially in the 75–84 age group. Similar results were obtained with FES-I. Additionally, most patients over 85 years old in our study were not living alone. Despite being a descriptive study with certain limitations, we suggest that BI and FES-I scores can be useful in assessing fall risk in older adults, particularly in the 75–84 age group.

Study limitations

Our study is a single center study. Only ED admissions were evaluated, and non-fall ED admissions were included as a control group. The study was conducted during the winter period, and the seasonal effects on falls could not be evaluated. Differences in the distribution of demographic data between the study and control groups may have led to biased results.

Conclusion

FES-I and BI showed no significant difference between fall and control groups across all age groups. However, in the 75-84 age group, both scales exhibited higher values in the fall group. We believe decreased self-sufficiency and mobility among participants over 75 years of age may have contributed to this outcome. The limited group size, focus on falls, and relatively low rate of living alone in patients over 85 years old may have influenced the similar results between patients over 85 years old and those under 75 years old.

Declarations

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Author contributions

Conceptualization, Y.A.A. and S.K.; Methodology, Y.A.A. and M.S.; Software, S.K.; Validation, M.S., F.K.A. and S.K.; Formal Analysis, S.K.; Investigation, S.K.; Resources, S.K.; Data Curation, M.S.; Writing – Original Draft Preparation, Y.A.A.; Writing – Review & Editing, Y.A.A., F.K.A. and M.S.; Visualization, F.K.A.; Supervision, Y.A.A. and M.S.; Project Administration, Y.A.A.

Conflicts of interest

We have no conflicts of interest to disclose.

Data availability

All data supporting the findings of this study are included in this published article and its supplementary information files.

Ethics approval

The ethics committee approval for the study was obtained from Ege University Clinical Research Ethics Committee (Date: 31.07.2019, No: 19-7T/7).

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ORIGINAL PAPER

Psychological consequences of war as a real threat to young people with post-traumatic stress disorder

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ABSTRACT

Introduction and aim. The consequences of Russia's invasion of Ukraine have led to a violation of the mental health of youth, children, and adults in Ukraine. Increased cases of mental disorders related to war trauma, including post-traumatic stress disorder (PTSD) due to violence, loss of family and friends, loss of normal life, fear, uncertainty, and constant stress, are leading to long-term PTSD, which can negatively affect their quality of life, such as physical, mental, and social well-being. Determination of symptoms of PTSD in students (men and women) of medical and technical higher education institutions during the war in Ukraine.

Material and methods. At the beginning of 2024, the survey was attended by 452 students of Ukrainian higher education institutions (medical and technical), of which 24.6% were male and 75.4% were female, aged 15–19 (65.8%), aged 20–28 (21.1%), and over 29 (13.1%). The methodology 'Identification of potentially traumatic events in life among applicants' was used to identify students with signs of PTSD out of a total of 452 surveyed students. The next step was to assess the degree of PTSD symptoms in 121 students of the total number of students who were identified as having PTSD symptoms using the 'PTSD symptoms list'. Research was conducted with the help of the STATISTICS program, by the Pearson's Chi-square method, Spearman's rank correlation coefficient (r_s).

Results. The severity of the consequences of the war was assessed based on the results of a survey among students of higher education institutions during the hostilities in Ukraine. Students who participated in hostilities or were in the war zone represented 45.9% of respondents from technical universities (TU) and 24.8% of the respondents from medical universities (MU). Students who experienced sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats) made up 18.9% of the students in the TU and 16.9% in the MU.

Conclusion. Sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats) among students in higher education institutions was experienced by 18.9% of respondents of technical specialization and 16.9% of respondents of medical specialization. The identified unfavorable features of students' mental health during hostilities may lead to long-term negative consequences in the future. Particular attention should be paid to risk groups, including female students.

Keywords. Full-scale war, posttraumatic stress disorder, students of higher education institutions of technical and medical profile

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Introduction

The Russian invasion of Ukraine since 2014 has caused a sudden increase in the number of vulnerable people exposed to war and other traumatic events. Loss of family and friends, violence, internal displacement, loss of personal housing, and jobs can lead to an increase in cases of mental disorders, depression, anxiety, and post-traumatic stress disorder (PTSD) among the Ukrainian population, especially among youth and children. Constant stress, fear, and uncertainty contribute to long-term consequences and impede the reconstruction of Ukraine. Kisarchuk found that out of 30% of people suffering from PTSD as a result of military actions, 20.0% need specialized help because they cannot overcome this disorder on their own.[1,2]

Young people around the world are particularly sensitive to military conflicts, and research of the consequences of their cognitive, emotional, and behavioral disorders is important. Living in war zones or under constant bombardment can create or exacerbate the risk of direct and indirect impacts on the physical, psychological/mental health of young people and negatively affect their physical, mental and social well-being. The negative effects of hostilities and migration are often manifested in the high prevalence of depression, anxiety disorders, and PTSD. Among adolescents aged 15–17 years who were traumatized by military operations, 53.7% were diagnosed with PTSD.[3-5]

According to global scientific data, the prevalence of PTSD among people who have suffered the consequences of severe psychotraumatic trauma, have been a combatant, a victim of violence, or have been in occupation or captivity (severe physical and psychological abuse, assassination attempts, murder, rape, etc.) reaches 100%.

Thus, according to scientific data, negative psychophysiological changes in the mental/mental health of trauma survivors were observed in 56.0% of people and minor symptoms due to trauma were observed in 44% of people. According to foreign researchers, the prevalence of PTSD among the world's population in recent years has ranged from 9% to 12%.[6,7]

Young people affected by war are a particularly vulnerable group for experiencing trauma and developing mental/mental health disorders. According to the results of studies, the indicators of PTSD criteria are high: 71.6% of adolescents had disorders in the emotional and volitional sphere; 62.9% of adolescents had disorders in the behavioral sphere, and the coefficient of academic performance, which characterizes cognitive disorders, is 25.1%.[8]

Aim

Therefore, the aim of the study was to investigate the initial signs of mental/mental health disorders and the diagnosis of posttraumatic stress disorder in students of higher education institutions of medical and technical specialization during the war in Ukraine.

Material and methods

At the beginning of 2024, the survey was attended by 452 students of Ukrainian higher education institutions (medical and technical), of which 24.6% were male and 75.4% were female, aged 15–19 (65.8%), aged 20–28 (21.1%), and over 29 (13.1%). The methodology 'Identification of potentially traumatic events in life among applicants' was used to identify students with signs of PTSD out of a total of 452 surveyed students. The next step was to assess the degree of PTSD symptoms in 121 students out of the total number of students who were identified as having PTSD symptoms using the "Checklist of PTSD symptoms."

Stage 1 – involved identifying signs of PTSD using the methodology «Identification of potentially traumatic events in life among applicants» among students of Vinnytsia National Medical University named after M.I. Pirogov. Pirogov Vinnytsia National Medical University (VNMU), Zaporizhzhia State Medical and Pharmaceutical University and students of Vinnytsia National Technical University (VNTU), Taras Shevchenko National University of Kyiv, of whom 24.6% were men and 75.4% were females, 65.8% were 15 19 years, 21.1% were aged 20–28, and 13.1% were over 29 years old.

Stage 2 included assessment of PTSD symptoms using the PCL-5 PTSD Symptom Checklist, which met the DSM-5 criteria for PTSD. The survey involved 121 applicants, of whom 19.0% were men and 81.0% were females, 44.6% were under 15 19 years old, 53.7% were over 20 28 years old and 1.7% were over 29 years old. The research was conducted with the help of STATISTICS program (Statsoft, Tulsa, OK, USA), using the Pearson Chi-square method and Spearman rank correlation coefficient (r_s). The quantitative values obtained from the questionnaire do not follow a normal distribution, so comparisons and relationships between them could not be calculated using parametric criteria (Student's, Fisher's). The most adequate method of analysis in this case is the non-parametric Whitney-Wilcoxon-Mann test or Pearson's chi-square test. The study has been approved by the Bioethics Committee of the University No 2023/09/01.

Results

According to our study, 48.6% of the students of technical universities and 32.8% of the students of medical universities witnessed a fire or explosion during the hostilities. 27.0% of the TU students witnessed traffic accidents and 22.4% of the MU students, while 24.3% and 26.8% were directly involved in the events. During hostilities, 16.2% of TU students witnessed armed at-

tacks and were in the combat zone, and 7.4% of MU students also witnessed armed attacks, while 5.9% of students were directly involved in the combat zone. According to TU survey, 27.0% of the students of the TU and 10.4% of the students of the MU witnessed severe suffering. Witnessing a sudden death among the TU students was 16.2% and 9.9% among the students of the MU (Table 1).

Table 1. Identification of Potentially Traumatic Events in the Life of Students of Higher Education Institutions of Ukraine by the Methodology 'Identification of Potentially Traumatic Events in the Life of Students", ($P \pm S_p$) %

Question	Medical professional institutions (MPIs)		Educational institutions of technical profiles (ETPs)	
	Witness	Participant	Witness	Participant
Natural disaster (flood, hurricane, earthquake)	8.9±2	7.4±1.9	10.8±5.1	10.8±5.1
Fire or explosion	32.8±3.3	18.4±2.7	48.6±8.2	29.7±7.5
Transport accident (road traffic accident, plane crash, train accident)	22.4±2.9	26.8±3.1	27.0±7.3	24.3±7
A serious accident at work, at home or while on holiday	9.4±2	4.4±1.5	–	10.8±5.1
Poisoning by toxic substances (including radiation)	–	10.9±2.2	–	10.8±5.1
Physical violence (e.g. being attacked, hit with a hand, object, kicked, beaten)	8.9±2	44.7±3.5	8.1±4.4	37.8±7.9
Armed assault (e.g., you were shot at, stabbed, threatened with a knife, gun, explosives)	7.4±1.8	5.9±1.6	16.2±6	–
Sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats of harm)	–	16.9±2.6	5.4±3.7	18.9±6.4
Other unwanted or unpleasant sexual experiences	–	25.8±3	–	13.5±5.6
Combat operations or stay in a war zone (as a military or civilian)	7.4±1.8	24.8±3	16.2±6	45.9±8.1
Captivity (e.g., as a result of kidnapping or capture, as a hostage or prisoner of war)	5.5±1	–	–	–
Life–threatening illness or injury	7.5±1.8	23.8±3	18.9±6.4	27±7.3
Severe suffering	10.4±2.1	23.4±2.9	27.0±7.3	24.3±7
Sudden violent death (e.g. murder, suicide)	7±1.7	–	10.8±5.1	5.4±3.7
Sudden death as a result of an accident	9.9±2.1	–	16.2±6	–
Serious injury or death caused by you to someone else	1.5±0.8	5.4±1.6	2.7±2.6	–

Among the students of the TU institutions, 45.9% of respondents were direct participants in hostilities or were in the war zone, and 24.8% of the MUs. 29.7% of the students of TU and 18.4% students of MU witnessed fire or explosions. 44.7% of the students in the TU and 37.8% of students in the MU experienced physical violence. Sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats) was experienced by 18.9% of the students in the TU and 16.9% of the students in the MU.

According to the results of our research on the gender peculiarities of traumatic events in the lives from students of Ukrainian higher education institutions, a high rate of 47.7% of male survivors of physical violence was found in the role of participant/victim compared to 42.8% of female survivors. Female students of higher education institutions 30.4% were in the combat zone and male students 18.2%. As participants, 26.8% of female students and 11.4% of male students of Ukrainian higher education institutions had unwanted or unpleasant sexual experiences. 23.2% of the female students and 22.7% of male students (Table 2).

Table 2. Identification of Potentially Traumatic Events in the Life of Students of Higher Education Institutions of Ukraine by the Methodology 'Identification of Potentially Traumatic Events in the Life of Students", ($P \pm S_p$) %

Question	Male persons		Female persons	
	Witness	Participant	Witness	Participant
Natural disaster (flood, hurricane, earthquake)	9.0±4.3	–	9.3±2	9.3±2
Fire or explosion	29.5±6.8	18.2±5.8	36.6±3.4	20.6±2.9
Transport accident (road traffic accident, plane crash, train accident)	22.7±6.3	27.3±6.7	23.2±3	26.3±3.1
A serious accident at work, at home or while on holiday	13.6±5.1	6.8±3.7	7.2±1.8	5.2±1.5
Poisoning by toxic substances (including radiation)	6.8±3.7	13.6±5.1	2.1±1	10.3±2.1
Physical violence (e.g. being attacked, hit with a hand, object, kicked, beaten)	11.4±4.7	47.7±7.5	8.2±1.9	42.8±3.6
Armed assault (e.g., you were shot at, stabbed, threatened with a knife, gun, explosives)	11.4±4.7	6.8±3.7	8.2±1.9	5.2±1.5
Sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats of harm)	–	–	3.1±1.2	20.1±2.8
Other unwanted or unpleasant sexual experiences	–	11.4±4.7	–	26.8±3.1
Combat operations or stay in a war zone (as a military or civilian)	11.4±4.7	18.2±5.8	8.2±1.9	30.4±3.3
Captivity (e.g., as a result of kidnapping or capture, as a hostage or prisoner of war)	–	–	–	–
Life-threatening illness or injury	6.8±3.7	29.5±6.8	9.8±2.1	23.2±3
Severe suffering	13.6±5.1	22.7±6.3	12.8±2.4	23.7±3
Sudden violent death (e.g. murder, suicide)	9.1±4.3	–	7.2±1.8	–
Sudden death as a result of an accident	11.4±4.7	6.8±3.7	10.8±2.2	–
Serious injury or death caused by you to someone else	–	13.6±5.1	–	–

Regarding changes in gender characteristics, the study found that they were predictive of most PTSD symptoms. To the greatest extent ($p < 0.001$), this was true for the following issues: "During the month, have you been disturbed by repeated, disturbing and unwanted memories of stressful experiences?" 23.04 ($p<0.001$), "Feeling of constant tension?" 20.52 ($p<0.001$), "Feeling upset when something reminds you of a stressful experience?" 19.26 ($p<0.001$), Strong physical reactions when something reminded you of a stressful experience (eg heartbeat, palpitations 17.72 ($p<0.001$)) (Table 3).

Table 3. Comparison of gender peculiarities in the study of PTSD symptoms of Ukrainian university students (comparison by Pearson's Xi- square (c

Question	c	p	*U*	p
During the past month, have you been bothered by recurring, disturbing, and unwanted memories of stressful experiences?	23.04	<0.001	19459.5	<0.05
Recurring and disturbing dreams about stressful experiences?	14.99	0.005	21129	<0.05
Sudden feelings or events as if the stressful experience is happening again?	11.19	0.024	21734.5	<0.05
Feeling upset when something reminds you of a stressful experience?	19.26	0.001	20539.5	<0.05
Strong physical reactions when something reminded you of the stressful experience (e.g., heart palpitations, difficulty breathing)?	17.72	0.001	20977.5	<0.05
Avoidance of memories, thoughts, or feelings associated with the stressful experience?	10.22	0.037	23622	>0.05
Avoidance of external stimuli (people, objects, places, etc.) that remind you of the stressful experience?	1.72	>0.05	25307.5	>0.05
Have trouble remembering important moments from a stressful experience?	8.82	>0.05	22598.5	<0.05
Strong negative beliefs about yourself, other people, or the world around you (e.g., "I'm bad")	5.28	>0.05	25051.5	>0.05
Self-blame or blame others for the stressful experience or what happened afterwards?	2.88	>0.05	24382.5	>0.05
Strong negative emotions such as fear, terror, anger, guilt, or shame?	11.02	>0.05	22424	<0.05
Loss of interest in the activity(s) that used to bring pleasure?	1.81	>0.05	25065.5	>0.05
A sense of remoteness or separation from others?	4.34	>0.05	23555	<0.05
Problems in experiencing positive emotions (eg, inability to feel joy or love)	2.31	>0.05	24961	>0.05
Irritation, outbursts of anger, aggressive behavior?	9.49	0.05	22986	<0.05
Are you take risks or do things that could be harmful?	11.95	0.018	25356.5	>0.05
To be "on the alert" or "on the lookout"?	3.98	>0.05	23978.5	>0.05
A feeling of constant tension?	20.52	<0.001	19786	<0.05
Difficulty concentrating?	4.47	>0.05	23908	>0.05
Have trouble falling asleep or waking up at night?	13.9	0.008	23119	<0.05

Table 4 shows the results of the evaluation of age-related characteristics for manifestations of PTSD.

It has been proven that 'Repeated, disturbing dreams about stressful experiences', 'Difficulty concentrating', 'Problems falling asleep or waking up at night' increase significantly ($p<0.05$) increase with age. The "feeling of constant tension" does not depend on age in a linear way. Other symptoms do not clearly depend on age.

Discussion

Scientists from different countries are actively conducting research on the onset, duration, diagnosis, and treatment of combat-related PTSD. The Ukrainian population has experienced a significant number of traumatic events caused by the ongoing hostilities in the eastern part of the country, the annexation of territories, and significant human losses that affected many families. These events had a negative impact on the life of all Ukrainian citizens, especially young people, causing constant anxiety and tension as predictors of emotional burnout and increased cases of stress disorders. Therefore, we analyzed scientific works on PTSD, where we identified the factors that cause these conditions during the military conflict with the Russian Federation, as well as reviewed guidelines for diagnosis, prevention, and treatment. [9,10]

Table 4. Age Peculiarities in the Study of PTSD Symptoms of Ukrainian Higher Education Students, (comparison by Pearson's Chi- square (^{2}), Spearman's rank correlation coefficient (r_s))

Question	c	p	r_s	p
During the past month, have you been bothered by recurring, disturbing, and unwanted memories of stressful experiences?	14.394	>0.05	-0.015	0.738
Recurring and disturbing dreams about stressful experiences?	10.255	>0.05	0.1	0.022
Sudden feelings or events as if the stressful experience is happening again?	14.935	>0.05	-0.051	>0.05
Feeling upset when something reminds you of a stressful experience?	7.9	>0.05	0.049	>0.05
Strong physical reactions when something reminded you of the stressful experience (e.g., heart palpitations, difficulty breathing)?	8.992	>0.05	0.028	>0.05
Avoidance of memories, thoughts, or feelings associated with the stressful experience?	14.212	>0.05	-0.015	>0.05
Avoidance of external stimuli (people, objects, places, etc.) that remind you of the stressful experience?	8.575	>0.05	0.065	>0.05
Have trouble remembering important moments from a stressful experience?	7.558	>0.05	-0.016	>0.05
Strong negative beliefs about yourself, other people, or the world around you (e.g., "I'm bad")	10.865	>0.05	0.046	>0.05
Self-blame or blame others for the stressful experience or what happened afterwards?	5.991	>0.05	0.009	>0.05
Strong negative emotions such as fear, terror, anger, guilt, or shame?	11.885	>0.05	0.031	>0.05
Loss of interest in the activity(s) that used to bring pleasure?	4.627	>0.05	0.05	>0.05
A sense of remoteness or separation from others?	9.066	>0.05	0.062	>0.05
Problems in experiencing positive emotions (eg, inability to feel joy or love)	4.811	>0.05	0.032	>0.05
Irritation, outbursts of anger, aggressive behavior?	12.667	>0.05	0.062	>0.05
Are you take risks or do things that could be harmful?	13.493	>0.05	0	>0.05
To be "on the alert" or "on the lookout"?	9.723	>0.05	0.021	>0.05
A feeling of constant tension?	21.89	0.005	0.058	>0.05
Difficulty concentrating?	23.109	0.003	0.096	0.027
Have trouble falling asleep or waking up at night?	14.322	>0.05	0.097	0.025
During the past month, have you been bothered by recurring, disturbing, and unwanted memories of stressful experiences?	1.152	>0.05	0.044	>0.05

In times of war, children and youth face various forms of violence, traumatic events, and many factors that affect their physical, emotional, social, and cognitive development. These challenges can pose serious threats to the mental/mental health of children and youth. Children exposed to war, flight, and internal displacement show a wide range of possible reactions to distress and stress, such as specific fears, dependent behaviors, prolonged crying, sleep disturbances, lack of interest in the environment, and psychosomatic symptoms.[11,12]

According to the United Nations Children's Fund (UNICEF), during the ATO/JFO period (2014-2022), 40.0% of Ukrainian children aged 7–12 witnessed war-related events, and 50% of children aged 13-18 witnessed war-related events (14% witnessed the operation of military equipment; 13% witnessed the aftermath of battles; 2.2% witnessed combat clashes; 4% and 15% witnessed violence and beatings of acquaintances; 6% and 5% witnessed threats of use of weapons).

Since the beginning of the war in Ukraine in 2014 (ATO/JFO), a retrospective analysis of the population of Kharkiv and Lviv revealed 23% of respondents with symptoms of PTSD among 65% of internally displaced persons (IDP). The prevalence rate was observed in different groups by sociodemographic characteristics.[13]

A significant proportion of Ukrainian students over 12 years of study have mental health disorders during the war: depressive symptoms (33.9–38.1%); anxiety symptoms (30.8–38.9%); PTSD (42.2-46.8%). The proportion of female students with mental disorders is 3–4 times higher than that of male students. The presence of chronic diseases, the educational field (52.8% of medical students have signs of depressive disorders compared to 31.5% in students of other educational fields ($p<0.001$)), and the fact of direct participation or witnessing of military events contribute to the increase in the level of mental disorders among students of Ukrainian higher education institutions.[13]

The prevalence of PTSD and traumatic events among young people as a result of the Russian invasion of Ukraine found that 68.7% of young people reported various traumatic events in their lives during hostilities, 39.7% experienced bowling events, and 38.0% cases of domestic violence. As a result, 70% of the respondents met the criteria for PTSD (DSM-5) and 31.0% for PTSD (ICD-11).[14,15]

Among 563 Ukrainian students who studied and lived in Kyiv during hostilities between December 2022 and January 2023, 91.5% of Ukrainian students reported one stressor related to the hostilities in Kyiv at the time, while 20.8% reported four or more stressors. Of the total number of respondents, 59.5% of Ukrainian students attributed stressors to military conflict, 54.5% to forced separation from family members, 53.3% to lack of housing, and 15.6% to the loss of a family member or friend. These students had 12.4% probable PTSD (ICD-11) and 11.2% general PTSD among respondents.[16]

According to an online survey of 98.0% of Ukrainian university students from western Ukraine, 86% had been in the war, 86% had seen combat directly, 49% reported symptoms of the insomnia, and 27% of students were diagnosed with PTSD. It was suggested that multidisciplinary integrated intervention programs should be used to treat PTSD in students and that they have a good effect.[17-20]

The identified unfavorable features of the mental/mental health of Ukrainian students during the war may lead to negative consequences in the future, in particular, provoke the emergence of new noncommunicable diseases: cardiovascular diseases, immune disorders, diabetes mellitus. Therefore, it is necessary to continue to monitor the behavioral factors and the state of mental/mental health of Ukrainian students, as well as to raise awareness of early signs of PTSD. Another important issue today is the improvement of preventive measures in higher education institutions in relation to mental / mental health. Particular attention should be paid to risk groups: female students, students who were direct participants or witnesses of military events.

Conclusion

The study of long-term psychological consequences of a full-scale war among students of Ukrainian higher education institutions students is a relevant scientific topic that requires in-depth analysis, monitoring, and rapid response and improvement of the mental state of Ukrainian students.

Among Ukrainian students in higher education institutions, mental/mental health disorders were diagnosed due to their direct participation in hostilities or stay in the war zone: 45.9% of respondents of technical higher education institutions and 24.8% of medical higher education medical institutions in Ukraine. Sexual violence (rape, attempted rape, coercion to perform any type of sexual act by force or threats) among students in higher education institutions was experienced by 18.9% of respondents of technical specialization and 16.9% of respondents of medical specialization. The identified unfavorable characteristics of mental / mental health during hostilities may further lead to long-term negative consequences in the future. Particular attention should be paid to risk groups, particularly female students.

Further prospects for solving the problem from Ukrainian PTSD among students of medical and technical higher education institutions include psychodiagnostics using the methods "Identification of potentially traumatic events in life among students" and "Checklist of PTSD symptoms' for early intervention, psychocorrection, psychotherapy (group, individual), reflexology and preventive measures to improve stress resistance in students.

Declarations

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Author contributions

Conceptualization, V.C., H.S. and M.S; Methodology, M.A.; Software, V.C. and M.A.; Validation, M.A.; Formal Analysis, V.C., M.A., V. K. and N. P.; Investigation,

V.C. and M.A. Resources, V.C.; Data Curation, V.C., M.A.; Writing – Original Draft Preparation, V.C., A.M. and N. P.; Writing –Review & Editing, H.S.; Visualization, H.S. and M.S.; Supervision, V.C.; Project Administration, V.C.; Funding Acquisition, V. K., V. C. and H.S.

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets used and/or analyzed during the current study are open from the corresponding author on reasonable request.

Ethics approval

All subjects gave informed consent to the inclusion prior to participating in the study. The study has been approved by the Bioethics Committee at the University No 2023/09/01.

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ORIGINAL PAPER

Overuse of ionizing radiation imaging by skull X-ray scans for minor pediatric head trauma

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ABSTRACT

Introduction and aim. The assessment of light head trauma in pediatric patients (GCS 14-15) often involves the use of skull X-rays for forensic reasons. This study aims to evaluate the necessity of radiographic imaging and reducing the overuse of X-rays, and developing Slovak guidelines for the appropriate use of X-rays and computed tomography (CT) in pediatric head trauma cases.

Material and methods. This retrospective descriptive study analyzed records from children with head trauma seen at trauma clinics over a period of one year. The study focused on the number of radiographic images (CT and X-rays) performed on pediatric patients and assessed the appropriateness of these imaging techniques.

Results. Out of 1168 pediatric patients with head trauma, 831 (71%) had simple head injuries, 295 (25.26%) had wounds in the head area, 17 (1.45%) had fractures, 23 (1.97%) had concussions, and 2 (0.17%) had intracranial hematomas. A total of 1097 (93.9%) children with head trauma underwent imaging: 1032 had X-rays and 65 had CT scans. The study found that only 3.42% of patients actually needed radiation.

Conclusion. The majority of pediatric head trauma cases were minor and not associated with brain injury, highlighting the overuse of radiographic imaging in these cases.

Keywords. epidemiology, guidelines, ionization, overuse, pediatric head trauma, radiological imaging

Introduction

Head injuries are a common reason for emergency room visits, especially among children.[1,2] These injuries can range from minor to severe and can be classified as closed or open (penetrating) head injuries. Closed head injuries involve a blow to the head without breaking the skull, while penetrating injuries occur when an object breaks the skull and enters the brain, often resulting from high-speed accidents or gunshot wounds.[1,2]

Severe head trauma is less common in younger children due to their smaller body size and lower fall speeds, which can typically be absorbed by their well-padded skin and elastic skeleton. However, assessing head trauma in children remains challenging, particularly in distinguishing between minor and clinically significant injuries.[1,3-5] In Slovakia, the Pediatric Glasgow Coma Scale (PGCS) is used to evaluate head trauma in children. For light head trauma (GCS 14-

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15), a skull X-ray in two views and, in some cases, a head CT scan are performed for forensic reasons (parents might complain to the Slovak health care surveillance authority for an insufficient examination of their child).[6] Simple or light head trauma in pediatric age (PGCS 14-15) means eye opening spontaneously, localized pain or without pain, crying, obeying commands, oriented and conversing, and using appropriate words. Further investigations are sometimes performed to alleviate family fears or due to family exaggeration of the trauma history. However, there is no standardized guideline similar to the Pediatric Emergency Care Applied Research Network (PECARN) guidelines to assist physicians in determining when to use CT scans.[7,8] The most common symptoms for severe head trauma are paleness, somnolence, and vomiting, caused by car accidents, including pedestrians, bicycle injuries, or falling from a height.[5]

The Appropriateness Criteria provide evidence-based guidelines for the use of CT scans in children with head trauma aims to minimize the risks associated with ionizing radiation.[8,9] While MRI or magnetic resonance spectroscopy (MRS) is an effective alternative, its use in children is limited due to the need for sedation or general anaesthesia.[10-12]

Skull X-rays of minor traumatic brain injury does not bring any benefit for diagnosis.[13] For the group aged 0-2 years, a skull X-ray is indicated only if a skull fracture is suspected and the CT scan is not indicated immediately for minor head injury.[14,15] Portable point-of-care ultrasound has high diagnostic precision and valid in children with skull fracture and closed blunt head trauma.[15,16] Also we can reduce the radiation of CT scans by monitoring the child with blunt head trauma and minor skull injury.[16-18]

Aim

The primary aim of this study is to reduce the overuse of X-rays in pediatric head trauma cases. By evaluating the incidence of head trauma and the use of radiographic imaging, the study seeks to create a database for further research and develop Slovak guidelines similar to PECARN to ensure effective and appropriate imaging practices.

Material and methods

The study was conducted based on a review of medical records of 3261 pediatric patients aged 1 to 17 years with trauma who were in trauma clinics in the emergency teaching department and the radiology department. Institutional forms were archived and evaluated in the year 2018. The trauma clinic of the University Hospital, with 1356 beds, is the second-largest hospital in Slovakia, providing a high-standard of healthcare to patients from the Kosice area and Eastern Slovakia. One-thousand one-hundred and sixty-eight patients had head trauma, which constituted 35.82% of all patients treated during that period. Information was used regarding age, types of injuries, the number head trauma images taken, and the severity of head trauma.

The assessments were based on radiological examination: reviews of X-rays of the skull and head, and CT, depending on indications.

Data analysis

Data were analyzed using Microsoft Excel, employing statistical features such as the Chi-square test, standard deviation (SD), and percentage comparison (Microsoft, Redmond, Washington, USA). The hospital's ethics committee reviewed and approved the study (Ethical Committee of the Louse Pasteur University Hospital, 2019/UK/6034).

Sample selection

The sample included all pediatric patients with head trauma seen at the trauma clinics during the study period. Patients were selected based on the criteria of head trauma and the need for radiographic imaging as recorded in their medical files.

Results

A total of 3261 pediatric patients (0–17 years) with trauma were evaluated in the trauma clinics, with a SD of 654.07. Among these, 1168 patients had head trauma (35.82%): 831 had simple head injuries, 295 had wounds in the head area, 17 had fractures: 10 in skull area, 4 in the fascial area and 3 nasal bone fractures, 23 had concussions, and 2 had intracranial hematomas (SD=355.51) (Fig. 1, Table 1).

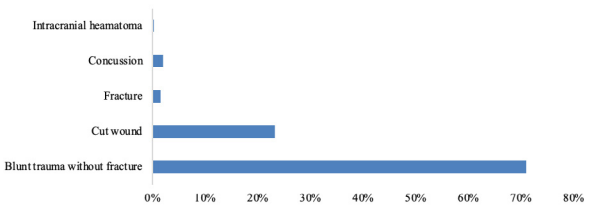

Fig. 1. Head trauma incidence due to severity

Table 1. Types of head trauma

	Blunt trauma	C/W	Fracture	Concussion	Intracranial hematoma
Skull trauma	753	210	10	23	2
Around eye trauma	30	44	0		
Ear trauma	2	3	0		
Max fax area	7	27	4		
Nose	39	11	3		
Total	831	295	17	23	2

Most children (1097 patients, 93.9%) with blunt head trauma underwent radiological imaging (X-ray or CT scan) following the hospital's guidelines. In the age group under 4 years, 738 had light head trauma (89%), and only 93 patients had head contusions in the 4–17 years age group. Cut wounds were observed in 187 patients under 4 years (63%), and 108 patients above 3 years. Seventeen patients had fractures: 14 were less than 4 years old (82%). Twenty-three patients had concussions: 8 were less than 4 years old (35%) and 15 were older than three years (65%). Two patients had intracranial hematomas (See table No. 2), both above three years old (Fig. 2, Table 2). All 42 patients with fractures, concussions, and hematomas were admitted to the hospital, along with 4 patients with blunt head trauma and cut wounds (C/W).

Table 2. Types of head trauma due to age group*

Age group	Light contusion	C/W	Fracture	Concussion	Intracranial hematoma
0–3 years old	738	187	14	8	0
4–17 years old	93	108	3	15	2

* C/W – cut wound

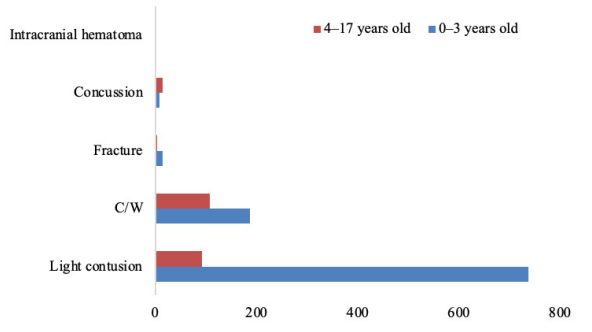

Fig. 2. Head trauma incidence due to age

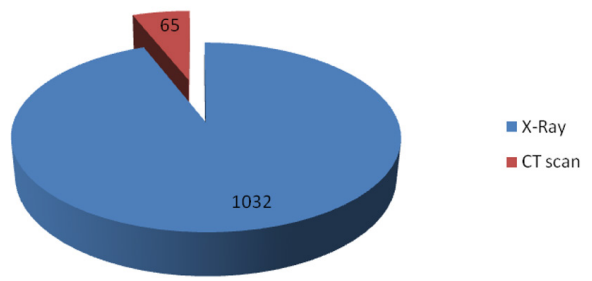

Fig. 3. Number of imaging of head trauma due to type

Among the 1168 patients, 1097 underwent radiation (X-ray or CT), representing 93.90% (see figure 3). One-thousand one-hundred and thirty-two underwent X-ray imaging (anteroposterior and lateral views) with an average ionizing radiation of 5 mGray per patient, and 65 underwent CT scans with an average ionizing radiation of 20.865 mGray per patient (the hospital's X-ray machine is old with hard copies only). Only 3.42% of patients actually needed radiation.

We used the "N-1" Chi-square test to compare the percentage of patients who were irradiated with the percentage of patients who needed radiation (PGCS≤13). The test result showed that the number of irradiated patients was significantly different from the number of patients needing irradiation. $X2(1)= 1913.777$, $p<0.001$. The percent difference is 90.50% with a confidence interval of 88.56–92.02% (Table 3).

The p-value is much below 0.05 (or 0.001), showing that the disparity between the observed and expected frequencies is statistically significant. This indicates that the real amount of irradiated patients deviates significantly from what would be anticipated based on adherence to the PGCS criteria for radiation use.

Table 3. Table of Chi-Square test applied

Category	Observed frequency	Expected frequency	Chi square statistic	Degrees of freedom	p
Irradiated	1097	1057			
Not Irradiated	71	111			
Total	1168	1168			
Chi square statistic			15.92813248	1	<0.001
Calculations					
Expected frequency (needing radiation)			90.5% * total patients		
Total patients			1168		
Calculation			(90.5/100) * 1168		
Expected frequency (needing radiation)			1057.24		
Expected frequency (Not needing radiation)			Total patients – expected frequency (needing radiation)		
Calculation			1168–1057.24		
Expected frequency (Not needing radiation)			110.76		
Degree of freedom			df=(r–1)×(c–1)		
			(2–1)×(2–1)=1×1=1		

Discussion

This study analyzed 1168 pediatric patients with head trauma in the emergency department. Most (71%) did not require medical care or assessment, having no neurological deficit (PGCS 15) with minor trauma, but were seen due to parental concern.[1] This is the first study in Slovakia to address the use of ionizing radiation in the assessment of pediatric head injury, which is a major strength.[1]

The clinical challenge in evaluating minor head trauma in pediatric patients (PGCS 15) is identifying those with clinically important traumatic brain injury while limiting imaging and radiation exposure. Neuroimaging, usually with CT or USG is highly sensitive for identifying brain injuries requiring acute intervention.[7,8,10,16,17] A study in Switzerland emphasized cost-effective and quick imaging.[11] Monitoring and raising awareness is the gold standard, which aligns with our findings about overuse of

skull X-rays.[11] A study which was conducted in the Czech Republic showed that in 93.2% cases, the skull X-ray was negative, which is similar to our study (96.58%). Skull X-rays of minor traumatic brain injury does not bring any benefit for diagnosis aligns with our findings and for an Iranian age group between 0-2 years. skull X-Ray was indicated only if a skull fracture was suspected. Furthermore the CT scan was not indicated immediately for minor head injury.[13,15]

An advanced portable point-of-care ultrasound had high diagnostic precision and was valid in children with skull fractures and closed blunt head trauma.[16,17] Also, we can reduce the radiation of CT scans by monitoring the child in the emergency room (ER) or in the department with blunt head trauma and minor skull injurys.[18,19]

Most infants and children with minor head trauma can be safely discharged home after careful clinical evaluation without undergoing imaging. However, the current lack of clear guidelines in Slovakia leads to the overuse of ionizing radiation. We propose new guidelines to limit X-ray and CT use to patients with significant injury risk. MRI and MRS, while effective, require sedation or anesthesia in young children (0–14 years).[7,8,12] On the other hand Nour et al. recommend skull X-ray scans only if a skull fracture is suspected for younger children than 2 years.[14]

A study which was done in Poland (1993–2002), showed that they had about 12 facial fracture annually (115 in 10 years).[20] Evaluation of pediatric head trauma by neuroimaging is an integral part.[21] But skull radiography can be removed from imaging guidelines.[21] It is recommended if a skull fracture is suspected for younger children than 2 years.[22]

Study limitations

This study had limitations, including the data from a single hospital. Also, realization of the ultrasonography of the pediatric brain is very difficult in our hospital.

Conclusion

Head trauma is common in childhood, with most cases being minor and not associated with brain injury. However, some low-risk children may have significant injuries. The Ministry of Health and Medical Chamber should review and create new guidelines like ACR and PECARN to limit unnecessary radiation exposure (X-rays) and support other valid methods like USG. A health awareness project on radiation risks and initial signs for seeking medical assistance in the ER is recommended.

Acknowledgment

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Declarations

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Author contributions

Conceptualization, G.A. and M.M.G.; Methodology, G.A.; Software, G.A. and A.L.; Validation, R.C., I.M. and V.F.; Formal Analysis, G.A.; Investigation, G.A.; Resources, M.M.G.; Data Curation, G.A.; Writing – Original Draft Preparation, G.A.; Writing – Review & Editing, G.A.; Visualization, G.A.; Supervision, G.A.; Project Administration, A.A.; Funding Acquisition, M.L.

Conflicts of interest

All authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors have no competing conflicts of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval

The study was reviewed, considered, and approved by the Ethical Committee of Louis Pasteur University Hospital (2019/UK/6034).

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ORIGINAL PAPER

Wound healing potential of *Apamarga Ksharodaka* (herbal alkaline water made from *Achyranthus aspera* Linn.) in excision rodent wound model

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ABSTRACT

Introduction and aim. Wound healing is a biological process that aims to restore tissue integrity and function. Despite medical advances, wound management remains challenging. Traditional medicinal preparations, like *Apamarga ksharodaka* (AK), offer promising therapeutic potential due to their phytochemical richness. This study evaluated wound healing and antimicrobial activity of AK. This study aimed to validate the traditional claim of AK's wound healing potential using an excision wound model.

Material and methods. An excision wound model was created using 24 male Wistar rats. A positive control group applied 5% w/w povidone-iodine (PI) ointment. Wound contraction (WC), epithelialization period (ET), wound closure day, and histopathology were assessed. Antibacterial activity was evaluated against *Escherichia coli*, *Streptococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*.

Results. AK showed slightly better wound healing than PI ointment, with significant results in WC rate, wound closure, and ET. Histopathology revealed normal skin and organ architecture. The minimum MIC was 6.25 mg/ml against *Pseudomonas aeruginosa with* a maximum inhibition zone of 15 mm.

Conclusion. AK is safe and effective for wound healing.

Keywords. antimicrobial activity, *Apamarga ksharodaka*, excision wound model, phytochemical richness, traditional medicinal plants, wound, wound healing

Introduction

Disruption of normal anatomical structure and function of the skin is called a "wound".[1] Healing is a series of events typically involving hemostasis, inflammation, proliferation, and remodeling with scar formation phases.[1] Clinical management strategies for wounds are aimed at preventing infection and accelerating healing. The management of wounds with a sterile dressing, the administration of antimicrobial, anti-inflammatory, analgesic drugs and promoters of wound healing augments the healing of wounds.[2]

Achyranthus aspera Linn. commonly known as *Apamarga* in *Ayurveda*, has been described as a divine medicine. *A. aspera* has rich medicinal values and is used as an important ingredient in various formulations. It has been reported to possess activities such as antihyperlipidemic, wound healing, hepatoprotective, antimicrobial, diuretic, antioxidant, anti-inflammatory lithotriptic,

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etc.[3] It is described in the Ayurvedic literature as an expectorant, carminative, digestive and blood-purifier.[4]

Kshara (herbal alkalizer in dry and powdered form) is considered a parasurgical instrument in Ayurveda. Healing and purification of wounds are special properties of *Kshara*.[5] External application of *Kshara* is indicated in many diseases such as skin disorders, nonhealing ulcers, poisoning, psoriasis, piles, fungal infections, worms, sinuses, mouth disorders, tumors, fistula, etc.[6] An intermediary aqueous preparation of *Kshara* is known as *Ksharodaka* (alkaline water). In Ayurved classics, the external application of *Ksharodaka* is recommended for various diseases, that is, in the treatment of goitre, and dandruff.[7] In the context of parasurgical procedures, *Ksharodaka* is mentioned for washing the wound for deworming.[8] *Apamarga kshara* is widely used to treat nonhealing ulcers and anorectal diseases. *A. aspera* Linn is the most commonly used plant for the preparation of *Kshara*. The ease of availability and its pharmacological characteristics are favorable for wound healing (antimicrobial, analgesic, anti-inflammatory, hemostatic, etc.) and exemplify an ideal agent for wound healing.

Aim

For the treatment of fresh excised wounds, *Apamarga Ksharodaka* (AK) is not commonly used and is also not mentioned in the classical ayurvedic literature, so in the present study, AK was selected to validate the traditional claim and attempts were made to evaluate the healing potential of AK in the excision wound model.

Material and method

Animals

Wistar Albino male rats, weighing 200–250 g were selected for the present study. The animals were received from the IMS animal house, Banaras Hindu University (BHU), Varanasi. Rats were kept in the Animal House of the Centre of Experimental Medicine and Surgery, IMS, BHU at 26±2°C and relative humidity of 44–56%, with light and dark cycles of 10 and 14 hours, respectively, for 1 week before and during the experiments. under standard conditions of temperature (22±2°C), humidity and 12-hour light /dark cycle, and relative humidity of approximately 50–55% with a normal diet and purified water *ad libitum*. The animals were acclimatized to standard experimental conditions for 7 days prior to initiation of the experimental study. The CPCSEA guidelines were strictly followed throughout the study. Before the experimental work, approval was obtained from the Institutional Animal Ethical Committee (IAEC) of IMS, BHU (Dean/2010-11/173 dated 23/07/2010). The experiments were carried out during the months from November to January.

Preparation of herbal formulation

The fresh mature *Apamarga panchanga* (all parts of *A. aspera* plant) was collected from the local supplier of Varanasi and weighed. Authentication was performed at the Department of Botany, IMS, BHU. The voucher sample has been kept in the herbarium. (Voucher specimen No. Amarantha 2023/03). *Apamarga Ksharodaka* (AK) was prepared using the classical method of *Ayurveda*.[9] The plant was washed with water to remove physical impurities and then placed for drying in sunlight. When it was completely dried, it was weighed again on the digital weighing machines. The dried plant was placed in a large earthen pot and completely burnt and grey ash was collected after self-cooling, free from stones, mud, and charcoal. The ash was collected in a glass vessel and 6 times of water was added to it, then the contents and left undisturbed for the next 24 hours. The liquid layers of the clear supernatant were collected via an outlet and filtered 21 times filtered using a 3-layer cotton cloth. This *Ksharodaka* was heated over a laboratory hot plate at 60° fixed temperature with intermittent stirring until it remained 1/3 of the total and turned sticky, transparent, and red.

Excision wound healing activity

Male Wistar rats with good health condition, weighing between 200–250 g were randomly divided into four groups (6 rats per group). Group I was a negative control group and did not receive any intervention, and group II was the positive control group that applied 5% pomidine iodine (PI) ointment. Group III was test group 1 received the test drug AK followed by 5% w/w povidone iodine ointment (PI) and Group IV was test group 2 applied AK only.

Excision wound model

The partial-thickness excision wound model was used as described by Morton and Malone.[11] Rats were subjected to ketamine anesthesia (IP) and the skin of the rats was shaved in the mid-scapular region and full-thickness skin wounds were established with a diameter of 2 cm diameter and 0.2 cm depth of punches. These wounds were generated by surgical removal of all skin layers (epidermis, dermis, and subcutaneous fat).

After full recovery from anesthesia, the animals were individually housed in cages. Topical administration of all drugs was conducted daily on wounds till they healed. Wound contraction was measured by planimetric measurement of the wound area. The duration of complete epithelization was determined by noting the days until the scab fell off and no raw wound behind. The percentage of wound contraction and epithelization period was also accessed for healing.

Method of application of test drug

The surrounding area of the wound was covered with a gauze piece to prevent the spread of the drug on healthy tissue. The test drug (AK) was dropped on the other gauze piece with the help of a dropper. Approximately 450 mg of gauze absorbed the test drug (AK) with complete soackage achieved by 10 drops (0.5 mL). AK was applied to the proposed lesion by probing up to the count of 100. As soon as the sign of proper cauterization appears (as described by Sushruta).6 AK is rapidly neutralized by dropping acidic fluid (lemon water) on the wound to neutralize the drug. The wound was washed out with distilled water and the dressing was done. The wound was monitored using signs from the relevant article, that is, after applying AK, the tissue of the treated area becomes purple or dark black, the cauterized lesion shrinks and pain and discharge are relieved.[10] Any adverse effect during the application of AK such as redness of the cauterized area, ulceration with purulent discharge, fever, severe pain, shock, etc. was carefully monitored to evaluate the safety of *Ksharodaka*.

Assessment of wound contraction

Wound contraction was evaluated by monitoring changes in the wound area using planimetric techniques, excluding the day of wounding. The sizes were traced onto transparent paper at specific intervals of 4 days (ie, on the zero, 4^{th}, 8^{th}, and 12^{th} days) and then on alternate days until complete wound healing occurred. These traces were noted onto a 1 mm^2 graph sheet to quantify the wound surface area. Using the following formula, the percentage of wound contraction was calculated.[11]

$$\text{\% Wound contraction} = \frac{\text{(Initial wound size - wound size on a specific day)}}{\text{Initial wound size}} \times 100$$

The duration needed for Escher to fall away without leaving a raw wound was used to monitor epithelialization time (ET).[12] The day the raw surface reaches the level of the skin and develops a scab on its upper surface is recognized as wound closure day.[13] To evaluate the quality of wound healing, an excisional biopsy of the healed skin was analyzed for histological evaluation.

Histopathology

After complete epithelization, wound tissues were excised and stored for 24 hours in 10% buffered formalin. Subsequently, histopathological analysis of regenerated skin tissue was performed following the same procedure as in liver, brain, heart, and kidney histopathological analysis.

In vitro antimicrobial activity

The antibacterial activity of AK was tested using the disc diffusion technique.[14] Muller-Hinton Agar (MHA) plates were developed by placing 15 ml of the molten medium into sterile Petri plates. The recently cultured bacteria were suspended in sterile saline to obtain 107 colony-forming units (CFU) per ml concentration. The suspension was then spread evenly on the surface of Mueller-Hinton agar (MHA) plates and allowed to air dry for 5 minutes. Then 5 µl drug in 300 mg/ml concentration was sited on 6-mm sterile discs of Whatman filter paper no.1. Subsequently, these discs were placed onto the agar surface to allow the compound to diffuse for 5 minutes. The plates were then incubated under specific conditions of 37°C for the 24 hours for cultures. After incubation, the zone of inhibition (ZOI) was measured near the discs with the help of a ruler.

Statistical analysis

Data were presented as mean ± standard error (SE). Statistical significance between the control and treated groups was evaluated using analysis of variance (ANOVA), followed by the student's t-test and post hoc test. A significance level of $p<0.05$ was considered significant. All statistical analyzes were performed using GraphPad Prism software (GraphPad Software, Boston, MA, USA).

Results

Excised wound healing activity

The progression of wound healing in all groups is shown in Figures 1 and 2. The wound contraction (WC) in control group rats was 12.22% to 87.78% from day 4 to day 16, in test group 1 it was 84.44% on day 16. Complete skin healing was observed on day 19 in both these groups. In the positive control group, wc was 96.67 % on day 16 and 95.54 % in test group 2, and skin normalization was observed on day 17 in both these groups (Table 1, Figures 1 and 3).

Table 1. Comparison of all groups for the percentage of WC at different time points

		% of wound contraction					Mean area of wound (mm^2)				
Groups (n=6 in each)	Summary	Day 4	Day 8	Day 12	Day 16	Day 21	Day 4	Day 8	Day 12	Day 16	Day 21
Negative control	Mean±S.E.	12.22± 2.68	45.56± 3.18	65.56± 3.62	87.78± 2.05	97.78± 1.41	547.38± 10.45	213.19± 8.06	88.52± 5.46	12.05± 1.19	1.05±0.21
Positive control	Mean±S.E.	8.89± 3.30	25.56± 5.82	73.33± 4.55	96.67± 2.28		590.86± 13.49	403.86± 12.80	57.62± 5.80	2.62± 0.65	
Test group 1	Mean±S.E.	32.28± 5.27	52.50± 5.92	66.11± 5.46	84.44± 3.14	94.44± 2.08	459.73± 18.87	136.71± 12.77	56.05± 9.96	13.62± 2.74	2.62±0.85
Test group 2	Mean±S.E.	17.51± 1.91	39.04± 2.71	70.10± 2.61	95.54± 2.85		632.55± 14.24	396.52± 9.54	100.05± 6.94	6.81± 1.51	

Epithelialization time

The positive control group (PI) (mean=12.83) showed a significant reduction (p=0.0418) in ET compared to the control (mean=14.83) and test Group 2 (AK) (mean=11.83) also showed a significant reduction (p=0.0201), while test Group 1 (AKPI) (mean=14.67) was not significant (p=0.9947) (Table 2).

Wound closure day

Significant results were observed in test group 2 (p=0.0110), positive control group (p=0.3081), and test group 2 (p=0.011), while test group 1 was not significant p=0.9735 (Table 2).

Table 2. Comparison of all groups with respect to ET and wound closure day

Groups (n=6 in each)	Summary	Epithelialization time	Wound closure day
Negative control	Mean±S.E.	14.83±0.60	12.33±0.76
Positive control	Mean±S.E.	12.83±0.48	10.67±0.21
Test group 1	Mean±S.E.	14.67±0.49	12.17±0.48
Test group 2	Mean±S.E.	11.83±0.31	9.67±0.33
p		0.0041	0.0314
F		9.024	10.69

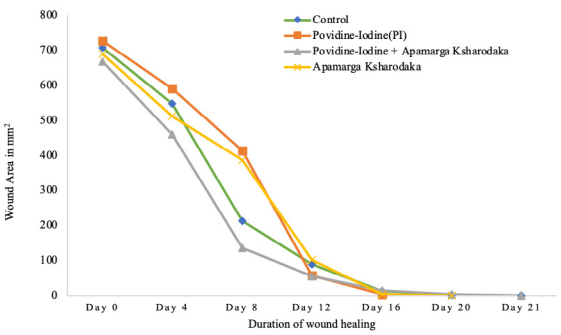

Fig. 1. Graphical presentation of wound healing activity

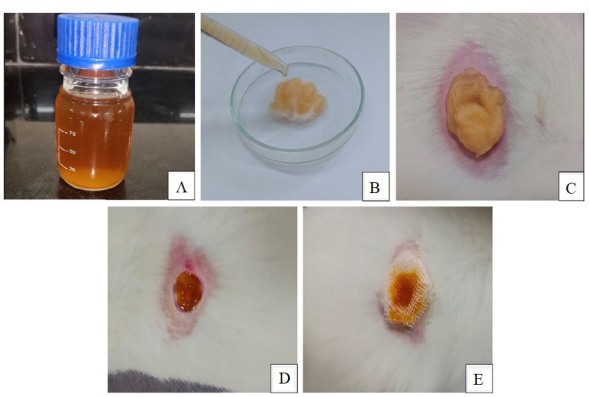

Fig. 2. A: Test drug (*Apamarga Ksharodaka*), B: *Cotton soaked with Apamarga Ksharodaka* (10 drops or 0.5 ml for complete soak), C: Wound dressing with *Apamarga Ksharodaka* in group 4, D: Dressing with 5% w w Povidone iodine ointment in group 2, E: The wound was covered with sterile cotton and gauze in group 2

Histopathology of healed skin and organs

Histopathological observations of the skin section for all groups showed normal blood vessels, dermal and epidermal layers. Histology of liver sections in all groups showed no histological abnormalities with visible hepatic lobule/lumen and well-preserved liver cells in hepatocellular architecture. All groups showed normal histology of the rat cardiac muscles. The brain sections in all groups showed no histological abnormalities and normal blood vessels and pyramidal cells. Histopathological observations for all groups showed normal histology of the rat kidneys (renal tubules, renal cells) was found (Fig. 4).

Antimicrobial analysis

Table 3. *In vitro* MIC-MBC value of AK against different bacteria (in mg/ml)*

Organism	MIC	MBC	ZOI
Escherichia coli	12.5	25	10 mm
Streptococcus aureus	12.5	25	10 mm
Pseudomonas aeruginosa	6.25	12.5	15 mm
Acinetobacter baumannii	25	50	6 mm

* MIC minimum inhibitory concentration, MBC minimum bactericidal concentration, ZOI zone of inhibition

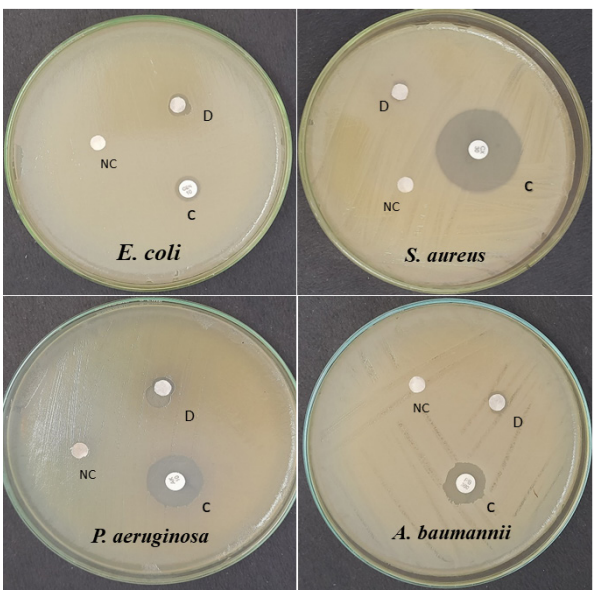

Fig. 5. Antimicrobial activity of AK by the Agar-plate diffusion method

For different bacteria, AK (5 µL drug at a 300 mg/mL concentration) showed the minimum MIC value (6.25 mg/mL) against the *P. aeruginosa* (Gram-negative) bacteria (negative Gram) compared to the other Gram-negative and positive bacteria. The maximum ZOI (15 mm) was expressed against *P. aeruginosa* (Table 3, Fig. 5).

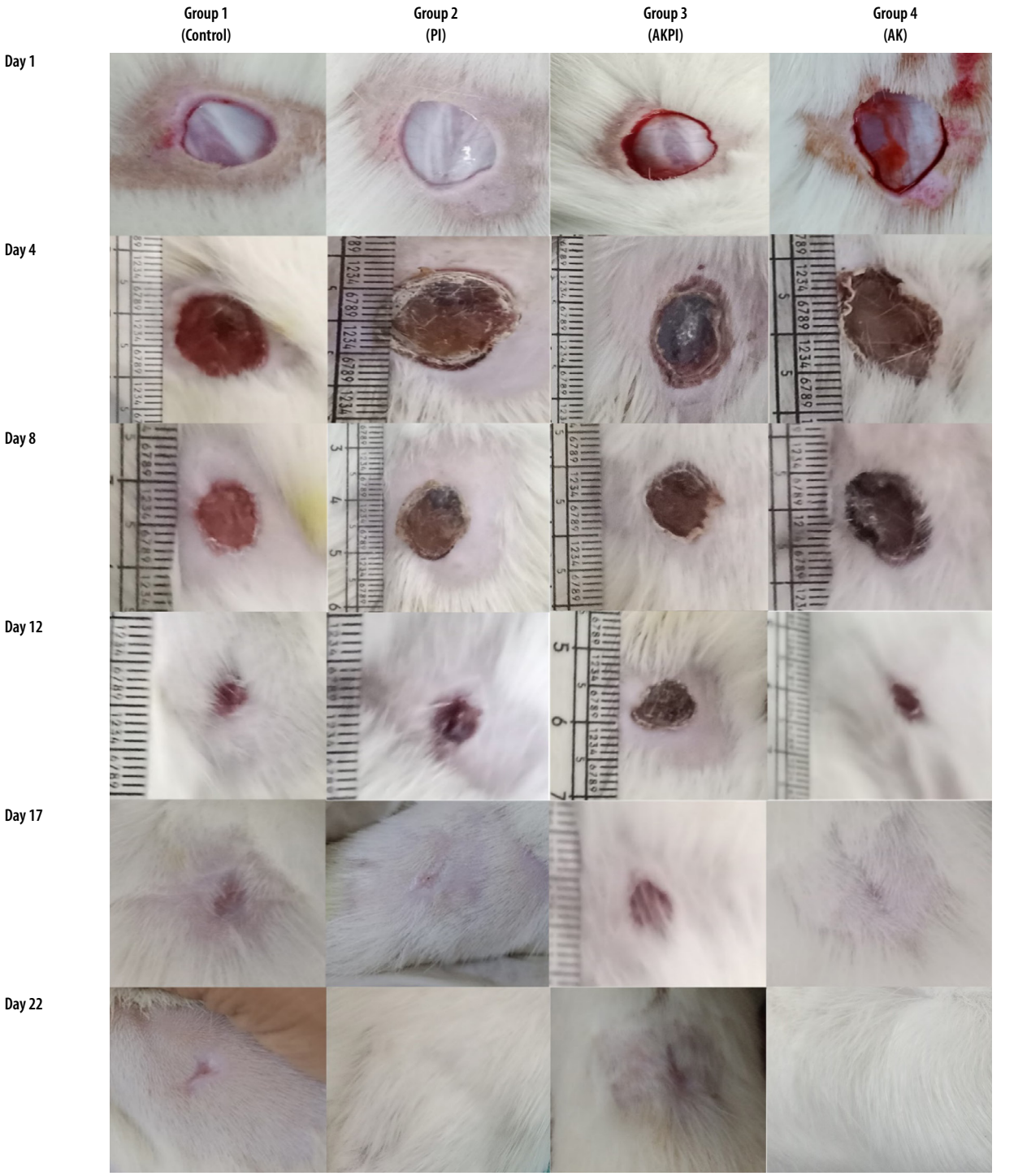

Fig. 3. Photographic representation of WC on different days (group 1 was negative control who received no intervention, group 2 was positive control who received PI ointment 5% w/w, group 3 was test group 1 who received AK and PIointment 5% w/w, and group 4 was test group 2 treated with AK)

Discussion

Wound management aims to enhance tissue regeneration and prevent associated risk factors like infection that directly influence the healing process. Wound healing progresses through distinct phases composed of complex cellular and molecular events. Initially, hemostasis occurs to stop bleeding, followed by an inflammatory phase in which immune cells clear debris and pathogens. Subsequently, proliferative processes promote tissue rebuilding, with fibroblasts producing collagen and new blood vessels forming. Finally, remodeling refines the structure and strength over time, completing the dynamic wound repair process.[1]

AK is an herbal formulation that is not commonly used for a fresh excised or incision wound. An acute dermal toxicity study was also performed to ensure its safety before starting the experiment in animals.

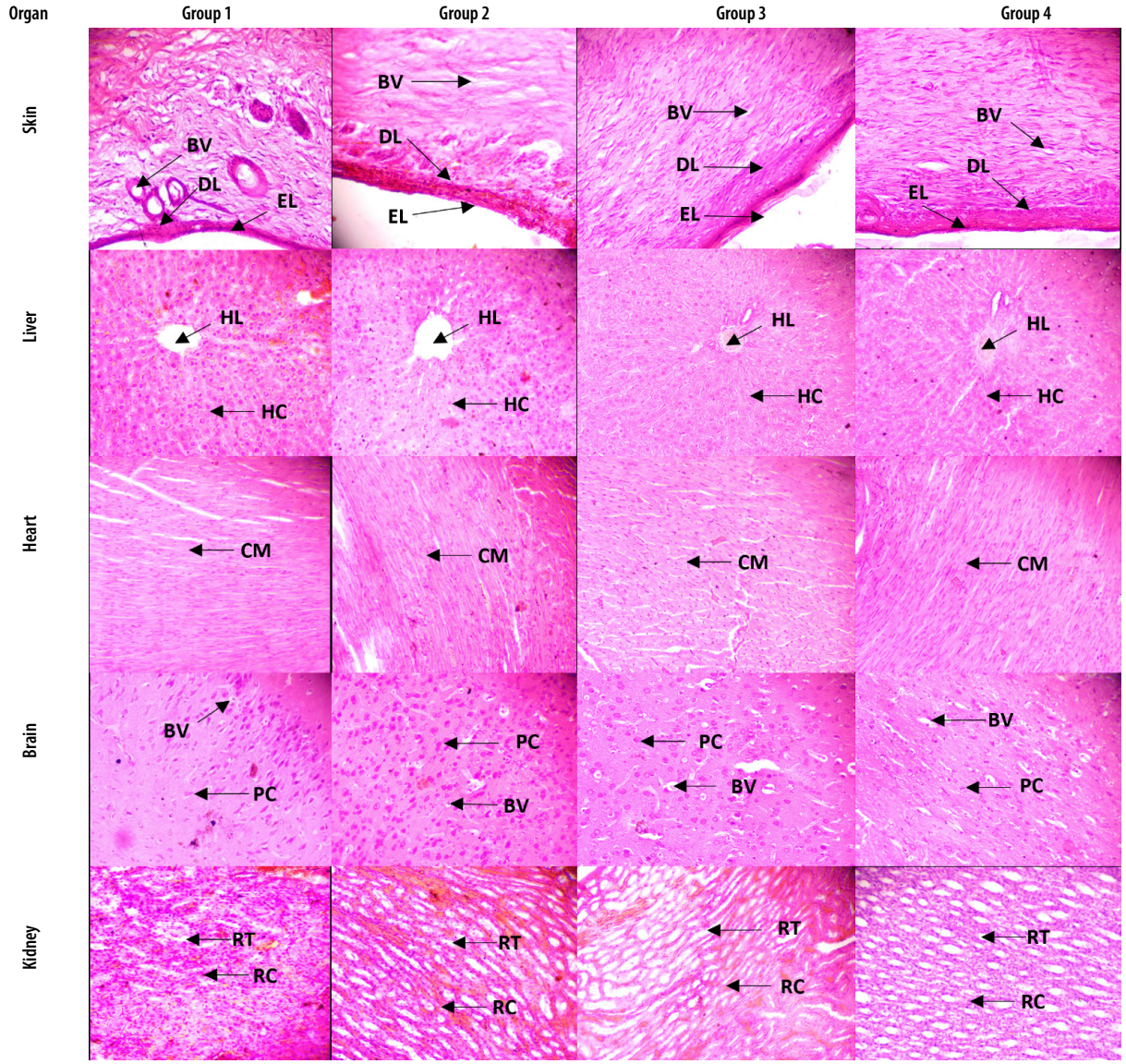

Fig. 4. Tissue sections of different organs and their histopathology on the 21st day after treatment (BV blood vessels, DL – dermal layer DL, epidermal layer, HL – hepatic lobule/lumen, CM – cardiac muscle, HC hepatocyte, PC – pyramidal cells PC, renal tubules, RC – renal cells RC)

WC refers to the process in which the size of the wound decreases due to the pulling together of the wound edges by specialized cells called myofibroblasts. Myofibroblasts are a type of fibroblast that contain contractile proteins, allowing them to exert forces and pull on the surrounding tissue. This cell contraction contributes significantly to reducing the overall size of the wound during the healing process, ultimately aiding in wound closure and scar formation.[15]

The results of this study demonstrated a significant effect of AK on promoting wound contraction, reducing epithelialization time, and a potent antimicrobial action. The results of wound contraction clearly showed differences in the rate and extent of healing between the groups. In the present study, the test drug (AK) and PI showed a significant WC rate ($p<0.0011$) (mean difference (MD)=2.667)) for AK and ($p<0.0045$) (MD=2.550) for PI itself compared to the control ($p<0.3080$) (MD=1.545). Wound healing consists of an orderly progression of a series of stages that establish the integrity of damaged tissues. Wound infections represent the invasion of tissues by the number of species of microorganisms. *A. aspera* has antibacterial and anti-inflammatory properties, and researchers have also claimed that it to be good for skin diseases and also a wound healer, cleanser and removes body toxins, etc.[16] The phytochemicals present in AK play a role in preventing the extension of the initial phase of wound healing by reducing infection, thus facilitating the process of wound contraction. The significant result of wound contraction for AK shows the healing potential through the topical route.

The drug *A. aspera* is delivered in the form of *Ksharodaka* (alkaline herbal water), which is highly alkaline (pH=12.75). High pH drugs create an environment that

supports the activity of certain enzymes involved in tissue repair, so they can contribute to wound healing. This alkaline environment helps deactivate harmful bacteria and enzymes that can delay healing. High pH solutions may help soften necrotic tissue and promote wound debridement. However, its use requires careful monitoring to avoid potential adverse effects on healthy tissue surrounding the wound.[17]

PI (mean=12.83, p=0.0418) and AK (mean=11.83, p=0.0201) showed a significant reduction in epithelialization time compared to control. In between all groups, AK showed the fastest epithelialization. Reduction in epithelialization period attributed to enhanced collagen synthesis, leading to increased skin tensile strength in excision wounds.[18]

Significant results were observed in AK (mean=9.87, p=0.0110) on wound closure day compared to control. Between all groups, AK showed the minimum wound closing time. Hemostasis involves platelet aggregation and clot formation, followed by inflammation with immune cell infiltration. During proliferation, fibroblasts synthesize extracellular matrix, leading to tissue reconstruction, and remodeling involves collagen reorganization for wound maturation.[19] Enhancement of collagen synthesis by fibroblasts, stimulation of neoangiogenesis, and increase of granulation tissue formation are attributed to the presence of phytochemicals in *A. aspera* (alkaloids, flavonoids, saponins, and tannins), which accelerates the process of tissue regeneration.

Bacterial skin infections commonly lead to other systemic diseases by disrupting the immune system and inducing inflammation, tissue impairment, and resulting in delayed wound healing. An antimicrobial study was performed to evaluate the antibacterial activity of AK. This pharmaceutical preparation can be used to treat topical infections of *P. aeruginosa*, *A.baumannii, S. aureus,* and *E. coli.* The MIC and MBC values, particularly against *P. aeruginosa,* are significant. AK was more effective against *P. aeruginosa* (MIC 6.25 mg/mL, MBC 12.5 mg/mL) with a zone of inhibition (ZOI) of 15 mm, followed by *E. coli* with a ZOI of 10 mm. This indicates the potential of AK to target both Gram-negative and Gram-positive bacteria, supporting its use in wound healing, where infections from these pathogens are common. The antimicrobial action of AK may have contributed to faster wound healing by preventing bacterial colonization and subsequent infection. Previous studies show that *A. aspera* possesses an antibacterial potential, which is confirmed in the current study by inhibition of bacterial growth. Sofowora reported that secondary metabolites (such as alkaloid, phenol, flavonoid, steroid, saponin, glycoside, oil, and tannin) in plant extract are traditionally used in the treatment of infectious wounds and have potential antimicrobial activity that justifies their traditional use for the treatment of various illnesses.[20] Yadav et al. found antibacterial activity in the aqueous extract of *A. aspera* against *Streptococcus mutans*.[21] A similar study was performed by Kaur et al., and found the antibacterial potential of chloroform and methanol shoot and root extracts against *Klebsiella sp.* and *Bacillus Substilis*.[22] Owk et al. proved the aqueous extract of plants has a maximum inhibition zone against *B. subtilis and S. aureus;* while considerable inhibitory activity against *Streptonryces pneumoniae*.[23] This inhibitory effect of extracts against human pathogens introduces this plant as a potential substance for the development of new drugs against pathogenic microbes.

Histopathological observations of the skin section for all the groups showed normal blood vessels, dermal and epidermal layers. Histopathological observations for all groups showed normal architecture of rat kidneys, liver, brain, lungs, and spleen cells. All groups did not show histological abnormalities (Fig. 4).

Many studies are carried out with various herbal medicines and plant extracts that have wound-healing properties.[24] Results indicate that the aqueous and ethanol extracts of *A. aspera* significantly promoted the tensile strength of the wound using an incision and excision wound. The aqueous extract has more superoxide scavenging and DPPH radical scavenging activity compared to the ethanolic extract.[25] A study revealed that the 80% methanol leaf extract of *A. aspera* with 10% chloroform fraction possessed a high degree of WC, a reduced period of epithelialization, and also had significant anti-inflammatory activity.[16] In this research, the wound healing activity in AK is supported by the reduction in the rate of WC, ET, histopathological evidence, potent antibacterial activity, no secondary infections, and no morbidity. This study also validates the traditional claim for AK for wound healing.

The result obtained from this study restores the facts explained by our Acharyas regarding the healing properties of *Ksharodaka.* This echoes the vast knowledge and deep insight of our Acharyas in designing *Ksharodaka* as a separate dosage form. The current observations can be considered as a basis for further studies on *Kshara Kalpana.* This study gives valuable considerable research that has focused on the validation of *Apamarga Ksharodaka* for wound healing activity.

Study limitations

The study was carried out on a relatively small number of animals (24 rats). A larger sample size could enhance the applicability of the findings, and testing on additional species may provide broader insights, especially to translate the results to human applications. The short duration of the study restricts insights into the long-term effects of AK. Variability in the traditional preparation of AK may affect the consistency of the result, so standardization of the formulation could help ensure more reliable and reproducible outcomes across different studies.

Future perspective

Future research should focus on conducting clinical trials with humans to test how safe and effective AK is for wound care and to identify any possible side effects. Comparative studies with other herbal and modern wound healing agents would provide information on their relative effectiveness. Optimizing dosage and standardizing formulation is essential for consistency, and exploring the molecular mechanisms underlying AK's effects could further clarify its therapeutic potential.

Conclusion

This study revealed that in the topical application of AK, there were no signs of dermal toxicity. The wound healing activity in AK was slightly more potent compared to PI ointment. AK showed pronounced antimicrobial and healing potential, so it may be suggested for treating various types of wounds. AK showed a significant improvement in WC rate, wound closing period, ET, and histopathological changes. This study shows that AK is a safe and effective drug for wound healing.

Declarations

Funding

The author declares that no funding was received for conducting this research.

Author contributions

Conceptualization, B.S. and S.K.S; Methodology, B.S. and S.K.S.; Software, A.K.G.; Validation, A.K.G. and C.P.; Formal Analysis, B.S., A.K.G. and S.K.S; Investigation, S.N.R; Resources, S.N.R and S.K.S; Data Curation, A.K.G. and S.S.M.; Writing – Original Draft Preparation, B.S. and A.K.G.; Writing – Review & Editing, B.S., A.K.G. and S.K.S; Visualization, A.K.G. and S.N.R.; Supervision, B.S. and S.K.S.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data are available on logical request.

Ethics approval

Ethical approval was obtained from the Institutional Animal Ethical Committee (IAEC) of IMS, Banaras Hindu University (Dean/2010-11/173 dated 23/07/2010).

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ORIGINAL PAPER

Spatial working memory in hypothyroidism – an observational study on different ranges of thyroid stimulating hormone

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ABSTRACT

Introduction and aim. Hypothyroidism is associated with cognitive impairments. Clinically, subclinical hypothyroidism (SCH) is very common and patients often experience symptoms such as forgetfulness or memory deficits. Despite achieving normal thyroid stimulating hormone (TSH) levels through levothyroxine (LT-4) treatment, patients still report persistent complaints of lack of memory. Previous imaging studies have shown abnormalities in some cognitive domains, particularly in spatial working memory (SWM), that are characteristic of SCH. Therefore, the current study aimed to investigate SWM function across different ranges of TSH in patients with SCH.

Material and methods. This cross-sectional study included a total of 136 participants. Group 1: 36 controls, group 2: 33 newly diagnosed patients with SCH (TSH levels ≥2.5 mIU/L), group 3: 32 patients with SCH (elevated TSH levels ≥4.0 mIU/L) having L-T4 treatment, group 4: 35 euthyroid (TSH levels <4.0 mIU/L) but ongoing LT-4 treatment. The SWM task was performed for an assessment of SWM function, using a computerized battery (Cambridge Neuropsychological Test Automated Battery-CANTAB).

Results. Our results report a statistically significant difference in the key parameters of SWM task among all groups.

Conclusion. Our findings indicate that patients with SCH show better performance in SWM when their TSH levels decrease with LT-4 treatment, in comparison to patients who were newly diagnosed. The present study suggests a TSH level of 2.5 mIU/L may be the optimal threshold for initiating LT-4 treatment in patients with SCH.

Keywords. hypothyroidism, memory, neuropsychological symptoms, spatial working memory, subclinical hypothyroidism

Introduction

Thyroid hormones play an important role and intervene in the development of the adult brain, exerting multiple effects on various processes such as neurogenesis, dendritic cell proliferation, glial development, synaptogenesis and myelination.[1] As the brain is a major target organ for thyroid hormones, it is known that cognitive impairment occurs along with thyroid dysfunction.[2]

Hypothyroidism cases have shown changes in brain metabolism. These alterations encompass emotional lability, characterized by slowed thought processes, diminished attention, apathy, occasional psychosis, and agitation. Additionally, cognitive functions may experience a decline, including general intelligence, memory, concentration, psychomotor skills, and executive functions.[3]

Subclinical hypothyroidism (SCH), i.e., an elevated TSH level with normal free thyroxine (fT4), is of particular importance due to its high prevalence and its association with cognitive impairment. Several studies have reported subtle impairments in certain cognitive do-

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mains; among the domains frequently affected is memory, particularly working memory (WM).[4–7] Working memory, the "sketchbook of rational thought", acts as a platform on which we manipulate and record thoughts.[8] It is capacity limited and denotes a multi-processing that makes task relevant information approachable to some sophisticated cognitive processes such as reasoning, decision making, reading comprehension and learning. Spatial working memory (SWM), i.e. the ability to recall the location of the object or occurrence of an event, is likely to be affected and is generally recognized to require greater attentional resources compared to textual or non-spatial memory.[9-11] Spatial working memory is generally regarded as a fundamental component that supports various higher cognitive functions, including. but not limited to, language learning, language comprehension, reasoning, and decision making. In addition, it shows strong connections with other cognitive behaviors, demonstrating its crucial role in cognitive processing.[12] In addition, imaging studies[13] have shown abnormalities in some cognitive areas, particularly in WM, that are characteristic of SCH. Functional neuroimaging provides a neuroanatomical basis for these reported deficits. Patients with SCH showed poor SWM and abnormal fMRI findings in frontal brain regions.[10]

Clinically, patients with SCH often express complaints, particularly in the cognitive and psychiatric areas; the most common complaints include memory impairment and neuropsychological symptoms (such as insomnia, depressive mood, fatigue, mental fogginess, and apathy).[13]

In addition, patients, in spite of being euthyroid (normal thyroid profile) after treatment, present neuropsychological symptoms. Also, despite achieving normal TSH levels (4 mIU/L), a considerable number of SCH patients undergoing levothyroxine (LT-4) treatment frequently report persistent forgetfulness and mental slowness. Talaie et al. reported that a patient with a TSH ≤4.0mIU/L and no symptoms should be considered to be in the euthyroid state.[14] However, if a patient reports neuropsychological symptoms (such as fatigue, insomnia, depression, and mental fogginess, apathy) a cut off value of TSH 2.5mIU/L should be considered as a guide for initiating LT-4 treatment.

Given the relevance of memory deficits in the SCH population, to the best of our knowledge, no prior study has been conducted at different ranges of TSH for the examination of SWM function.

Aim

The current study was designed to investigate the impairment of SWM function in patients with SCH and euthyroid patients (on LT-4 treatment) compared to newly diagnosed cases (with neuropsychological symptoms) and controls, and also sought to address the gap in the existing literature by investigating the potential direct relationship between TSH levels and depressive symptoms. The findings of this study have important implications for clinical practice, particularly regarding the trajectory of hypothyroidism and the potential long-term cognitive challenges that patients may face.

Material and methods

Study design, participants and clinical evaluation

This cross-sectional study was conducted between May 2022 and July 2023, and included 136 participants, of which 100 were patients with SCH. The patients were recruited from the endocrine outpatient department of multiple health centers in the city as well as through local general medical practices. Written permission was obtained from each consultant physician for the recruitment process. The protocol of this study was approved by the Institutional Ethics committee of Guru Nanak Dev University, Amritsar (302/HG). It is the part of a larger research program which is registered under Clinical Trial Registry of India, CTRI/2022/04/042319. It was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients before study procedure.

Additionally, 36 healthy controls were selected from a cohort of volunteers residing within the local community. These healthy subjects exhibited normal thyroid function results, accompanied by a favorable medical history, indicating good overall health. They also had no reported neurological or significant cardiovascular disorders, or any other unmanaged chronic ailments.

Thirty-three patients were recruited who had complaints of neuropsychological symptoms such as fatigue, forgetfulness, insomnia, and depressive symptoms, and they were directed to undergo thyroid profiling. Upon review of the laboratory findings indicating TSH levels ≥2.5 mIU/L, the consultants established a diagnosis of SCH for these newly identified cases. Thirty-two patients with elevated TSH levels and continued LT-4 who visited due to hypothyroidism symptoms were also enrolled, as were thirty-five euthyroid individuals who were seen for follow-up.

Inclusion criteria: Patients diagnosed with SCH were identified through thyroid function tests and self-reported symptoms. Those who reported neuropsychological symptoms (such as fatigue, forgetfulness, insomnia, and depressive symptoms) and had a TSH 2.5 mIU/L or higher were considered newly diagnosed cases of SCH by the consultants. Patients with symptoms of hypothyroidism (such as fatigue, dry skin, cold intolerance and weight gain) or uncontrolled TSH levels (≥4.0 mIU/L) were included in the study as having elevated TSH levels. Patients with TSH levels below 4.0 mIU/L who were seen for follow-up were classified as euthyroid.

Subjective health and laboratory measures

The patients were interviewed about their subjective complaints, with a focus on any complaints that had developed or worsened since their thyroid pathology. A self-developed assessment form, encompassing all hypothyroidism and neuropsychological symptoms, was administered to the patients. Each subject underwent a clinical and biochemical evaluation of their thyroid function. The serum levels of TSH in mIU/L, free triiodothyronine (fT3) in ng/mL, and free thyroxine (fT4) in µg/dL were measured using the mini VIDAS automated immunoassay system. These assays were performed by a certified medical technologist. Blood samples were collected from patients in a fasted state between 8:00 AM and 10:00 AM.

Any medical comorbidities, personal history of psychiatric disorders, family history of psychiatric disorders, and use of other medications (psychiatric and non-psychiatric drugs) were noted. The patient's medical history included information about their current L-T4 treatment, including specific doses and duration, as well as any previous thyroid replacement therapy and neuropsychological symptoms.

Exclusion criteria: Patients any other neurological disorders or psychiatric disorders; history of antipsychiatry drugs; family history of dementia; any other systemic diseases such as myocardial infarction, hypertension or diabetes mellitus; vitamin B12 deficiency; any drug addiction.

Participants were enlisted on the basis of the biochemical evidence of hypothyroidism and medication history and grouped as follows:

- group 1: Controls with no history of thyroid dysfunction
- group 2: Newly diagnosed cases with TSH levels ≥2.5mU/L and presenting with neuropsychological symptoms but no past history of thyroid dysfunction
- group 3: Patients with the current history of hypothyroidism symptoms (TSH levels ≥4.0 mIU/L) who were currently undergoing L-T4 treatment
- group 4: Euthyroid patients (TSH levels ≤4.0 mIU-/L) and currently undergoing LT-4 treatment

Objective depression and cognitive function assessment

Global cognitive status was evaluated using the Mini Mental State Examination (MMSE). The maximum score on the MMSE is 30. There are three categories for cognitive impairment severity: 0–17 represents severe cognitive impairment, 18–23 represents mild cognitive impairment, and 24–30 represents no cognitive impairment.[15] Participants were eligible for recruitment if they achieved a score of 24. Depression was assessed by Hamilton depression rating scale (HDRS) that is a specific scale for assessment of depressive symptoms, consisting of 17 items. Eight items are scored on a 5-point scale, ranging from 0=not present to 4=severe. Nine are scored from 0–2.[16] Total score of 0–7=normal, 8–13=mild depression, 14–18=moderate depression, 19–22=severe depression, >23=very severe depression were considered.

Socioeconomic status was assessed using the Kuppuswamy scale.[17] The total score ranges from 3 to 29 and it classifies families into 5 groups, "upper class (I), upper middle class (II), lower middle class (III), upper lower (IV) and lower socio-economic class (V) on the basis of a) occupation of the head of the family, b) education of the head of the family, c) total monthly income of the family."

SWM function task

All the recruited subjects were administered the computerized neuropsychological test for SWM from the Cambridge Neuropsychological Test Automated Battery (CANTAB). It is a fair neuropsychological authenticated and reliable computerized test battery, used to assess executive functions.[18,19] The extensive subsets of CANTAB allow measuring various cognitive subdomains of executive functions such as SWM, new learning, short-term memory, attention through a single battery, in comparison to traditional neuropsychological tests. In current study, it is believed that the use of CANTAB battery for the assessment of SWM function would accurately document changes.

The evaluation of the battery performance was conducted in a controlled and noise free environment, specifically a quiet room.

Participants were instructed to search through a number of boxes on the screen to find which hid a yellow token. There was a guideline to govern this search, which said that if a token was found inside a specific box, it would not be found there again during that trial. As a result, as they moved through each trial, the participant needed to remember which boxes the tokens had already been located in. Trials increased in difficulty according to the number of boxes (4, 6 and 8). Following performance indices were recorded:

1. Spatial working memory between error (SWMBE): The number of times the subject incorrectly revisits a box in which a token has previously been found. Calculated across all assessed four, six and eight token trials.
2. Spatial working memory between errors SWMBE4 boxes: The number of times a subject revisit a box in which a token has previously been found. Calculated across all trials with 4 tokens only.
3. Spatial working memory between errors SWMBE 6 boxes: The number of times the subject revisits a box in which a token has previously been found. Calculated across all trials with 6 tokens only.

4. Spatial working memory between errors SWMBE8 boxes: The number of times the subject revisits a box in which a token has previously been found. Calculated across all trials with 8 tokens only.
5. Spatial working memory double error (SWMDE): The number of times a subject commits an error that is both a within error and a between error. Calculated across all assessed four, six and eight token trials.
6. SWM Strategy (6-8 boxes): The number of times a subject begins a new search pattern from the same box they started with previously. If they always begin a search from the same starting point, we infer that the subject is employing a planned strategy for finding the tokens. Therefore, a low score indicates high strategy use (1=they always begin the search from the same box), a high score indicates that they are beginning their searches from many different boxes. Calculated across assessed trials with 6 tokens or more.
7. Spatial working memory total error, SWMTE: The total number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the subject, i.e., between errors + within errors - double errors. Calculated across all assessed four, six and eight token trials.
8. Spatial working memory total errors SWMTE4 boxes: The number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the subject, i.e., between errors + within errors - double errors. Calculated across all trials with 4 tokens only.
9. Spatial working memory total errors SWMTE6 boxes: The number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the subject, i.e., between errors + within errors - double errors. Calculated across all trials with 6 tokens only.
10. Spatial working memory total errors SWMTE8 boxes: The number of times a box is selected that is certain not to contain a token and therefore should not have been visited by the subject, i.e., between errors + within errors - double errors. Calculated across all trials with 8 tokens only.
11. Spatial working memory within errors (SWMWE): The number of times a subject revisit a box already shown to be empty during the same search. Calculated across all assessed four, six and eight token trials.

The level of education was recorded for each participant in CANTAB, (level 1, left formal education before age 16; level 2, left formal education at age 16; level 3, left formal education at age 17–18; level 4, undergraduate degree or equivalent; level 5, Master's degree or equivalent; level 6, PhD or equivalent).

Statistical analysis

The sample size estimation was conducted using G* Power (3.1.9.2). The average proportion of exposed cases (i.e. TSH mean value influencing cognition process in hypothyroidism) was ρ=0.27, with a desired absolute error of d=0.05 and a power of β=0.90.[20] Further analysis were done by using SPSS version 27.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA). Descriptive statistics were used to describe the basic characteristics of the study population. The normality of the cognitive data was assessed using the Kolmogorov-Smirnov test. Due to the violation of the assumptions of normality of variance, non-parametric tests (the Kruskal-Wallis test and Spearman correlation test) were employed. Between-group comparisons were conducted using the Kruskal-Wallis test. General linear models (GLM) and repeated measures factorial ANOVAs were used to investigate the effect of working memory load for conditions to demonstrate overall group effects. Greenhouse-Geisser corrected statistics were used to report the repeated measures ANOVAs. Confounding factors such as age, gender difference, level of education, and socioeconomic were controlled for in the overall group effects using a linear regression model, we inspected whether these factors influenced the dependent measures. Additionally, to explore the potential impact of the duration of LT-4 treatment on SWM with the reduction in TSH levels, group 4 (euthyroid patients, n=35) was divided into two subgroups: 4a (ongoing LT-4 for 1 or more than one year, n= 29) and 4b (ongoing LT-4 treatment for 10 or more than ten years, n=6). These subgroups were then analyzed for analysis of one of the key parameters of the SWM task: SWMTE.

Results

The present study analyzed data from 136 subjects, including 100 patients and 36 controls. Descriptive values of the clinical characteristics and demographics of the study population (as shown in Table 1). Between group differences were found in total error, between errors, and double error, which are the key parameters of the SWM task (Table 2). Further analysis was conducted to determine if there was a significant effect of load on between search errors. A repeated measures ANOVA with a 3 load (4, 6, 8 boxes) by 3 group designs showed a significant effect of load ($F(2, 1.32)=141.21$, $p\leq0.001$). However, the interaction between group × load result was not significant ($F(6,3.9)=1.26$, $p=0.27$). This effect was observed in all groups was due to the increase in load from 4 to 6 boxes rather than from 6 to 8 boxes (Fig. 1). Socioeconomic status was found as a predictor variable and influenced the measures of dependent variables: SWM Total errors ($F=5.49$, $p=0.02$) and SWM strategy scores ($F=7.23$, $p=0.008$), as assessed by a linear regression model. This was suggesting that socioeconomic status has an impact

on working memory. Another aspect we investigated was the effect of the duration of LT-4 treatment on SWM in the euthyroid group. A notable trend was observed (Fig. 2), the group 4b making fewer errors compared to group 1, indicating better performance in SWM function after 10 or more years of LT-4 treatment.

Table 1. Clinical characteristics and demographics of the study population*

Variable	Group 1 n=36	Group 2 n=33	Group 3 n=32	Group 4 n=35
Age (years)	36±9.2	36.9±0.65	38.6±11.1	38.9±9.79
Gender	M=6; F=30	M=4; F=29	M=1; F=31	M=2; F=33
Level of education	3.8±0.77	3.6±0.65	3.6±1.03	3.51±0.81
Socioeconomic status	20.5±3.24	19.75±3.77	20.8±4.29	18.9±4.84
MMSE	29.4±0.73	29.18±2.06	28.09±2.05	27.57±2.44
HDRS	1.97±1.62	5.09±3.71	4.28±3.21	2.68±2.24
TSH (mIU/L)	1.6±0.72	4.04±1.40	13.30±20.99	2.94± 0.94
T3 (pmol/L)	1.57±1.43	1.16±0.58	1.04±0.56	1.07±0.50
Free T4 index (µg/dL)	7.71±2.42	8.47±1.69	8.95±1.65	9.09±1.41
Years of medication	–	–	4.62±3.69	3.85±3.25
Neuropsychological and other symptoms				
Fatigue	52.78%	72.72%	87.5%	57.14%
Weight Gain	16.6%	33.3%	68.7%	11.4%
Insomnia	22.4%	42.4%	59.3%	48.5%
Memory loss	08.3%	78.78%	81.2%	42.8%
Cold intolerance	05.5%	27.2%	31.2%	25.7%
Dry skin	11.1%	21.2%	34.3%	31.4%
Hearing loss	5.5%	18.1%	43.7%	11.4%

* M – male, F – female, MMSE – Mini Mental State Examination, HDRS – Hamilton Depression Rating Scale, TSH – Thyroid Stimulating Hormone, group 1 – controls, group 2 – newly diagnosed cases, group 3 – patients with elevated TSH levels (on going-LT-4), group 4 – euthyroid (but on LT-4)

Table 2. Comparison of Spatial working memory (SWM) task parameters among all groups*

Variables	Group 1 (n=36), mean rank	Group 2 (n=33), mean rank	Group 3 (n=32), mean rank	Group 4 (n=35) mean rank	p
SWMTE	56.35	74.26	80.73	64.39	0.05
SWMBE	55.78	74.64	81.02	64.36	0.04
SWMWE	70.25	69.18	75.25	59.89	0.22
SWMDE	67.96	70.80	77.56	58.60	0.04
SWMTE4 boxes	57.61	67.33	77.03	73.00	0.11
SWMBE4 boxes	57.61	67.33	77.03	73.00	0.11
SWMTE6 boxes	61.14	76.00	80.53	58.00	0.04
SWMBE6 boxes	60.29	76.20	80.47	58.74	0.04
SWMTE8 boxes	57.79	71.61	77.11	68.71	0.21
SWMBE8 boxes	56.11	72.53	75.06	71.44	0.16
SWMS	67.19	67.83	78.31	61.50	0.36

* SWM – spatial working memory, TE – total error, BE – between error, WE – within error, DE –double error, group 1 – controls, group 2 – newly diagnosed cases, group 3 – patients with elevated TSH levels (on going-LT-4), group 4– Euthyroid (but on LT-4)

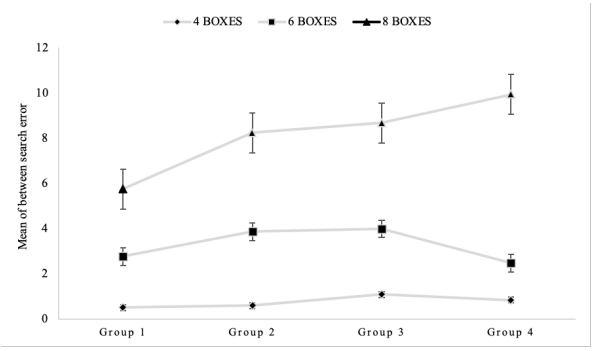

Fig. 1. Shows mean of between search error with load (4 to 6; 6 to 8 boxes) within groups, group 1 – controls, group 2 – newly diagnosed cases, group 3 – patients with elevated TSH levels (on going-LT-4), group 4 – euthyroid (but on LT-4)

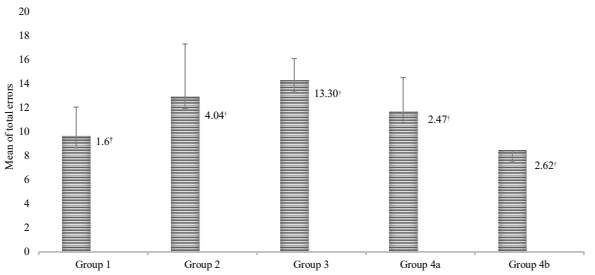

Fig. 2. Shows the mean values and standard deviation of total error in spatial working memory task among all groups, † – mean value of TSH, group 1 – controls, group 2 – newly diagnosed cases, group 3 – patients with elevated TSH levels (ongoing LT-4), group 4a – euthyroid (on LT-4 for 1 or more than one year but less than 5 years), group 4b – euthyroid (on LT-4 for 10 or more years)

Discussion

The specific impairment in WM in individuals with SCH is not well understood. Previous research has consistently shown that patients with SCH exhibit significant impairments in SWM performance.[21,22] However, the exact range of TSH levels at which memory impairment occurs is still unclear.

Our clinical observations indicate that patients on LT-4 can be broadly categorized into three groups:

1. Newly diagnosed patients reporting symptoms.

2. Diagnosed patients who are either noncompliant or poorly compliant with their dosage, resulting in poorly controlled TSH levels.

3. Compliant patients who regularly follow up with their clinician to regulate medication dosage and maintain TSH levels within normal limits.

Based on this clinical picture, patients with subclinical hypothyroidism (SCH) were classified into these three categories. Therefore, the present study aimed to investigate deficits in SWM function in patients with SCH who are currently on LT-4 treatment, as well as newly diagnosed cases, and compare them to a control

group. To objectively assess SWM function, we utilized a computerized battery with touchscreen technology. Our results, confirmed by psychological testing, showed that the patient groups had statistically significant differences ($p \leq 0.05$) in key parameters of the SWM task when compared to the control group.

We discovered that group 4 (with a mean TSH level of 2.94 mIU/L) performed better with fewer errors in the SWM task compared to group 3 patients (with mean elevated TSH levels of 13.3 mIU/L). This enhancement in memory skills can be attributed to the decrease in TSH levels resulting from appropriate treatment. These findings suggest a connection between TSH and WM. However, we also found that SWM deficits were more prevalent in group 4 compared to group 1, indicating that this memory domain does not fully recover to a euthyroid state.

Interestingly, we found that out of 35 euthyroid cases of group 4, 6 patients had been administered LT-4 for a duration of 10 or more years. These patients of group 4b showed a notable reduction in total errors compared to group 4a (who had been on LT-4 for one or more than one year but less than 5 years). Contrary to this, a clinical trial conducted by a study in patients with SCH found that there was an improvement in SWM function in the Wechsler memory scale after 6 months of LT-4 treatment with a reduction in TSH value with a mean and standard deviation of 3.96±1.23 mIU/L.[21] Also, Correia et al. reported that there was improvement in SWM function in the n-back task after 6 months of LT-4 treatment in patients with SCH.[23] One study conducted on animal models found that the administrating LT4 daily for three months significantly improved spatial memory in older mice.[24] The varying results among studies on the impact of LT-4 treatment on SWM in the SCH population may be attributed to differences in assessment methods or study design.

On the other hand, group 2 made more between search errors (SWMBE) and double errors (SWMDE) but fewer errors than group 3. This might be because mild SCH impairs memory and causes neuropsychological symptoms in these patients. It is important to determine whether the observed enhancement in WM among patients is a direct result of restoring of normal endocrine function or an indirect consequence of alleviating neuropsychological symptoms. Though beyond the scope of this study, this area may be the target of future neuroimaging studies. In contrast to the impairment for between search errors and double errors, the patient groups taken entirely did not make more within search errors (SWMWE) than controls ($p \geq 0.05$). This may be because the patients experience a lack of short-term memory but can retain information from a previous search to avoid between search errors. However, they may struggle to maintain information during the same search, resulting in within search errors.[25] Furthermore, the error rates increased with load, with the greatest impairments occurring with the initial increase from 4 to 6 boxes within all groups. Working memory had 'capacity constraints', meaning performance deteriorated with increased task load, indicating it was close to or surpassed its capacity.[26] Leung et al. found similar results on the correlation between spatial memory networks and memory load.[27]

We found that group 3 was less likely to use an efficient strategy (scores of SWMS), compared to the other three groups. However, patients in group 4 patients were more likely to use an efficient strategy compared to group 1 and group 2. The CANTAB SWM task measures executive WM, and it is believed to reflect planning ability and the ability to choose effective response sequences by integrating information.[25] Our findings, suggests that the participants' specified socioeconomic status had an impact on the dependent variable's measurements of SWM task. A total of 61% of our study participants had an undergraduate or equivalent level of education, and 78.5% of participants belonged to the upper middle class (II) socioeconomic status. After reviewing the literature, we found a relevant explanation for these results. According to Leonard et al., socioeconomic status may have a selective impact on WM, which relies on the hippocampus and prefrontal cortex, while having only a minor effect reliant on procedural memory which is dependent on striatum.[28] It is important to note that the subjects in our study were limited to individuals who had completed graduate education exclusively. In previous literature, it has been reported that individuals who have achieved higher levels of education often exhibit a tendency to dedicate more time and effort to intellectually demanding activities.[29] This prolonged exposure to cognitively stimulating environments is believed to have a positive impact on brain structure and function, leading to enhanced neurological development, such as an increase in synaptic density, or a more efficient utilization of existing brain networks.[30] In addition, these neuroprotective effects, the continued practice of cognitive skills may facilitate the development of compensatory strategies that help maintain cognitive abilities.[31] However, we did observe a trend of enhanced strategy implementation across all study groups. Notably, among the euthyroid patients receiving LT-4 treatment for a duration of 10 or more years, lower scores for strategy used were recorded compared to the control group, indicating a heightened utilization of strategic approaches in these patients. This suggests that prolonged administration of LT-4 in these patients positively influenced their performance on the SWM task, implying improved WM in conjunction with TSH levels.

Our findings suggest that early and regular screening for SWM function, along with TSH evaluation, is necessary for individuals who experience symptoms such as insomnia, depressive mood, fatigue, mental fogginess and apathy, it may be appropriate to initiate LT-4 treatment for patients with SCH at a TSH level of ≥2.5 mIU/L. In such cases, an initial dose of LT-4 may be beneficial.

Some limitations of the study: First, the subjects were not evaluated for their dosage of LT-4 medication, which could potentially affect their cognition. This is an important factor to consider in the study. Second, the mean value of the HDRS scale was 5.09 in newly diagnosed patients with SCH (group 2), indicating that depression was not diagnosed in these patients. However, it is worth noting that a high percentage (81.8%) of these patients pronounced depressive symptoms, as shown in Table 1. This could potentially have an impact on their performance in SWM task. Unfortunately, due to limitations, we were unable to analyze the effect of depressive symptoms on the SWM task performance.

To summarize, this is the first study to demonstrate that euthyroid patients who have been taking medication (LT-4) for a decade experience more significant enhancement in their SWM function compared to those who have been on the medication for one or more years. Additionally, recent research has established a more appropriate threshold for initiating medication for hypothyroidism. Our study clearly indicates that the patients with TSH levels ≥2.5 mIU/L exhibit impairments in SWM function. Therefore, instead of adopting a "wait and watch" for neuropsychological symptoms, treatment could be initiated earlier at a threshold value of 2.5 mIU/L.

Conclusion

The results of current study demonstrates that in patients with SCH, the performance of SWM exhibits enhancement as a result of the decrease in TSH levels subsequent to LT-4 treatment. However, there was a significant reduction in SWM function among newly diagnosed patients with SCH having TSH levels of 2.5 mIU/L or higher. Our research indicates that a TSH level of 2.5mIU/L may be the optimal point to begin LT-4 treatment for patients with SCH. Further well-designed randomized controlled trials with longer follow-up periods are needed to gain a deeper understanding more insights of the pathogenesis and natural history of SWM in SCH across these ranges of TSH included in the current study. These trials should also investigate the effectiveness as well as the efficacy of LT-4 treatment.

Declarations

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Author contributions

Conceptualization, S.S. and S.K.; Methodology, S.K.; Software, S.K.; Validation, S.S., S.K., A.P. and A.S.; Formal Analysis, S.K.; Investigation, A.P.; Resources, A.S.; Data Curation, S.K.; Writing – Original Draft Preparation, S.K.; Writing – Review & Editing, S.S.; Visualization, S.K.; Supervision, S.S.; Project Administration, S.K.; Funding Acquisition, A.S.

Conflicts of interest

No conflict of interest was reported by the author(s).

Data availability

Data files are available with the first author of the article.

Ethics approval

This study was approved by the Institutional Ethics Committee of Guru Nanak Dev University, Amritsar (302/HG).

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ORIGINAL PAPER

A comparative study of pharmacological, nonpharmacological, and combined methods of induction of labor

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ABSTRACT

Introduction and aim. Induction of labor (IOL), a common obstetric procedure, aims to induce labor. The study defined labor induction success as true uterine contractions and classified delivery outcomes as vaginal, instrumental, and cesarean. A higher Bishop score predicts a vaginal delivery. The objective was to compare cervical status, induction success, cesarean section rates, and normal delivery rates of pharmacological and non-pharmacological IOL methods.

Material and methods. In this study, 296 pregnant women admitted to the labor room were divided into three groups: those who received pharmacological agents (25 µg/50 µg misoprostol or dinoprostone 5 g gel to start labor), those who were given non-pharmacological agents (Foley's catheter and membrane stripping to start labor), and those who were given both non-pharmacological and pharmacological agents (Foley's catheter and membrane stripping followed by oxytocin to start labor).

Results. Although a 92.5% induction success rate, the use of non-pharmacological methods alone led to a rate of 49.06% cesarean section rate. Combined with a pharmacological agent such as oxytocin, it achieved almost the same success rate (91.43%) as a pharmacological method of inducing labor (18.57%). This resulted in a lower rate of cesarean section than pharmacological and nonpharmacological methods (p=0.002).

Conclusion. Nonpharmacological IOL methods alone led to higher cesarean rates despite improved cervical status. Combining them with pharmacological agents such as oxytocin resulted in higher normal delivery rates and fewer cesarean sections, indicating a more effective approach for improving delivery outcomes.

Keywords. Bishop score, cervical ripening, cesarean section, induction of labor, PV findings, success rate

Introduction

The process of labor induction is a common obstetric intervention aimed at facilitating childbirth in cases where spontaneous labor is not forthcoming or poses a risk to maternal or fetal health.[1] There has been a notable increase in labor induction rates in recent years, particularly in developed countries.[2,3] The growing global prevalence of cesarean sections has linked this increase to a higher incidence of maternal and newborn complications in subsequent pregnancies.[4] Cervical status significantly influences the effectiveness of labor induction, requiring a thorough assessment to optimize results.[5] Bishop score (BS) was initially developed with a minimum score of 6, indicating a favorable cervix, while higher scores are

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associated with successful induction outcomes.[6] BS is a standardized tool for evaluating cervical favorability before induction, with scores above 5 generally indicating favorable conditions for successful induction.[7-9] The timing of induction techniques also affects their efficacy, underscoring the need for tailored strategies.[10,11] Factors such as previous cesarean deliveries and cervical length further influence induction success.[12,13]

Ultrasonography and elastography could be a more advanced method than just using the traditional Bishop score. Research by Młodawski et al. shows the repeatability and reproducibility of ultrasonographic parameters, indicating that these parameters can effectively function as reliable alternatives to the Bishop score used for labor induction.[14] Alaa and Hak's study further supports this, revealing a stronger correlation between successful labor induction outcomes and transvaginal ultrasound measurements of cervical length than the BS.[15] This suggests that cervical length could serve as a more precise predictor of effective induction, reinforcing the notion that integrating objective ultrasound assessments may enhance the precision of cervical evaluations in clinical practice, as highlighted in Demir's study, which compares the effectiveness of both methods in determining the need for cervical ripening before labor induction.[16] Several studies challenge the assertion that transvaginal ultrasound (TVS) cervical length measurements are a superior alternative to the BS. Bayoumy et al. found that while posterior cervical angle (PCA) and cervical length offer some predictive value, they do not significantly outperform the Bishop score in predicting successful induction of labor (IOL).[17] This suggests that reliance solely on TVS may overlook the nuanced insights provided by the Bishop score, which remains relevant despite its subjectivity. Liu et al. also noted that a multifactorial approach that includes maternal and obstetric factors improves prediction accuracy, showing that cervical length alone is not enough. 18 Research by Alaa and Hak further supports combining Bishop's score and cervical length to predict successful induction.[15] Furthermore, Al-Adwy et al. found that optimal IOL prediction accuracy was greater than 99.5%.[19] On the other hand, Hemmatzadeh et al. emphasized the significance of the BS, particularly when combined with other clinical parameters, indicating that it remains a valuable tool for evaluating labor induction.[20] Although TVS provides objectivity, BS still holds clinical relevance when integrated into a comprehensive predictive model.

An unfavorable cervix at admission significantly increases the risk of cesarean delivery, regardless of whether labor is induced or not. This underscores the importance of BS in determining the appropriate phase of labor for cesarean delivery, particularly in nulliparous women who are at increased risk for cesarean sections when faced with prolonged pregnancies and unfavorable cervical conditions.[21] Rishitha et al. offer important information on the correlation between cervical status and the rate of cesarean deliveries, especially about labor induction.[22] The study indicates that women with an unfavorable cervix (BS<5) exhibit a significantly higher rate of cesarean delivery rate compared to those with a favorable cervix. Cesarean delivery is indicated for women who have reached full cervical dilation but experience an arresting second stage of labor lasting more than two hours, suggesting that prolonged labor without progress is a critical factor for intervention.[23]

Aim

This implies that inadequate preparation of the cervix for labor induction leads healthcare providers to perform cesarean deliveries more frequently. This evidence shows the significance of cervical assessment in deciding the mode of delivery.

Material and methods

We conducted a prospective observational cohort study at Rising Medicare Hospital, a tertiary hospital in Kharadi, Pune, Maharashtra, India, from 11 March 2021 to 12 September 2023. The study was approved by the ethical committee of the local hospital with approval number ECR/1578/Inst/MH/2021. We strictly followed ethical guidelines. All group members completed the informed consent form and kept all data collected anonymous. 680 women in all who were admitted to the labor room between 11 March 2021 and September 12, 2023, were enrolled in the study. Among them, 187 women were refused participation, while 197 who did not meet the study inclusion criteria were left out. Although, 296 qualified women with comparable clinical and demographic characteristics were finally taken into account for the study and divided into 3 groups: The first group, called "pharmacological methods," included women with a better Bishop score who were induced with 25 μg misoprostol tablets, and women with Bishop scores less than 5 were given 50 μg misoprostol tablets for induction. We administered both tablets vaginally, positioning them in the posterior vaginal fornix for optimal absorption. whereas women who were induced by inserting dinoprostone 0.5 mg (cerviprime gel), which comes in a disposable syringe containing 3 g of clear gel, were introduced into the cervical canal just below the internal os.

The second group, named 'non-pharmacological (mechanical) methods," included women induced using the Foley transcervical catheter, filled with 30 cubic centimeters (cc), is equivalent to 30 milliliters (mL) of normal saline, inserted into the cervical canal, placed just above the internal os, and inflated to facilitate cervical dilation. Another nonpharmacological intervention is done by stripping the membrane through the insertion of a sterile

gloved finger into the cervical canal and advancing it until it reaches the internal cervical os, performing a circular motion with the fingertip to separate the amniotic membranes from the lower uterine segment.

The third group, named 'the combined methods group", employed a mixture of pharmacological and non-pharmacological methods for the IOL. This cohort comprised women who underwent induction using a transcervical Foley's catheter (14 F) filled with 30 cc normal saline, and administered oxytocin at an initial dose of 2 mU every 15 minutes, gradually increasing to a maximum of 40 mU via the intravenous route. Additionally, included women induced by membrane stripping in conjunction with oxytocin administration under the same dosing regimen. The patients were advised to remain in the supine position for 30 minutes after administering all the interventions by the physician. A physician repeated the dose every six hours, up to three maximum doses in a 24-hour period, until they achieved the desired Bishop score and uterine contraction. Healthcare providers perform cesarean delivery if the cervix has not achieved at least 5 cm of dilation and 90% effacement after 36 hours of cervical ripening or 12 hours of activation.[24,25] Healthcare providers will perform fetal monitoring throughout the induction procedure. Labor will start and kept going for at least 12 hours unless certain medical conditions occur, such as signs of fetal distress (persistent slowing or bradycardia), chorioamnionitis (intrapartum temperature 38°C with tenderness in the uterus, foul smelling discharge, or tachycardia in both the mother and baby), arrest in cervical dilation (no change for more than 2 hours during the active phase), arrest in descent (no change in fetal station for more than 1 hour at 8 cm dilation), or failure in descent during the second stage of labor.

Data were collected by structured observations by the investigator. Demographic data was collected from electronic database viz.; care-expert and E-hat hospital software, partograph, and ANC reports of the women. The collected data were recorded in the Excel sheet. Data analyzes were done by using IBM SPSS Statistics version 20 (Armonk, NY, USA), using logistic regression with significance set at $p<0.05$. We expressed the results as an odds ratio and / or a 95% confidence interval.

Statistical tests were performed to present the following

The Pearson correlation coefficient was used to determine the relationship between categorical variables and labor outcomes among participants in the pharmacological, nonpharmacological and combined intervention groups. In the data analysis, categorical variables are compared with Pearson's Chi-square test, and the importance of independent variables is assessed with likelihood ratio tests. Logistic regression models utilize McFadden's pseudo-R2, Cox and Snell R2, and Nagelkerke R2 to determine explained variation, while multivariate logistic regression examines relative risks for ineffective labor induction techniques, and binomial logistic regression assesses event probabilities like BS results. Statistical significance is determined at $p<0.05$, with correlation measured by Kendall's Taub and changes in proportions evaluated using the McNemar-Bowker test.

Kendall Tau (also known as Kendall's tau rank correlation coefficient) is used for assessing the association between a nominal variable and an ordinal variable.

In the current retrospective observational study, there is no blinding or randomization, as the physician performs the induction according to the indication. Thus, the chances of bias become zero.

The primary outcome measure for this study was the effect of cervical favorability on the success rate of delivery.

Secondary outcome measures were the rate of cesarean deliveries and the onset of active labor (cervical dilation of 4 cm or greater).

Sampling criteria

Inclusion criteria

All pregnant women admitted to the labor room and who were expected to undergo induction.

Exclusion criteria

Less than 18 years of age and less than 37 weeks of gestation, scarred uterus (previous surgery on the uterus, i.e., cesarean delivery), twins, triplet pregnancy or multiple birth pregnancy, breech presentation, fetal anomalies.

Results

This study conducted a comprehensive evaluation to compare the IOL methods in terms of Bishop score and success rate of administering induction doses. Of the 296 women, 58.45% received pharmacological treatments such as misoprostol 25 g, misoprostol 50 g, and dinoprostone gel 0.5 g gel (Fig. 1). On the other hand, 17.91% of women received nonpharmacological methods like Foley's catheter and membrane stripping, while 23.65% received a combination of these methods, including the pharmacological agent oxytocin and a non-pharmacological strategy that involved either stripping a membrane or a balloon catheter.

During the labor induction process, cervical favorability and the rate of successful induction are determined by evaluating cervical status through a per vaginal (PV) examination and BS.

We define the success of labor induction as the commencement of labor pains that are continuous, periodic (at regular intervals), and moderate to strong in intensity.

The outcome of inducing labor is determined by the type of birth that occurs. There were two indicators: the first was the success rate of IOL, the second was the fail-

ure of IOL, and the third was the women's abortion from the IOL process. Ultimately, the second indicator shifted the women to a cesarean section.

PV findings before administration of labor induction

Figure 1. Initial status of the cervix

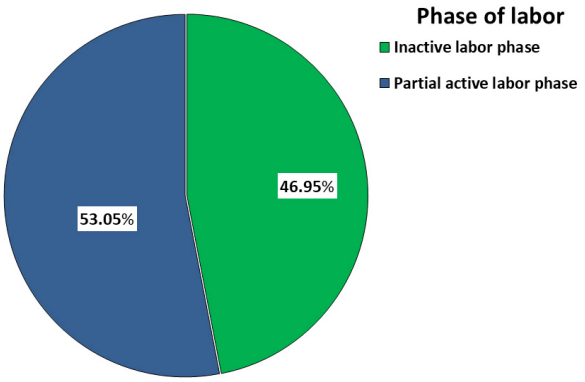

Fig. 1. More than 50% of the patients were in the partial active phase of labor before administering the IOL method to induce it.

PV findings 6 hours after administration of the 1st dose of labor induction

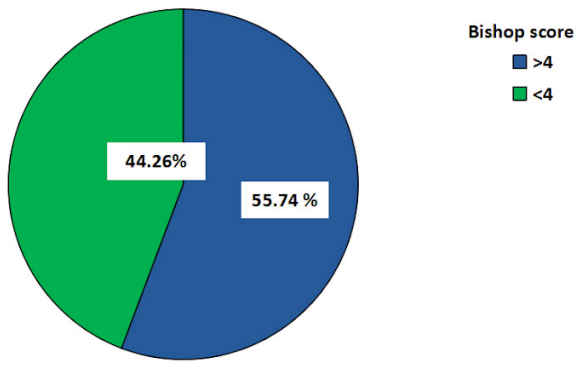

Fig. 2. BS 6 hours after initial dose of IOL

A BS of more than four (>4) showed that 55.74% of the patients had successfully progressed to active labor after the first labor induction intervention. On the other hand, a Bishop score of less than four (<4) indicates that an induction response was not successful in 44.26% of cases, when the patient did not reach active labor (Fig. 2).

The favorability of the findings of the PV examination increased after 6 hours of administration of the first induction dose. This suggests that the first dose contributes to a positive change in the PV examination finding outcomes.

Success ratio of the first dose for induced labor

The success of labor induction is characterized by the onset of labor contractions that are continuous, periodic (occurring at regular intervals) and of moderate to strong intensity. This definition establishes a success ratio for the induction process.[26] People often assess the success of labor induction in conjunction with the delivery mode and they commonly use the rate of cesarean sections as an essential indicator to evaluate the efficacy of the induction strategy.[27]

Table 1. Maternal parameter

Maternal parameters	Pharmacological		Non-pharmacological		Combined		p
Maternal age groups	**Count**	**%**	**Count**	**%**	**Count**	**%**	0.07
18–25	46	15.54	13	4.39	16	5.41	
26–30	75	23.34	21	7.09	32	10.81	
31–35	45	15.2	17	5.74	17	5.74	
36–40	7	2.36	2	0.68	5	1.69	
Maternal height groups							0.035
140–150	24	8.11	6	2.03	7	2.36	
151–160	92	31.08	26	8.78	47	15.88	
161–170	52	17.57	21	7.04	16	5.41	
171–180	5	1.69	0	0	0	0	
Maternal weight groups							0.035
Low weight: <50 kg	0	0	0	0	0	0	
Normal weight: 50–70 kg	82	27.7	23	7.77	33	11.15	
High weight: 71–90 kg	90	30.41	30	10.14	36	12.16	
Very high weight: >90 kg	1	0.34	0	0	0	0	
BMI groups							0.03
Underweight: BMI<18.5	0	0	0	0	0	0	
Normal weight: BMI 18.5–24.9	28	9.46	10	3.38	8	2.7	
Overweight: BMI 25–29.9	79	26.69	23	7.77	39	13.18	
Obese (Class I): BMI 30–34.9	50	16.89	17	5.74	20	6.77	
Obese (Class II): BMI 35–39.9	16	5.41	3	1.01	3	1.01	
Morbidly Obese (Class III): BMI≥40	0	0	0	0	0	0	
Is patient in active labor?							<0.001
No	94	31.76	29	9.79	16	5.41	
Yes	79	26.69	24	8.11	54	18.24	

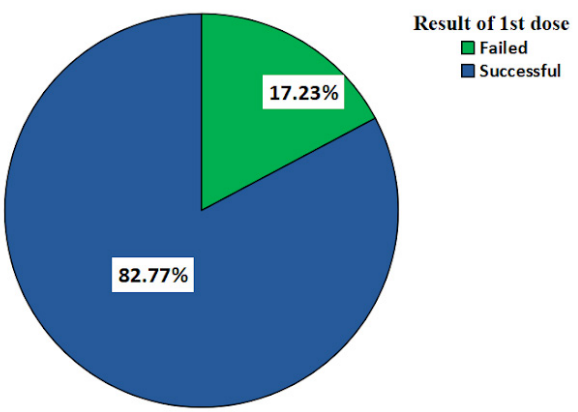

Fig. 3. Result of the first dose of IOL

The first dose was administered to the participants and 82.77% of the patients experienced labor 6 hours after induction, while 17.23% of the patients reported no labor pain starting after the induction (Fig. 3).

Differences in maternal parameters such as height, weight, and BMI, along with the status of active labor, are significant between induction methods, particularly with a higher likelihood of active labor in combined methods (Tables 1 and 2).

Table 2. Variables in the equation

	B	S.E.	Wald	df	p	Exp (B)	95% CI for Exp (B)	
							Lower	Upper
Age	-0.085	0.031	7.360	1	0.007	0.918	0.863	0.977
Height	-0.333	0.158	4.431	1	0.035	0.717	0.526	0.977
Weight	0.365	0.173	4.434	1	0.035	1.441	1.026	2.024
BMI	-0.927	0.427	4.715	1	0.030	0.396	0.171	0.914
Active labor	-1.226	0.264	21.585	1	0.000	0.293	0.175	0.492
Constant	56.479	24.922	5.136	1	0.023	337530454578440200000000.000		

From the above table, we get the fitted logistic regression model as follows:
Log odds=56.479+(-1.226*active)+(-0.927* BMI)+
+(0.365* weight)+(-0.333* height)+(-0.085*age)

Methods of IOL-wise BS of first dose

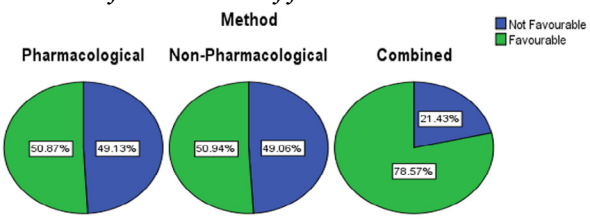

Fig. 4. IOL wise Bishop score 6 hours after the first dose

Six hours after administering the first dose, the combined IOL methods demonstrated the highest favorable Bishop score of 78.57%, which ranged from "7 cm cervix dilated, station -2, 70-80% effacement" to "fully dilated, fully effaced station 0," while the pharmacological and nonpharmacological IOL methods yielded similar results (50.87%) and (50.94%), respectively, with a p<0.001 (Fig. 4).

Methods of IOL-wise success rate of first dose

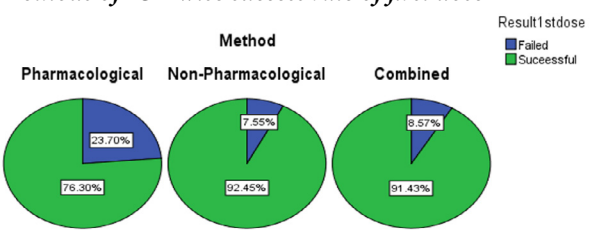

Fig. 5. Methods of IOL-wise success rate 6 hours after first dose

Six hours after administering the first dose, the non-pharmacological methods of IOL showed a higher success rate of 92.45%, while combined methods yielded equivalent results of 91.43% compared to pharmacological methods of IOL of 78.30%, with a p-value of 0.002 (Fig. 5).

Comparison of the induction-wise mode of delivery

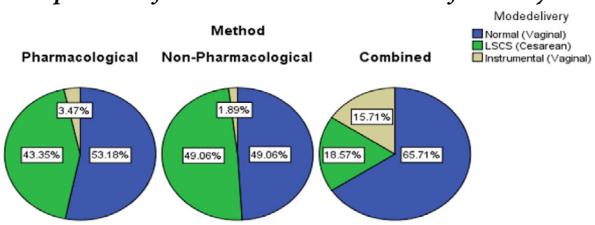

Fig. 6. Comparison of the induction-wise mode of delivery

Combined methods of IOL showed the highest normal delivery rates, 65.71%, compared to pharmacological, 53.18% and non-pharmacological, 49.06%, with negligible instrumental (vaginal) delivery rates (Fig. 6).

Only 18.57% of the patients underwent a cesarean section using combined IOL methods, while both pharmacological and non-pharmacological IOL methods had higher cesarean rates of 43.35% and 49.06%, respectively.

Table 3. Case processing summary*

Methods	Count	Marginal percentage
Pharmacological	173	58.4%
Non-pharmacological	53	17.9%
Combined	70	23.6%
Valid	296	100%
Missing	0	
Total	296	
Sub-population	296[a]	

* [a] The dependent variable has only one value observed in 296 (100%) subpopulations

Pharmacological methods are the most frequently used, followed by combined and non-pharmacological methods. All observations are valid, with no missing data (Table 3).

Table 4. Model fitting information

Model	Model fitting criteria			Likelihood ratio tests		
	AIC	BIC	-2 Log likelihood	Chi-square	df	p
Intercept only	574.014	581.394	570.014			
Final	551.823	706.818	467.823	102.191	40	<0.001

We evaluated the model's fitness using the chi-square statistic. The chi-square value was 102.191, and the p<0.001 is less than the significance level of 0.05. This shows that there is a relationship between the different methods of induction and other independent variables such as vaginal findings and BS in the final fitted model (Table 4).

Table 5. Goodness of fit

	Chi-Square	df	p
Pearson	567.121	550	0.298
Deviance	467.823	550	0.995

Here, p = 0.298 is greater than the 0.05 level of significance (Table 5). Therefore, we are unable to reject the null hypothesis at the 5% level of significance. That is, we concluded that the given model fits the data very well.

Table 6. Pseudo R-Square

Cox and Snell	0.292
Nagelkerke	0.342
McFadden	0.179

The Cox and Snell R^2=0.290 value suggests that the model explains approximately 29.2% of the variance in the dependent variable, but it cannot reach a maximum of 1, which limits its interpretation (Table 6).

Nagelkerke R^2=0.342 is an adjusted version of Cox and Snell's R^2, rescaled to cover the full range (0 to 1). It indicates that the model explains 34.2% of the variance.

The McFadden value suggests that the model explains 17.9% of the variance and is typically lower than other pseudo-R^2 values, but still a reasonable fit.

These values provide different perspectives on the model's fit, with Nagelkerke showing the highest adjusted explanation of variance.

Discussion

The present study investigates the intricate mechanisms underlying labor induction techniques, particularly the relationship between BS and delivery outcomes.

Although nonpharmacological IOL methods achieved more favorable cervical conditions, we observed a higher incidence of cesarean sections, suggesting that these interventions do not consistently lead to better outcomes of delivery. In contrast, combined IOL methods demonstrated the highest rates of normal delivery and were equally effective in enhancing cervical status or Bishop scores while significantly reducing cesarean section rates. The study found that 46.95% of the patients had poor cervical conditions before IOL strategies were used. These included a closed os, a posterior cervix, and a high fetal head. This meant they had a low Bishop score and a lower chance of going into labor on their own (Fig. 1).

However, 53.04% of patient were in a somewhat or partially active labor phase, with a cervix that was 1 cm dilated, 40% effaced and the fetal station at -2, which means that the conditions for labor were good (Fig. 1). These findings corroborate the existing literature that highlights the challenges associated with low BS. Studies frequently link a BS below 6 to reduced chances of spontaneous labor and an increased likelihood of cesarean delivery during IOL.[28-30] This is particularly pertinent as studies indicate that patients with unfavorable cervical conditions are less likely to experience spontaneous labor, necessitating surgical interventions for delivery.[29] Research has shown that a moderate BS increases the likelihood of successful induction with fewer cesarean deliveries.[19] Advanced methods such as ultrasound and elastography may provide more accurate assessments than the traditional Bishop score.[14]

In this study, 55.74% of the patients exhibited a favorable BS of more than four (>4) following the initial dose of labor induction, suggesting successful progression to active labor (Fig. 2). This finding aligns with Grobman et al.'s research, which indicates that a favorable BS, defined as greater than four, correlates with a higher likelihood of successful labor induction and a lower cesarean delivery rate. Specifically, their findings demonstrated that the patient with a favorable Bishop score experienced more favorable outcomes compared to those with an unfavorable score, consistent with the observation that 55.74% of the patients in this study achieved a favorable score after induction.[31] This correlation underscores the importance of the Bishop score in predicting successful labor outcomes. Researchers who looked at different methods to induce labor, such as the Foley catheter and pharmacological agent such as oxytocin, found that nonpharmacological methods can work well even with lower BS (4-5), while pharmacological methods usually need a higher threshold (6 8) for the best success rates.[31] Studies show that a BS of 5 is a strong indicator of the need for a cesarean section due to failed attempts to induce labor. This shows how important the initial cervical status is.[31-33]

In this study, 44.26% of the patients did not meet the favorable threshold, with a BS of less than four indicating a lower probability of progressing to active labor (Fig. 2). This finding is consistent with the systematic review by Kolkman et al., which emphasized the BS's predictive capacity of BS for labor induction success.[28] A study by Iftikhar shows that using both BS and transvaginal sonography together can help predict whether or not an induction will work. This suggests that BS is useful, but it may not be the only thing that determines whether or not an induction will work.[34]

Among the 296 patients, 82.77% successfully induced labor, while only 17.23% did not progress six hours after the initial dose (Fig. 3). The high success rate fits Msumi's research, which shows that cervix conditions of the cervix during induction have a big effect on how many babies are born alive.[35] The importance of cervical ripening for good results is emphasized. This correlation indicates that a properly prepared cervix is a significant predictor of successful labor induction, consistent with the results presented.

Research by Bekru and Yirdaw demonstrated that a BS of five or less is a major risk factor for failed induction, revealing that a significant percentage of patients with low BS experienced unsuccessful labor induction.[36] This aligns with the observed failure rate of 17.23%, suggesting that certain clinical scenarios could potentially predispose patient to an unsuccessful induction. This highlights the importance of cervical status in forecasting induction success, potentially elucidating the failure rates observed in the present study.

A study by Haavaldsen et al. looked at the increasing use of labor induction and how it affects the outcome of pregnancies. They found that although overall induction rates have increased, the risks of bad outcomes, such as failed induction, are still a big concern. This underscores the importance of careful patient selection and monitoring during the induction process to mitigate the risk of failure.[37]

Kim et al. identified that factors such as maternal age, parity, and initial BS significantly influence labor induction success.[38] Research indicates that specific maternal characteristics can predict the likelihood of successful vaginal delivery after induction, emphasizing the importance of individualized assessment prior to the induction process.

Table 1 compares maternal parameters across pharmacological, non-pharmacological and combined labor induction methods, highlighting significant differences in height, weight, BMI, and active labor status. Most pharmacological patients fell within the 26–30 age group and the 151–160 cm height range, with a significant portion categorized as high weight (71–90 kg) and overweight (BMI 25–29.9). Combined methods resulted in a higher proportion of patients in active labor, while fewer patients fell into the very high-weight or morbidly obese BMI categories across all groups. Statistically significant differences ($p<0.05$) indicate that combined induction is associated with active labor status, while specific height and weight groups are more prevalent in pharmacological cases. The data supports the hypothesis that maternal factors such as height, weight, and BMI significantly affect active labor across induction methods. Table 1 links certain maternal characteristics with labor induction outcomes.

Hirshberg et al. found a negative correlation between maternal weight and cervical dilation rate after induction, suggesting that a heavier patient may experience longer labors. This supports the idea that maternal factors influence active labor potential in induction strategies.[37] Rogaleli and Awang found that shorter maternal height increases cesarean sections and prolongs obstructed labor, implying that maternal height may affect labor mechanics and induction success.[39]

Ghazali's study highlights the impact of maternal BMI on the length of induced labor, noting that higher BMI is associated with slower labor progression and higher cesarean birth rates.[40] This association is significant as it suggests that patients with higher BMIs may face more challenges in inducing labor, affecting their chances of achieving active labor. The results support maternal weight and BMI as critical considerations in the development of induction techniques (Table 1). The meta-analysis by Sotiriadis et al. supports the assertion that maternal factors significantly affect induction outcomes, finding that maternal BMI and height influence labor outcomes after elective induction.[41]

The logistic regression model showed that active labor cut the chances of unsuccessful induction by 70.7% (Exp(B)=0.293), which is a solid result ($p<0.001$) with a confidence interval of 0.175 to 0.492. The significant value of the constant term Exp (B) value indicates a baseline effect on the outcome when all predictors are zero, with the intercept of the model being significant ($p=0.023$). A strong correlation was discovered between active labor and successful outcomes, with the probability of a favorable outcome for the patient not in active labor being approximately 29.3% lower than for those who had a statistically significant difference ($p<0.001$) (Table 2). This effect has a narrow confidence interval (17.5% to 49.2%), indicating the reliability of the estimate.

A good Bishop score of 78.57% within six hours of administration was observed in several studies (Fig. 3), which supports the results (Fig. 4) of combined IOL methods. These studies highlight the advantages of utilizing a combination of nonpharmacological and pharmacological approaches. A systematic review by Chen et al. compared the use of Foley catheters, misoprostol, and dinoprostone for cervical ripening. They found that mechanical methods, such as the Foley catheter, combined with pharmacological agents, such as oxytocin, made the cervical area more ready for delivery, leading to more vaginal deliveries and fewer cesarean deliveries.28 Numerous studies have shown that the use of a Foley catheter followed by oxytocin improves Bishop scores, increases vaginal birth rates, and decreases cesarean section rates.[26,27,42,43,44] This study revealed that nonpharmacological approaches to IOL demonstrated a more favorable Bishop score of 92.45% compared to the pharmacological method 76.3%. Additionally, combined methods showed a favorable Bishop score of 91.43% after initial administration (Fig. 5). This suggests that, while combining both methods can lead to more favorable outcomes, careful consideration of the associated risks is necessary. Juncu's systematic review emphasized the importance of evaluating various IOL methods, including pharmacological and nonpharmacological methods, to determine their effectiveness in different clinical scenarios.[45] While combined methods can produce better outcomes, the review found that op-

timizing induction success rates also requires consideration of individual patient factors and clinical contexts.

Conclusion

The findings show that a more effective method may involve a combination of nonpharmacological and pharmacological methods, potentially enhancing Bishop's score and delivery outcomes, increasing the likelihood of successful vaginal delivery.

Declarations

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Author contributions

Conceptualization, P.S.U. and V.N.M.; Methodology, L.R.V.; Statistical Software P.S.U.; Validation, P.S.U., V.N.M. and L.R.V.; Formal Analysis, P.S.U.; Investigation, P.S.U.; Resources, P.S.U.; Data Curation, P.S.U.; Writing – Original Draft Preparation, P.S.U.; Writing – Review & Editing, P.S.U.; Visualization, P.S.U.; Supervision, P.S.U. and V.N.M.; Project Administration, P.S.U.; Funding Acquisition, P.S.U.

Conflicts of interest

No conflicts of interest.

Data availability

Data related with induction of labor were collected by the structured observations and demographic data were collected from electronic database viz.; care-expert and E-hat hospital software, partograph and ANC reports of the patients. The collected data were recorded in the excel sheet.

Ethics approval

The study protocol was approved by the local ethics committee of the Rising Medicare Hospital. The permission was granted from the director and professor in charge of the hospital. Registration No: ECR/1578/Inst/MH/2021, registration date is 30 September 2021.

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ORIGINAL PAPER

Efficacy of furosemide in patients with chronic kidney disease with residual renal functions in hemodialysis and non-hemodialysis patients

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ABSTRACT

Introduction and aim. Chronic kidney disease (CKD) affects kidney function, characterized by albuminuria or reduced estimated glomerular filtration rate (eGFR), and is influenced by factors such as etiology, pathogenesis, intensity, and progression. According to data from the literature, the efficacy of furosemide has not been much researched much in CKD patients. The study evaluates the efficacy in chronic kidney disease patients, regardless of hemodialysis, and compares its diuretic effect based on the administration route.

Material and methods. A prospective observational study was conducted in a tertiary healthcare facility for 6 months (October 2021 to March 2022). 100 CKD patients who met the criteria were enrolled in the study. Data on study-relevant parameters, such as route of administration (ROA), hemodialysis frequency, hospital stay, blood urea, serum creatinine, sodium, and potassium, were collected. Pearson's chi-square test was used to evaluate the association between parameters. One-way ANOVA was applied to analyze the significant association between ROA and urine output.

Results. Of all the study samples, 72% received intravenous furosemide and 28% received furosemide orally. There was a significant difference in eGFR and urine output on admission and discharge days. There was an increase in urine output when the patient received furosemide and improvement in eGFR was found. A significant association was also observed between systolic blood pressure, sodium, and potassium.

Conclusion. The study found no significant differences in furosemide efficacy in CKD patients, regardless of ROA, hospital stay, or frequency of hemodialysis, indicating similar effectiveness.

Keywords. chronic kidney disease, efficacy, hemodialysis, route of administration

Introduction

Chronic kidney disease (CKD) refers to a variety of diseases that impact the structure and function of the kidneys. The diversity in the manifestation is influenced by factors such as etiology, pathogenesis, intensity, and pace of progress. CKD is characterized by albuminuria or a reduced glomerular filtration rate (GFR) [below 60 mL per min/1.73 m^2] for three months or more, regardless of clinical diagnosis.[1] CKD is characterized by initial phases of kidney damage, in which more than 50%

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of the kidney tissue is damaged, leading to elevated serum creatinine and reduced kidney function. This is the reason for the need for kidney transplantation and treatment focused on restoring the health of patients with kidney disease without risking the donor's health.[2] One of the most pressing global public health concerns is CKD. In general, the estimated frequency of CKD is 13.4% (11.7–15.1%), and the estimation of end-stage kidney disease (ESKD) that requires renal replacement therapy is between 4.902-7.083 million.[3] In 2017, globally, 1.2 million people died from CKD. The worldwide increase in the total age mortality rate from CKD was 41.5% between 1990 and 2017, although there was no remarkable difference in the age-related mortality rate.[4]

In India, the current estimated value of ESKD is 229 million people and annually, more than 100,000 new cases begin kidney replacement programs. The screening and early evaluation of kidney disease (SEEK) India cohort study found a 17.2% prevalence of CKD, with 6% having stage 3 or severe CKD. The prevalence of stages 1, 2, 3, 4, and 5 of CKD was 7%, 4.3%, 4.3%, and 0.8%respectively.[5] Diuretics play a key role in the management of CKD.[6] Although all drugs have their benefits and side effects, they commonly cause fluid, electrolyte abnormalities, and acid-base disturbances. Diuretics are used to modify interdialytic weight gain in patients with residual renal function. High-dose loop diuretics are recommended in patients who undergo hemodialysis on non-hemodialysis days. Furosemide in high doses has been found to be effective in high doses is efficient in both acute and chronic kidney disease. It is also helpful in correcting fluid overload.[7] Nearly 500 monogenetic causes of CKD have been recognized primarily in pediatric populations. A limited number of studies on monogenic causes in adults with CKD.[8] Most patients with CKD generally do not show any symptoms in the early stages. In the advanced stages of CKD, symptoms like weight loss, anorexia, pedal edema, shortness of breath, fatigue, hematuria, polyuria, insomnia, pruritus, headache, numbness, vomiting, and muscle cramps can be seen.[9] Hypertension: one of the leading causes of CKD.[10] Other risk factors: kidney stones, cirrhosis, atherosclerosis, bladder cancer, scleroderma, systemic lupus erythematosus, and kidney infection.[11] Diuretics play a crucial role in the management of CKD. However, all drugs have their benefits and side effects. In general, they cause fluid, electrolyte abnormalities, and acid-base disturbances.[6] Diuretics are used to modify interdialytic weight gain in patients with residual renal function. High-dose loop diuretics are recommended in patients undergoing hemodialysis on nonhemodialysis days.[12] Furosemide is often preferred in the treatment of CKD due to its potent diuretic effects, which help manage fluid overload and hypertension commonly associated with CKD. Key reasons include its potent diuresis, which effectively promotes sodium and water excretion, relieving symptoms of volume overload.[13] Additionally, it helps lower blood pressure, which is crucial for managing CKD management to slow disease progression.[14] Furosemide remains vital for managing fluid overload in the early to moderate CKD stages. However, in patients with severe CKD and dialysis, clinicians may need to consider other approaches to fluid management.[15]

Aim

The study assesses the efficacy of furosemide in the treatment of patients with chronic kidney disease, regardless of hemodialysis, and compares its diuretic effect based on the administration route.

Material and methods

Study design and study settings

An observational cross-sectional study of all eligible patients with chronic kidney disease was conducted in the nephrology department of a South Indian hospital and research center from October 2021 to March 2022. The institution's research ethics committee approved the study. The demographics and parameters relevant to the study were collected, such as age, sex, hospital stay, hemodialysis frequency, route of hemodialysis, furosemide administration route, blood pressure, urine output, blood urea, serum creatinine, sodium, and potassium. The selection of furosemide dosage was mainly based on the patient's body weight of the patient and kidney function. The study has been approved by the Human Ethics Committee of Mahavir Hospital and Research Center, with reference no. ECR01/450/Inst/AP/11/03/22.

Inclusion criteria

Patients over 18 years old, Individuals belonging to any class, caste, or sex, Patients diagnosed with CKD receiving furosemide, and both hemodialysis and nonhemodialysis patients were included.

Exclusion criteria

The patients were prescribed diuretics other than furosemide. Patients who had undergone a kidney transplant and pregnant women were excluded from the study, and patients who were not ready to give their consent to participate in the survey.

Sample size

A total of 130 patients diagnosed with CKD, regardless of hemodialysis, were assessed, of which 30 patients were excluded according to exclusion criteria. In our study, around 70% of the participants had hypertension as a comorbidity and around 48% to 59% of the participants had diabetes and cardiac abnormalities as a comorbidity. The baseline characteristics were obtained for each patient.

Table 1. Association among parameters on admission and discharge

		n	Correlation	p
Pair 1	Systolic BP on admission and systolic BP discharge	100	0.105	0.296
Pair 2	Diastolic BP on admission and diastolic BP on discharge	100	0.006	0.956
Pair 3	EGFR at admission and EGFR mL/min/1.73 m^2 on discharge	100	0.646	<0.001
Pair 4	Serum creatinine at admission and serum creatinine on discharge	100	0.155	0.123
Pair 5	Blood urea on admission and blood urea on discharge	100	-0.099	0.325
Pair 6	Sodium on admission and sodium on discharge	100	-0.041	0.688
Pair 7	Potassium on admission and potassium on discharge	100	-0.078	0.443
Pair 8	Fluid output (mL) on admission and fluid output (mL) on discharge	100	0.390	<0.001

Table 2. Mean, standard deviation, and associations of parameters using one-way analysis of variance (ANOVA).

	Paired differences					t	df	p
	Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
Systolic BP on admission – systolic bp discharge	14.29	46.145	4.614	5.133	23.446	3.097	99	0.003
Diastolic BP on admission diastolic BP on discharge	2.69	67.738	6.774	-10.751	16.13	0.397	99	0.692
EGFR at admission EGFR ml/min/1.73 m^2 on discharge	-2.51	21.703	2.17	-6.816	1.796	-1.157	99	0.250
Serum creatinine at admission – serum creatinine on discharge	1.072	5.291	0.529	0.022	2.121	2.026	99	0.045
Blood urea on admission – blood urea on discharge	-4.14	67.476	6.748	-17.529	9.249	-0.614	99	0.541
Sodium at admission – sodium on discharge	4.942	17.104	1.71	1.548	8.336	2.889	99	0.005
Potassium on admission – potassium on discharge	0.429	1.37	0.137	0.158	0.701	3.134	99	0.002
Fluid output (mL) at admission – fluid output (mL) on discharge	-236.03	916.53	91.653	-417.89	-54.17	-2.575	99	0.011

Statistical analysis

Data were analyzed using SPSS (version 28.0, IBM, Armonk, NY, USA). In this statistical analysis, the dependent variables were route of administration (ROA) of furosemide, hemodialysis, estimated glomerular filtration rate (eGFR), sodium, potassium, blood pressure (BP), and urine output. The independent variables were demographics, hospital stay, comorbidities, serum creatinine, and blood urea. Pearson's chi-square test evaluated the association between ROA, hemodialysis (HD) frequency, and hospital stay. To analyze the significant association between the parameters taken on the day of admission and discharge, a one-way analysis of variance (ANOVA) was used.

Results

The present study compared the relevant admission parameters with the discharge parameters. As shown in the table, there was a significant difference in eGFR and fluid output (Tables 1 and 2).

There was a significant association between systolic BP, sodium, and potassium on admission day and discharge day, as indicated by a one-way ANOVA test of significance ($p \leq 0.05$, Table 3).

Table 3. Analysis of variance (ANOVA) for urine output of IV furosemide in HD and non-HD

	Paired differences					t	df	p
	Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
Urine output of IV furosemide in HD urine output of iv furosemide in non-HD	-871.657	1086.169	183.596	-1244.769	-498.545	-4.748	34	<0.001

There was a significant association between the urine output of participants on HD receiving IV furosemide and the urine output of participants not on HD receiving oral furosemide, as indicated by a one-way ANOVA test of significance ($p<0.05$, Table 4).

Table 4. ANOVA for oral furosemide urine output in HD and non-HD Patients in the study*

	Paired differences					t	df	p
	Mean	Std. deviation	Std. error mean	95% confidence interval of the difference				
				Lower	Upper			
Urine output of oral furosemide in HD urine output of oral furosemide in non-HD	-1050.769	520.359	144.322	-1365.219	-736.319	-7.281	12	<0.001

* HD hemodialysis, Non-HD – non-hemodialysis patients

There was a significant association between the urine output of participants on HD who received oral furosemide and those not on hemodialysis who received oral furosemide, as indicated by a one-way ANOVA test of significance ($p<0.05$, Table 4).

The frequency of HD was higher in participants who received 40 mg of oral furosemide than in participants who received 20 mg of oral furosemide. There was no significant association between oral dose and frequency of HD at $p=0.8$ (Table 5).

Table 5. Correlation of oral dose furosemide with frequency of HD patients

		Frequency of HD						Total
		No	Once a week	Twice a week	Thrice a week	Daily	SOS	
Oral dosage	No	37	3	7	18	6	1	72
	20 mg	3	0	1	1	0	0	5
	40 mg	9	3	4	6	1	0	23
Total		49	6	12	25	7	1	100

χ^2=5.6, df=10, p=0.8

Table 6. Oral dose frequency correlated with HD frequency of HD

		Frequency of HD						Total
		No	Once a week	Twice a week	Thrice a week	Daily	SOS	
Oral dose frequency	No	37	3	6	18	6	1	71
	OD	1	0	1	2	0	0	4
	BD	11	3	4	4	1	0	23
	TID	0	0	1	1	0	0	2
Total		49	6	12	25	7	1	100

χ^2=11.81, df=15, p=0.63

* OD once daily, BD – twice daily, TID – thrice daily

The frequency of HD was found to be higher in participants who received oral furosemide twice a day, followed by once a day and three times a day. There was no significant association between oral dose frequency and frequency of HD at p=0.63 (Table 6).

Table 7. Dose of IV bolus correlated with frequency of HD

		Frequency of HD						Total
		No	Once a week	Twice a week	Thrice a week	Daily	SOS	
IV bolus dose	No	12	3	5	7	1	0	28
	20 mg	7	1	2	2	2	1	15
	40 mg	16	2	3	11	3	0	35
	60 mg	14	0	2	5	1	0	22
Total		49	6	12	25	7	1	100

χ^2=13.71, df=15, p=0.54

Table 8. IV bolus dose frequency correlated with HD cross-tabulation

		Frequency of HD						Total
		No	Once a week	Twice a week	Thrice a week	Daily	SOS	
IV bolus dose frequency	No	12	3	5	7	1	0	28
	OD	0	1	0	1	0	0	2
	BD	26	2	3	11	3	1	46
	TID	8	0	3	2	1	0	14
	SOS	3	0	1	4	2	0	10
Total		49	6	12	25	7	1	100

The frequency of HD was found to be higher in participants receiving 40 mg of IV furosemide, followed by participants receiving 60 mg of IV furosemide and 20 mg IV furosemide. There was no significant association between oral dose and frequency of HD at p=0.54 (Table 7).

The frequency of HD was found to be higher in participants who received an IV bolus twice a day, followed by three times a day, when necessary, and once a day. There was no significant association between the frequency of IV bolus and frequency of HD at p=0.037 (Table 8).

Discussion

CKD is a prominent contributor to global mortality. CKD ranks among the top five leading causes of death in multiple nations. CKD was ranked as the eighth leading cause of death in India, according to global burden of disease. The main purpose of our study was to evaluate the efficacy of furosemide in improving renal function in patients with chronic kidney disease. Our study consists of 130 patients diagnosed with CKD irrespective of hemodialysis, of which 30 patients were excluded based on exclusion criteria, making the participation rate 80%. Our study includes 41% (41) females and 59% (59) males, compared to the study conducted by Sanjay et al., where the percentage of males was predominant (55%).[16] In this present study, half of the participants (50%) were in between the 30–60 year age group, 45%were above 60 years, and 5% were below30 years, concluding that the highest number of people suffering from CKD belonged to the middle age group (30–60 years). In the current study, half of the participants (51%) were undergoing maintenance HD and the other half (49%) were not on hemodialysis. The percentage of participants who undergo hemodialysis is 6% once a week, 12% twice a week 25% three times a week, and 1% as required. In our study, the sum of participants staying in the hospital for 1–10 days was 70%, while the sum of participants staying there for 10-20 days was 30%. All participants were prescribed furosemide; most (72%) received intravenous furosemide and 28% received furosemide orally. Among participants receiving oral furosemide, 5% were prescribed a dose of 20 mg and 23% were prescribed a dose of 40 mg; The frequency percentage of BD (23%) was higher than OD (3%) and TID (2%). As mentioned above, most participants have been prescribed IV furosemide; of these, a higher number of patients received a 40 mg dose (35%), followed by a 60 mg dose (22%), and a 20mg dose (15%), the frequency percentage of participants taking BD (46%) was higher than OD (2%), TID (14%), and as needed (10%). In our current study, the main comorbidities were found to be hypertension (74%), diabetes (59%), heart disease (48%), and edema (25%). Parameters relevant to the study at admission were compared with those at discharge; There was a significant difference in eGFR and urine output; Similar results were seen in other studies.[17] We found that the mean systolic and diastolic blood pressure, serum creatinine, sodium concentration and

potassium concentration decreased slightly, and estimated glomerular count, blood urea nitrogen, and fluid output were increased; similar results were seen compared to other studies.[18] There was a significant association between systolic bp, sodium and potassium on admission day and day of discharge, indicated by a one-way ANOVA test of significance ($p<0.05$), compared to the study conducted by Bunyoung et al., where there was a significant increase in BP, sodium, and potassium.[19] In our present study, mean urine output was lower in participants receiving IV furosemide in HD patients compared with non-hemodialysis; we also found no significant difference in urine output among participants receiving IV furosemide undergoing HD compared to those who did not undergo HD. There was a significant association between the urine output of participants on HD receiving IV furosemide and those not on HD receiving oral furosemide, as indicated by a one-way ANOVA test of significance ($p<0.05$). In this study, the average urine output of the participants receiving oral furosemide in HD patients was lower than that in non-HD. We found a significant difference in the performance of participants who received oral furosemide in HD patients compared to non-HD patients. Improvement in diuresis was observed in patients not on HD. The result showed a significant association between the urine output of participants on HD receiving oral furosemide and those not on HD, as indicated by a one-way ANOVA test of significance ($p<0.05$). The frequency of HD was higher in participants who received 40 mg of furosemide orally, followed by participants who received 20 mg of furosemide orally. There was no significant association between oral dose and frequency of HD at $p=0.8$. Hemodialysis was found to be more frequent in participants who received oral furosemide twice a day, followed by once a day and three times a day. At $p=0.63$, there was no significant association between oral dose and HD frequency. The frequency of HD was higher in participants who received 40 mg of IV furosemide, followed by 60 mg and 20 mg. There was no significant association between the IV dose and the frequency of HD at $p=0.54$. The frequency of HD was found to be highest in participants given intravenous furosemide twice daily, followed by three times daily, as needed, and once daily. There was no significant association between the frequency of IV furosemide and the frequency of HD at p=0. 037. The sum of hospital stays for patients receiving oral furosemide was 18 for 1–10 days and 10 for 10–20 days. For participants who received IV furosemide, the percentage of hospital stay was 52% for 10 days and 20% for 10-20 days. There was no significant association between the route of administration and hospital stay at $p=0.43$. The sum of hospital stay for participants who received 20 mg of oral furosemide was 3 for 1–10 days, 2 for 10–20 days, and for participants who received 40 mg of oral furosemide, it was 15 for 1–10 days and 8 for 10–20 days. There was no significant association between oral dose and hospital stay at $p=0.72$. The sum of hospital stays for participants receiving oral furosemide once a day was 3 for 1–10 days and 1 for 10–20 days; twice a day, it was 14 for 1–10 days and 9 for 10–20 days, thrice a day it was 1 for 1–10 days and 1 for 10–20 days. There was no significant association between the frequency of oral dose and hospital stay at $p=0.63$. The total hospital stay days of patients receiving 20 mg IV furosemide was 9 for 1–10 days and 6 for 10–20 days; for 40 mg, it was 26 for 1–10 days and 9 for 10–20 days, 60 mg it was 17 for 1–10 days 5 for 10–20 days. There was no significant association between IV dose and frequency of hospital stay days. The total hospital stay days of participants receiving IV furosemide once a day for 1–10 days was 2, and for 10–20 days was 0; twice a day, it was 30 for 1–10 days and 16 for 10–20 days, and for thrice a day it was 10 for 1–10 days and 0 for 10–20. There was no significant association between IV dose frequency and the sum of hospital stays at $p=0.19$. In our study, the evaluation of side effects was not included as an objective. The KDIGO guidelines emphasize personalized treatment plans, recommending diuretics for volume overload while closely monitoring renal function and electrolytes.[20] Optimization strategies are dosing adjustment, monitoring protocols, and combination therapies such as the use of thiazide diuretics in conjunction with loop diuretics.[21]

Study limitations

The main limitation of the study was the small sample size. The study was limited by a limited sample size, which may affect the generalizability of the findings. Resource limitations: insufficient resources impacted the scope of the investigation, potentially limiting the depth of the analysis. Adverse effects: the study did not adequately address the adverse effects associated with the intervention, which is crucial for a comprehensive understanding of its safety profile. Supportive therapy information: there was a lack of detailed information on supportive therapies, which could influence treatment outcomes and recommendations.

Conclusion

CKD refers to a variety of diseases that impact both the structure and function of the kidney. The variability in the manifestation of the illness is influenced by factors such as etiology, pathogenesis, severity, and pace of development. It is one of the leading causes of death. It is prevalent in both men and females; higher testosterone levels in men can cause a loss in kidney function, thereby leading to a higher risk of CKD. The result did not show a significant association between ROA correlated with hospital stay and the Route of administration route

correlated with hemodialysis frequency. In our study, we do not have sufficient data to conclude any significant difference in the outcome caused by changes in the dose of furosemide. It were found that there was not many differences and the efficacy of furosemide was similar in CKD patients regardless of ROA. It does not show much significance in improving renal function. The results of this study conclude that the administration of furosemide to patients with chronic kidney disease with residual dieresis could improve urinary volume, sodium and potassium regardless of hemodialysis.

Declarations

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Author contributions

Conceptualization, P.A.K, S. and M.B.; Methodology, S.N.F and N.F.; Software, S.N.F and N.F.; Validation, P.A.K., A.F.F. and S.T.; Formal Analysis, P.A.K., A.F.F. and S.T.; Investigation, S., S.T. and M.B.; Resources, S., S.T. and M.B.; Data Curation, S., S.T. and M.B.; Writing – Original Draft Preparation, P.A.K, S., S.T., M.B., A.F.F., S.N.F. and N.F.; Writing – Review & Editing, P.A.K, S., S.T., M.B., A.F.F., S.N.F. and N.F.

Conflicts of interest

There are no conflict of interest involved in the study.

Data availability

Due to privacy concerns, the data are not publicly available, but can be accessed upon reasonable request from the corresponding author with a signed data access agreement.

Ethics approval

The study has been approved by the Human Ethics Committee of Mahavir Hospital and Research Center, with reference no. ECR01/450/Inst/AP/11/03/22.

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ORIGINAL PAPER

Protective and ameliorative effects of *Picrorhiza kurroa* rhizome extract against drug-induced liver injury in rats model

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ABSTRACT

Introduction and aim. The study evaluated the hepatoprotective and antioxidant properties of *Picrorhiza kurroa* rhizome extracts in rats, assessing their ability to scavenge free radicals and protect against liver damage.

Material and methods. Liver damage was observed in Wistar rats after seven days of oral paracetamol (PCM) and azithromycin (AZM) combination therapy, with serum biomarkers evaluated for effect.

Results. During the DPPH experiment, the antioxidant DPPH assay on rats' livers revealed that the ethanol extract of *P. kurroa* demonstrated free radical scavenging activity. The crude ethanol extract of *P. kurroa* showed a 15.62% yield and 48.62 IC_{50} values in an antioxidant DPPH experiment. Long-term treatment reduces liver toxicity by balancing biochemical factors. When compared to the group that received only PCM and AZM, the rats treated with *P. kurroa* crude extract showed a significant decrease in alkaline phosphatase, aspartate aminotransferase, glutamate pyruvic transaminase, and bilirubin ($p<0.001$) while showing an increase in protein and albumin at all doses ($p<0.05$). In addition, it was reproved by *in vivo* antioxidant parameters such as superoxide dismutase, lactate dehydrogenase, catalase, and glutathione, which were also examined to verify its strong hepatoprotective effect.

Conclusion. The study found that the ethanolic extract of *P. kurroa* rhizome has the potential to protect against liver damage caused by PCM and AZM due to its complementary anti-oxidant properties.

Keywords. antioxidant activity, hepatoprotective effects, liver damage, *Picrorhiza kurroa*

Introduction

Although drug-induced hepatotoxicity is well understood, it remains a significant public health concern. A serious problem is the glutamate pyruvic transaminase (SGPT) issue. The two primary areas affected by the obstacles presented by this condition are the development of novel pharmaceuticals and the removal of promising pharmaceuticals from the market. Troglitazone, a potentially effective antidiabetic medication, was taken off the market in a matter of years due to severe liver damage that occurred when the drug was being administered, as per the report by Niknahad and Fisher et al.[1,2] Significant idiosyncratic hepatotoxicity is thought to result from its sulfate conjugate's inhibition of bile SGPT transport from hepatocytes.[1,3] Hepatotoxicants can be classified into two categories: those that cause direct harm to the liver (intrinsic hepatotoxicity) and those that cause idiosyncratic hepatotoxicity, which is the toxicant that mediates an immunological response (hypersensitivity). Direct or intrinsic hepatotoxicity typically has a constant onset phase,

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is dose-dependent, and can be replicated in several animal models. For example, the hepatotoxicity caused by acetylpara-aminophenol (APAP) varies in dose.[4] Lack of repeatable animal models, dose-independent and host-dependent effects on the immune system, and genetic variability contribute to idiosyncratic drug-induced hepatotoxicity.[5,6] Troglitazone and chlorpromazine are two examples of idiosyncratic hepatotoxins.[6,7] Although not entirely understood, the mechanisms of action are known. Hepatotoxicants work in various ways, some directly interfering with the function of vital cellular structures such as the nucleus, mitochondria, endoplasmic reticulum, and plasma membrane. Numerous hepatotoxicants attach themselves to enzymes and mitochondrial membranes, affecting cellular respiration and energy metabolism.[8] Several hepatotoxicants operate as direct inhibitors and uncouplers of the mitochondrial electron transport chain.[9,10] Lipid peroxidation, redox recycling, and disruption of calcium homeostasis are other well-known modes of action.[10] All hepatotoxins bring on numerous different clinical and histological manifestations of liver injury. Certain biochemical markers, such as albumin levels, bilirubin protein, alkaline phosphatase (ALP), glutamate oxaloacetate transaminase (SGOT), and SGPT, can be used to diagnose liver injury (Fig. 1).[11-13] Increases in bilirubin levels are markers of overall liver function, while elevated serum enzyme levels are considered pertinent indicators of hepatic toxicity.[14-16] A rise in bilirubin to more than twice the upper limit of normal, accompanied by an elevation in transaminase levels, is thought to be a warning sign of hepatotoxicity.[17]

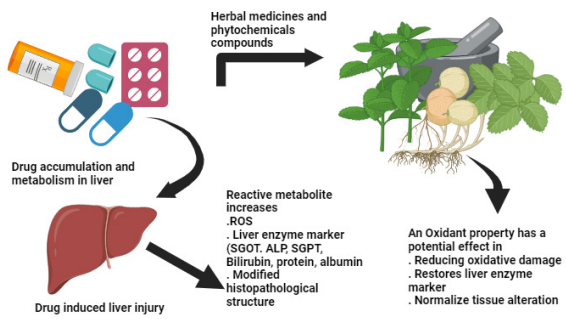

Fig. 1. Liver injury and protective effect of herbal plant

Hepatocellular and cholestatic toxicity are the two primary categories of hepatotoxicity, each with a unique mechanism of harm.[18] Although cholestatic injury is typified by predominantly initial ALP level elevations that precede or are relatively more prominent than increases in serum aminotransferase, hepatocellular or cytolytic injury involves primarily initial serum aminotransferase level elevations that typically precede increases in bilirubin levels and modest increases in ALP levels. In general, injuries of a mixed type that incorporate cholestatic and hepatocellular processes happen.[19] Selecting the type of hepatotoxic liver injury depends partly on the SGPT to ALP ratio value. When hepatocellular damage is prevalent, the ratio is larger than or equal to five (≥5), but when cholestatic liver damage is predominant, the ratio is less than or equal to two (≤2). When liver damage is combined, the ratio falls between two and five. In nonclinical investigations, SGPT, SGOT, and bilirubin in combination are often advised to evaluate hepatic damage in rodents and non-rodents. Since mitochondrial membrane damage, which is intracellular to the cell/plasma membrane, is required for an elevated SGOT level, SGPT is considered a more sensitive and specific biomarker of hepatocellular injury than SGOT. However, SGOT and SGPT are only cytoplasmic enzymes readily released into the bloodstream when hepatocyte cell/plasma membranes are damaged.[20,21]

Herbal therapies have demonstrated effective alternatives to hepatotoxic drug side effects. Only four plants have been scientifically clarified using internationally accepted standard protocols to develop evidence-based SGPT alternative herbal hepatoprotective drugs,[22,23] out of the many herbs and herbal medicines reported to have hepatoprotective effects.[24-26] A potent herbal hepatoprotective agent, silymarin, a flavonolignan derived from *Silybum marianum* (milk thistle), protects the liver from damage through antioxidative, antilipid peroxidative, anti-inflammatory, membrane-stabilizing, immunomodulatory, and liver-regenerating mechanisms.[27,28] Typically, oral Silymarin is prescribed at 600 mg/day, or 200 mg thrice daily, for medical therapeutic purposes. This study will employ an oral daily dose of 50 mg/kg of silymarin for treatment, since the 200 mg milk thistle plant extract typically includes 140 mg of silymarin or 70% silymarin concentration. In light of the ongoing search for more potent hepatoprotective agents, we plan to assess the hepatoprotective effects of the crude ethanolic rhizome extract of *Picrorhiza kurroa*, also known as the mango plant, in a mouse model by following the internationally recognized standard scientific protocol.[29] Kutki, also known as *P. kurroa* Royle ex Benth, is a member of the Scrophulariaceae family. North Burma, West China, South-ESGOT Tibet, and the Himalayan region (Garhwal to Bhutan) are home to the perennial herb *P. kurroa*. It grows spontaneously in organic soils and on rock fissures in alpine areas. Though considered an important medicinal herb, it is mainly used in traditional medicine to treat snake bites, fever, jaundice, liver problems, and malaria. *P. kurroa* has anti-microbial, anti-oxidant, anti-bacterial, anti-mutagenic, cardio-protective, hepato-protective, anti-inflammatory, anti-diabetic, anti-malarial, anti-ulcer, anti-cancer, and nephroprotective pharmacological qualities.[30,31] For herbal/botanical products sold commercially, ensuring that the raw material used is real *P. kurroa*. Overusing *P. kurroa* for medicinal purposes has jeopardized the plant's conservation status in several places. The

plant population is severely impacted by the widespread use of this remedy by locals to treat a wide range of ailments.[32-35] Additionally, the purpose of this work is to examine the hepatoprotective, ameliorative and antioxidant properties of the crude ethanolic rhizome extract against azithromycin and paracetamol-induced hepatotoxicity in a mouse model.

Aim

This study aimed to evaluate the protective and ameliorative effects of *P. kurroa* rhizome extract against drug-induced liver injury in a rat model.

Material and methods

Sampling and authentication of plant extracts

From July to September 2023, fresh *P. kurroa* rhizomes were harvested from hill crops at the Bimla Logistics Om complex in Raja Rani Vihar, Haldwani (UK), India. Botanists verified the taxonomic identification. Mr. R S. Jayasomu, the Chief Scientist Head of RHMD, and Dr. Sunita Garg, the Former Chief Scientist Head of RHMD, CSIR-National Institute of Science Communication and Information Resources (NISCAIR), verified the plants. The ethical number for animal experiments is added in the form of CPCSEA (1204/PO/Re/S/08/CPCSEA) Animal Welfare, Swami Vivekanand Subharti University Meerut.

Physico-chemical characteristics

Plant extraction method

To remove any earthy elements, the *P. kurroa* rhizome was thoroughly cleansed with water. After letting it air dry in the shade at room temperature, it was carefully weighed and machine-ground into a fine powder using a mixer grinder. Using a Soxhlet device, the powdered material was subjected to solvent extraction with ethanol (99.9%) at 50–60°C for 48 hours at room temperature. To dry the extract, an oven preheated to 40°C was utilized. The crude extract was kept dry and sealed in an airtight container for the next experiment.

Physical-chemical property evaluation

Using the Unani Pharmacopoeia of India, different physicochemical properties were determined.[36] The following chemical and physical attributes have been evaluated: drying loss, total amount of ash, sulfate ash, water-soluble ash, and acid-insoluble ash.[37]

Preliminary phytochemical analysis

A preliminary phytochemical examination was performed using recognized techniques to determine the presence or absence of specific phytoconstituents.[37]

Assay to determine antioxidant activity (DPPH)

Using the standard protocol described by Braca A. et al. the 2, 2-diphenyl1-picrylhydrazyl (DPPH) assay was used to assess the free radical scavenging activity of the ethanol extract of the *P. kurroa* rhizome *in vitro*.[38] The proportion of radical scavenging activity (RSA) was calculated using the following formula: RSA=[(A0 - A1)/A0]×100 where A0 is the absorbance of the control and A1 is the absorbance of the samples after thirty minutes. The IC_{50} value indicated the plant extracts' ability to scavenge free radicals. The IC_{50} value is the concentration of a sample (in μg/mL) that inhibits 50% of the DPPH radical.[39]

Pharmacological studies

Experimental animals

We chose all sexes of Wister albino rats weighing 180–220 g. The experiments were carried out according to the CPCSEA criteria for the use and care of experimental animals and the moral principles approved by the Institutional Animal Ethics Committee (IACE) guidelines for animal care. The ethical number for animal experiments is added as CPCSEA (1204/PO/Re/S/08/CPCSEA) Animal Welfare, Swami Vivekanand Subharti University Meerut.

Assessment of acute toxicity

The acute oral toxicity was evaluated at a test limit test dose of 2000 mg/kg following the parameters described in the Organization for Economic Cooperation and Development (OECD)-425.

Evaluation of hepatoprotective activity

The PCM and AZM-induced liver damage method was modified and utilized to evaluate the hepatoprotective effect *in vivo.* This method has been reported in several studies.

Experiments and procedures

There were six groups (n=6) of the animals. Wistar albino rats (150–250 g body weight) of both sexes (male and female) were used. Work carried out on accredited CPCSEA (1204/PO/Re/S/08/CPCSEA) Animal Welfare, Swami Vivekanand Subharti University Meerut.[40] Oral administration of Silymarin (50 mg/kg) and *P. kurroa* (200 and 400 mg/kg) was performed for 14 days. To cause liver damage, PCM (250 mg/kg, p.o.) and AZM (200 mg/kg, p.o.) were administered continuously for 7 days, from the 7th to the 14th day, 2 hours after the test, in addition to the usual drug administration.[41-44]

The following is a summary of the experimental groups:

Group 1: Normal control (seven days of 0.5 percent CMC administration to rats),

Group 2: Toxicant control (Rats were administered 0.5% CMC for seven days in conjunction with 250 mg/kg of PCM and 200 mg/kg of AZM),

Group 3: Silymarin (Rats received 50 mg/kg of Silymarin suspension (p.o.) for 14 days along with toxicants (7 days' worth of 250 mg/kg PCM and 200 mg/kg AZM),

The rats in groups 4 and 5 were given a 14-day course of treatment consisting of 200 and 400 mg/kg p.o. extract of *P. kurroa*, along with a combination of toxicants PCM (250 mg/kg) and AZM (200 mg/kg) for 7 days.

On the fourteenth day, the animals were anesthetized with diethyl ether. The blood was then extracted and coagulated and serum was separated to determine enzyme activity. A section of liver tissue was immediately placed in 10% formalin for histological examination.

Estimation of serum biochemical parameters

Biochemical indicators include serum levels of SGOT. As directed by the manufacturer, we measured serum levels of total bilirubin (TB), alkaline phosphate (ALP) and SGPT using commercial enzyme biochemical diagnostic kits (Sigma-Aldrich (Merck KGaA), Darmstadt, Germany).

Antioxidant activity (in vivo)

To assess *in vivo* antioxidant activity, the levels of SOD, CAT, LDH, and GSH were measured in blood plasma and liver tissue.

1. Superoxide dismutase (SOD) activity: The nitro blue tetrazolium (NBT) reduction method, as outlined by Marklund and Marklund, was used to measure the SOD activity. NBT, xanthine oxidase, and tris-HCl buffer were all present in the reaction mixture. The NBT decrease was tracked at 560 nm.[45]
2. Catalase (CAT) activity: By detecting the breakdown of hydrogen peroxide (H_2O_2), as explained by Aebi, CAT activity was evaluated. At 240 nm, a reduction in absorbance was noted.[46]
3. Lactate dehydrogenase (LDH) activity: LDH activity was assessed with a standard commercial enzymatic test kit in accordance with the manufacturer's instructions. The transformation of pyruvate to lactate was observed at 340 nm.[47]
4. Reduced glutathione (GSH) level: Ellman's reagent method was used to measure GSH levels. At 412 nm, the yellow-colored complex's development was measured.[48]

Absorbance measurements were conducted utilizing a Thermo Fisher Scientific Multiskan GO microplate reader (Waltham, MA, USA), which is outfitted with a UV-visible spectrophotometer.

Histopathological studies

Liver tissue was embedded in paraffin, fixed for 24 hours in 10% formalin, and then sectioned into 5-mm-thick pieces using a rotary microtome. The sections were stained with hematoxylin-eosin dye under a microscope to examine the liver's histology.

Statistical analysis

Data were expressed as means±standard errors of mean (SEM). The analysis was performed using the software application GraphPad Instat 3 (GraphPad Software, La Jolla, California, USA). Each experiment was completed, and all data are shown as mean±SD. A one-way ANOVA with Post Hoc Tukey's test assesses the significance of mean differences: a=extremely significant, b=significant, and ns=not significant.

Results

The percentage yield of crude extract

Table 1 presents the crude ethanol extract yield percentage was found.

Table 1. The crude ethanol extract's yield percentage was found

Fraction	Color of extract	Yield of extract (g)	Yield of extract (%)
Ethanol	Brown	24.6	15.62

Physicochemical property analysis

As indicated in (Table 2), the inorganic components of the ash are essential reference points for determining the validity and purity of the drug. The consistency, kind, color, and yield of each plant material extract were also examined.

Table 2. Physicochemical analysis of *P. kurroa*

Physicochemical parameters	% w/w
Ash value	
Total ash	13.6
Acid insoluble ash	1.57
Water soluble ash	7.25
Extractive value	
Ethanol 95%	2.544
Loss on drying	8.345

Preliminary phytochemical research

Alkaloids, glycosides, flavonoids, tannins, steroids, saponins, and quercitin are some phytochemicals that can be found in plant materials. Table 3 shows that the ethanolic extract of *P. kurroa* contained all the phenolic and flavonoid content.

In vitro antioxidant activity assays

Plants with antioxidant qualities have medicinal uses. Due to this, the well-known DPPH technique was used to detect free radical scavenging activity in the ethanolic extract of *P. kurroa*, as Table 4. 87.15±0.12 percent of DPPH radical scavenging activity is found in the ethanolic extract of the rhizome of *P. kurroa*, depending on the dose. Whereas the standard ascorbic acid had an IC_{50} of 35.20 g/mL, the extract was 48.62 g/mL (Fig. 2).

Table 3. A preliminary phytochemical screening was performed on the ethanolic extract of the *P. kurroa* rhizome to assess its effectiveness*

Constituents	Test	Observation	Inference of the ethanolic extract of the *P. kurroa* (rhizome)
Saponins	Frothing	Frothing persists for 15 min.	+
Alkaloids	Mayer's	White-cream ppt	+
	Draggondorf's	Orange ppt	+
	Wagner's	Reddish-brown ppt	+
Flavonoids	$FeCl_3$	Green or violet ppt	-
	Shinoda	Orange-red ppt	+
Tannins	Lead subacetate	Cream ppt	+
Steroids and terpenes	Lieberman-Buchard	Blue-green color at interphase	-
	Salkowski	Reddish color	+
Carbohydrates	Molish's	Reddish ring	+
	Fehling's	Red	+
Phenols	$FeCl_2$	Bluish black color Pin blood	+
Glycosides	Legal's	Red ppt	+
	Fehling's	Red ppt	+

* (+) – present, (-) – absent

Tables 4. Free radical scavenging activity of 1,1-diphenyl-2 picrylhydrazyl (DPPH) for ethanolic extract of *P. kurroa*

Concentration (µg/mL)	DPPH scavenging activity (% inhibition)	
	Standard (ascorbic acid)	Ethanol extract of *P. kurroa*
10	29.22±0.32	19.12±0.30
20	39.73±0.07	29.25±0.19
40	57.71±0.81	43.36± 0.19
60	66.34±0.94	57.71±0.53
80	85.75±0.56	77.54±0.25
100	95.23±0.17	87.15±0.12
	$Ic_{50=35.20}$	$Ic_{50=48.62}$

Acute oral toxicity study of the ethanol extract of P. kurroa (rhizome) in rats

Table 5. Acute oral toxicity study of the ethanol extract of *P. kurroa* in rats

S.N.	Response	Animals	
		Before treatment	After treatment
1	Alertness	Normal	Normal
2	Grooming	Absent	Absent
3	Restlessness	Absent	Absent
4	Touch response	Absent	Absent
5	Torch response	Normal	Normal
6	Pain response	Normal	Normal
7	Tremors	Absent	Absent
8	Convulsion	Absent	Absent
9	Gripping strength	Normal	Normal
10	Corneal reflux	Present	Present
11	Writing	Absent	Absent
12	Pupils	Normal	Normal
13	Salivation	Normal	Normal
14	Skin colour	Normal	Normal

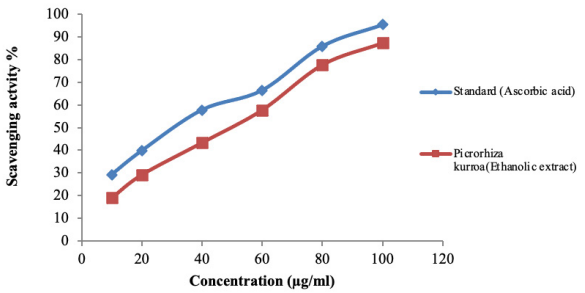

Fig. 2. Comparison of DPPH Scavenging activity between *P. kurroa* and standard (ascorbic acid)

Hepatoprotective parameter estimation

Table 6. Impact of *P. kurroa* on liver enzymes against PCM and AZM-induced liver toxicity in rats*

Group	SGPT (IU/l)	SGOT (IU/l)	ALP (IU/l)	Bilirubin (mg/dl)	Protein (Gm/dl)	Albumin (gm/dl)
Normal (control)	31.5± 1.6	33± 1.3	144.25± 1.48	0.8± 0.10	7.521± 0.09	4.986± 0.2
PCM (250 mg/kg) ±AZM (200 mg/kg)	107.76± 6.5^a	196± 8.6^a	427.6± 70.1^a	1.0245± 0.02^a	4.96± 0.4^a	4.726± 0.3^a
PCM (250 mg/kg) ±AZM (200 mg/kg)±Silymarin (50 mg/kg)	44.4 ± 4.6^{ns}	58.24± 4.6^{ns}	146.85± 2.3^{ns}	0.751± 0.07^{ns}	7.852± 0.3^b	4.94± 0.3^{ns}
PCM (250 mg/kg ±AZM (200 mg/kg)±*P. kurroa* (200 mg/kg)	62.24± 3.2^b	143.24± 11.2^b	154.4± 4.5^b	0.8132± 0.02^b	6.675± 0.3^b	4.876± 0.4^b
PCM (250 mg/kg ±AZM (200 mg/kg)±*P. kurroa* (400 mg/kg)	59.6± 5.2^{ns}	71.74± 4.9^{ns}	145.24± 1.7^b	0.55± 0.05^b	7.7± 0.3^{ns}	5.467± 0.09^b

* all data are presented as mean±SD, the significance of mean differences is determined using a one-way ANOVA with Post Hoc Tukey's test, a – highly significant, b – significant, ns – not significant

Table 7. Protective effects of *P. kurroa* on hepatic antioxidant enzyme activities against PCM and AZM-induced hepatotoxicity in rats*

Group	SOD (U/mg of protein)	CAT (µM/min/mg of protein)	LDH (U/L)	GSH (µ/mg of protein)
Normal (control)	46.42±5.5	196.44±15.95	155.89±39.17	20.32±1.13
PCM (250 mg/kg)±AZM (200 mg/kg)	9.97±2.09^a	32.97±9.85^a	703.94±28.87^a	6.87±1.65^a
PCM (250 mg/kg) ±AZM (200 mg/kg)±Silymarin (50 mg/kg)	43.94±3.49^{ns}	177.43±38.33^{ns}	171.845± 33.26^b	18.34±3.34^b
PCM (250±AZM (200 mg/kg)±*P. kurroa* (200 mg/kg)	35.69±7.83^a	127.52±7.98^b	234.13±36.22^b	11.17±3.30^a
PCM (250±AZM (200 mg/kg)±*P. kurroa* (400 mg/kg)	41.02±7.69^{ns}	160.45±5.70^b	180.65±28.56^b	15.48±2.01^{ns}

* all data are presented as mean±SD, the significance of mean differences is determined using a one-way ANOVA with Post Hoc Tukey's test, a highly significant, b – significant, ns not significant

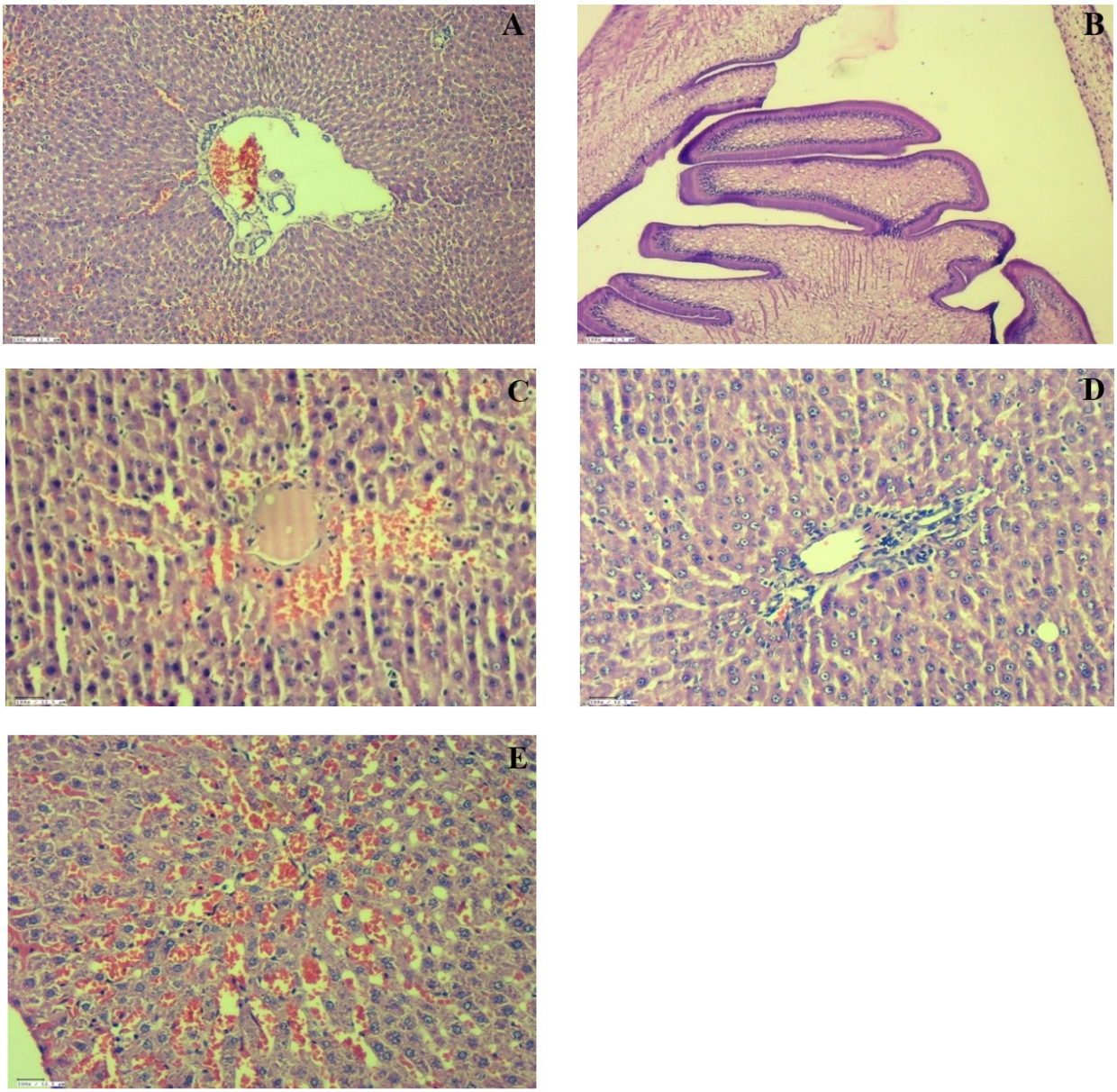

Fig. 3. Effects of *P. kurroa* on histopathological changes induced by PCM and AZM in rats, A: control group, B: animals treated with PCM (250 mg/kg) and AZM (200 mg/kg), C: animal treated with PCM (250 mg/kg) and AZM (200 mg/kg) and Silymarin (50 mg/kg), D and E: animals treated with PCM (250 mg/kg) and AZM (200mg/kg) and *P. kurroa* (200 and 400mg/kg) (H&E,200X)

Histopathological evaluation

The cellular architecture of liver tissue in each group of rats was examined using histopathological examination, and the results are shown in Figure 3. The photomicrograph of the liver revealed normal hepatic cell architecture, with transparent cytoplasm, slightly dilated central veins, normal kupffer cells, and normal prominent nuclei in every cell (Fig. 3A). Figure 3B illustrates the deformed architecture and a vast area of necrosis in the liver tissue of the PCM and AZM control groups. Figure 3C of the Silymarin group's -3 also revealed no necrosis and fewer inflammations in the liver cells. Following pretreatment with 200 mg/kg and 400 mg/kg of *P. kurroa* extract, respectively, animal groups 4 and 5 displayed more of the liver tissue's normal architecture with the SGOT amount of inflammation (Figures 3D and 3E). All histological observations were performed with hematoxylin and eosin (H&E stain, 200X) techniques, with 200 magnification power, and corroborated the production of hepatotoxicity by PCM in combination with AZM and the hepatoprotective action of *P. kurroa* extract.

Discussion

In the current study, we demonstrated the antioxidant approach of *P. kurroa* and Silymarin for hepatotoxicity induced by PCM and AZM: a a histological and biochemical study would typically interpret the findings, relate them to the existing literature and suggest im-

plications for future research and clinical practice.[49,50] Here is a structured discussion based on what the article probably covers: the exposure of PCM and AZM exposure to the rats' consequence in a vital increase in serum SGOT, SGPT, ALP, bilirubin, protein and albumin for *P. kurroa* when levels were compared from the control Group in Table No. 6. In group II, IV and V, the lethal activity of PCM and AZM was steadily reversed in the rats by demonstrating the vital reduction into the serum SGOT, SGPT, ALP and Bilirubin. Important increments were found in the protein and albumin levels. Group III, administration of Silymarin (standard) demonstrated a significant decrease in marker enzymes of the liver; however, an important increment was found in protein and albumin levels.[51]

To further understand the hepatoprotective activity of *P. kurroa*, this investigation verified the activity and potency of antioxidant enzymes, for example, SOD, CAT, and LDH, in addition to the levels of GSH in the animal's liver. Antioxidant enzymes such as CAT and GSH were regarded as major protective systems for protection from oxidative damage. Administration of PCM and AZM caused vital alterations in SOD, CAT, LDH, and GSH when they were compared with rats of the normal group. The data obtained demonstrated the effects of PCM and AZM-induced liver toxicity on the liver's functions parameter; for example, SOD, CAT, LDH, and GSH could be efficiently balanced by the ethanolic extract of *P. kurroa* rhizome.[52,53] The study shows that both *P. kurroa* and Silymarin significantly ameliorate liver damage caused by PCM and AZM. The reduction in liver enzyme levels and improved histological outcomes indicate that these natural antioxidants (effectively counteract hepatotoxicity). This supports the hypothesis that *P. kurroa* and Silymarin have potent hepatoprotective properties.[50] The findings align with previous research highlighting the hepatoprotective effects of Silymarin, which has been extensively studied and used clinically for liver disorders. However, the study adds value by providing new information on the protective effects of *P. kurroa*, particularly in the context of drug-induced liver injury.

The combination of *P. kurroa* and Silymarin may offer enhanced protection compared to either agent alone, suggesting a potential synergistic effect. This is consistent with other studies showing that the combination of different antioxidants can result in better therapeutic outcomes.[54] The protective effects observed in this study can be attributed to the antioxidant properties of *P. kurroa* and Silymarin. *P. kurroa*, rich in picrosides, likely exerts its effects by scavenging free radicals, reducing oxidative stress, and modulating inflammatory pathways. On the contrary, silymarin stabilizes hepatocyte membranes, inhibits lipid peroxidation, and enhances protein synthesis, promoting liver cell regeneration. The reduction in oxidative stress markers and the preservation of hepatic architecture in the treated groups further support the notion that both *P. kurroa* and Silymarin mitigate the damaging effects of NAPQI (from PCM metabolism) and azithromycin-induced oxidative stress.[55]

The histological analysis showing reduced necrosis, inflammation, and fatty changes in the liver tissue of treated groups correlates well with the biochemical findings. This concordance reinforces the conclusion that *P. kurroa* and silymarin prevent biochemical alterations and protect the structural integrity. Normalization of liver enzymes, coupled with histological improvement, suggests that *P. kurroa* and Silymarin facilitate the recovery of liver function, which is crucial for overall health of the organism. Given the widespread use of PCM and AZM, the hepatoprotective effects of *P. kurroa* and Silymarin could have significant clinical implications. These findings suggest that *P. kurroa* and Silymarin could be considered adjunct therapies in patients receiving potentially hepatotoxic drugs, especially in individuals with preexisting liver conditions or those at higher risk of drug-induced liver injury. The study also underscores the importance of exploring natural antioxidants as safer alternatives or complements to synthetic drugs in managing liver toxicity. Although the study provides strong evidence for the hepatoprotective effects of *P. kurroa* and silymarin, some limitations should be acknowledged. For instance, the study was conducted in animal models, and thus, the results may not directly translate to humans without further clinical trials. Furthermore, the exact molecular mechanisms underlying the protective effects of *P. kurroa* and Silymarin could be further elucidated through more detailed studies, such as those involving gene expression analysis or advanced imaging techniques.[56] Future research could explore the long-term effects of treatment with *P. kurroa* and Silymarin, potential dose-response relationships, and the efficacy of these compounds in human clinical trials. Moreover, investigating the impact of *P. kurroa* and Silymarin on other forms of drug-induced hepatotoxicity or in combination with other hepatoprotective agents could be valuable. The exploration of different natural products with similar antioxidant properties could also lead to the discovery of new therapeutic agents for liver protection.[57,58]

Conclusion

The study concludes that *P. kurroa* and Silymarin significantly protect against PCM and AZM-induced hepatotoxicity. These findings reinforce the therapeutic potential of natural antioxidants for liver protection and suggest a promising avenue for the development of adjunctive therapies in hepatology. This discussion integrates the study findings with broader scientific knowledge, highlights the implications for clinical prac-

tice, and suggests avenues for further research. In summary, this study has shown that the rhizome extract of *P. kurroa* exhibits strong hepatoprotective properties against paracetamol and azithromycin in rats. In addition, phenolic compounds and flavonoids have been reported in plants on the basis of phytochemical data. Their potential as antioxidants may account for their hepatoprotective properties.

Declarations

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Author contributions
Conceptualization, M.K. and V.R.; Methodology, V.R. and S.K.; Software, M.K.; Validation, M.K., V.R. and A.K.M.; Formal Analysis, M.K.; Investigation, V.S.; Resources, V.R.; Data Curation, M.K.; Writing – Original Draft Preparation, M.K. and V.R.; Writing – Review & Editing, V.R.; Visualization, V.R.; Supervision, V.R.; Project Administration, V.R.; Funding Acquisition, V.R.

Conflicts of interest
The authors declare that they have no potential conflicts of interest regarding the research, authorship, and publication of this article.

Data availability
All data generated or analyzed during this study are included in the published article. Any additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval
The ethical number for animal experiments is added in the form of CPCSEA (1204/PO/Re/S/08/CPCSEA) Animal Welfare, Swami Vivekanand Subharti University Meerut.

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ORIGINAL PAPER

Public knowledge of cancer in southern Poland

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ABSTRACT

Introduction and aim. Due to the constantly growing number of cancer cases in Polish society, our study aimed to check the respondents' knowledge of the broadly understood topic of cancer. The aim was to check the knowledge of Polish society and compare it according to age, gender, level of education, place of residence, and marital status.

Material and methods. Our study was held in Podkarpackie and Malopolskie voivodeship where participants completed a questionnaire containing 31 questions on the topic of cancer. The survey was completed by 360 people, 248 women, and 112 men. The study locations were diverse and the interviewees came from different backgrounds, age groups, and education.

Results. The study revealed generally high awareness of cancer among 360 respondents, with 93% of whom recognized the importance of early detection, but significant gaps were identified in knowledge about prevention methods, including vaccinations (36%) and viral causes (50%). Older adults and those with lower educational levels showed significantly lower awareness ($p<0.05$), highlighting the need for targeted educational initiatives.

Conclusion. The survey we conducted clearly shows that the state of knowledge about cancer in Polish society is not yet fully satisfactory. The conclusion that arises from our survey concerns the dissemination of knowledge about primary cancer prevention.

Keywords. cancer knowledge, cancer prevention, health education, southern Poland

Introduction

Cancers are a broad group of diseases that include focal lesions with a good prognosis and malignant systemic processes, where long-term survival is rare. Tumors have affected multicellular living things for more than 200 million years, and there is evidence of cancer among the ancestors of modern humans dating back well over a million years. Unlike infectious diseases, parasites, and many environmental diseases, cancers are not primarily caused by some entity foreign to our bodies. Their destructive agents are human cells that have, as it were, lost control of themselves and have been recruited and, to some extent, transformed into pathological organisms or building blocks of tumors.[1]

Since the mid-1960s, the number of cancer cases in Poland has increased approximately 2.5 times and continues to rise. The reasons for this phenomenon are related to factors such as an increase in the average life expectancy

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and changes in lifestyle, such as tobacco smoking, an unhealthy diet, and lack of physical activity.[2] While the Polish healthcare system has achieved some progress in the field of cancer treatment, access to modern therapies and research remains a challenge. Combating cancer in Poland requires an integrated approach that includes prevention, early detection, effective treatment, and support for patients and their families. Government and health organizations continue to work to improve oncological care through investments in advanced diagnostics, targeted therapies, radiation therapy, and the development of preventive programs. However, fighting cancer requires the participation of all of society, education about healthy lifestyles, and regular screening tests to achieve positive results in combating this serious disease. Early detection of malignant tumors is crucial for effective treatment and an improved prognosis. Therefore, regular preventive screenings and early response to concerning symptoms are essential to reduce the risk associated with this serious disease.[3]

In 2020, the Polish National Cancer Registry received information on nearly 146,200 new cancer cases and 99,900 deaths from them. Compared to 2019 registry, malignant tumors are still the second leading cause of death in Poland, causing 21.8% of deaths in men and 20% of deaths in women in 2020. They represent a significant health problem primarily in young and middle-aged people. This phenomenon is particularly evident in the female population, where for several years, cancer has been the most common cause of death before the age of 65, accounting for 28.3% of deaths in young women and 41.6 % of deaths in middle-aged women.[4] As claimed by the European Cancer Inequalities Registry from 2023 cancer mortality in Poland is higher than the EU average, and the cause of this may be the delay in diagnosis and restricted access to the best treatment. For that reason, the Polish National Oncological Strategy has taken steps to improve quality of health care benefits by: increasing the amount of health care providers to reduce the waiting time for diagnosis, investing in popularization of healthy eating habits, conveying the importance of physical activity that is key to primary prevention, increasing funding for more technologically advanced systems of secondary prevention, as well as financing more research to find a way for the most effective treatment and diagnostics available.[4,5]

It has been shown that about 30–50% of cancers are preventable. According to forecasts based on the GLOBOCAN study, the upward trend is expected to continue and by 2030, new cancer cases in Poland could exceed 220,000 individuals. Malignant tumors have become a serious threat to Polish society, and if the growing trend in the incidence of cancer persists, they may soon become the leading cause of death before the age of 65, surpassing deaths due to cardiovascular diseases.[4,5]

That is why it is so important to have knowledge in society about cancer, its prevention, and mechanisms of formation, as prevention is definitely better than cure. There are many cancer education and prevention campaigns available in our country that reach a wide audience.[5] The widespread European Cancer Code and the 12 ways to be healthy contained therein are an excellent source of knowledge about cancer. In the media, such as television, radio, and the Internet, there are articles, informational programs, and advertisements related to the risks of cancer and treatment options. However, the level of awareness in society can vary. Some people may be more aware of the risks and importance of prevention, while others may have limited knowledge on this topic.

Aim

The aim of our paper was to evaluate whether the citizens of Podkarpackie and Malopolskie voivodeships in Poland know the basic facts about this diverse and devastating group of diseases.

Material and methods

We created a survey consisting of 31 closed questions, which was completed by 360 people, including 248 women and 112 men. This gives us 6 metrics and 2 main elements, which are the survey and the group of respondents.

The study was conducted in various social groups, including, among others: factors: age, gender, residence, education, occurrence of cancer in the family. Consent was obtained by conducting a survey. Surveys were sent online to people interested in participating in the study.

Data were collected from different age groups from 14 to 65 years old, which differed in gender, education, place of residence, and marital status. Data collection and organization were performed using Excel. The study was carried out in both paper form in various centers and in the form of online surveys. The geographical area covers two voivodeships: Podkarpackie and Malopolskie.

The questionnaire was designed in accordance with the principles of survey methodology to ensure its reliability, completeness, and acceptability to respondents. The questions were created based on research in the previous literature to ensure their validity. The research team analyzed the questions to ensure that they were understandable and not misleading to the respondents. The questionnaire was tested in a small group of respondents as part of a pilot study. This was to assess whether the questions were understandable and the time needed to complete the questionnaire was acceptable. The comments collected were used to modify the questionnaire.

Statistical analysis was performed using Statistica 13.1 (Statsoft, USA). The Pearson χ^2 test was used to examine interactions between qualitative variables. The significance level of $\alpha=0.05$ was assumed. Abbreviations and symbols used: p – statistical significance value, if $p<0.05$ – there is a significant difference between the groups.

Our study obtained a positive opinion from the Bioethics Committee of Rzeszow University (Resolution number: 2022/101 date; 07 December 2022).

Table 1. Demographic characteristics of the respondent group

Demographic feature	Category	Number (n)	Percentage (%)
Age	14–17	114	32%
	18–24	106	29%
	25–34	6	2%
	35–50	28	7%
	51–64	42	12%
	65+	64	18%
Gender	Male	112	31%
	Female	248	69%
Marital status	Married	87	24%
	Single	229	64%
	Divorced	9	2%
	Widow	31	9%
	In separation	4	1%
Professional situation	Student	215	60%
	Full-time	50	14%
	Retired	2	0.5%
	Unemployed	5	1%
	Entrepreneur	6	2%
	Pensioner	79	22%
	Hired work	2	0.5%
Cancer history	Yes	22	6%
	No	338	94%

Table 2. Knowledge of cancer prevention

Question	Answer	Number (n)	Percentage (%)
Do you think that viruses can cause cancer?	Yes	180	50%
	No	121	33.6%
	Don't know	59	16.4%
Do you believe that mental attitude can speed up recovery?	Yes	252	70%
	No	57	15.8%
	Don't know	51	14.2%
Was cancer knowledge adequately conveyed during your education?	Yes	139	38.6%
	No	220	61.1%
	Don't know	1	0.01%
Are there vaccines that can prevent/reduce cancer risk?	Yes	121	33.6%
	No	109	30.2%
	Don't know	130	36.1%
Can obesity increase the risk of developing cancer?	Yes	220	61.1%
	No	84	23.3%
	Don't know	56	15.5%

Results

The study involved 360 respondents, diverse in terms of age, gender, marital status, education, professional situation, and history of cancer (Table 1). The majority of the respondents (94%, n=338) had no personal experience with cancer, while 6% (n=22) reported having or currently having cancer. In turn, 61% (n=219) of the respondents admitted that their family had a history of cancer, which may influence perception of risk factors and knowledge of the disease. The age structure of the respondents was varied, but the largest group were people aged 50–64 (29%, n=106). Knowledge of cancer in the study group was generally good, although there were significant gaps in some areas. As many as 93% of respondents agreed with the statement that early detection of cancer increases the chances of survival. However, only 46% of the respondents were convinced that early detection of cancer can lead to complete cure, which indicates a lack of full understanding of the importance of prevention and early diagnosis. More than 90% (n=360) of the respondents believed that a family history of cancer increased the risk of developing cancer, indicating a high awareness of the genetic determinants of the disease. Significant gaps in knowledge concerned prevention based on vaccinations and the association of obesity with cancer. Only 36% of respondents were aware that there are vaccines that can prevent some types of cancer, such as cervical cancer. Furthermore, only 50% of respondents knew that viruses can cause cancer, which indicates a need for education on viral risk factors for cancer, such as the HPV virus. Most respondents (61%, n=220) were aware that obesity can lead to the development of cancer, but still as many as 39% had limited knowledge on this subject or were not sure. The analysis showed a varied level of knowledge depending on demographic factors. Older respondents (over 65 years of age) and those with a lower level of education showed significantly lower levels of knowledge about cancer compared to younger and better educated participants ($p<0.05$). Furthermore, people with a family history of cancer were characterized by a greater awareness of risk factors and available methods of cancer prevention, suggesting that direct experience with the disease has a positive impact on the level of oncological knowledge. The analysis of the relationships between knowledge and demographic factors included variables such as age, marital status, professional situation, and personal and family history of cancer. The results indicate that age, education, and personal experience with cancer are significant predictors of the level of knowledge about cancer (Table 2). Respondents who had personal experience with cancer, both in their own life and in their family, had a higher level of knowledge about risk factors, prevention, and available treatments. In summary, the results of the study indicate a satisfactory level of knowledge among respondents regarding basic information about cancer, but significant gaps concern prevention, in particular vaccinations and factors related to obesity. The groups with the lowest level of knowledge are older people and those with lower levels of education, which suggests the need to direct educational activities to these populations (Table 3).

Table 3. Prognostic factors related to knowledge about cancer prevention

Factor	Category	Question	Answer	Number (n)	Percentage (%)	p
Age	<65	Do you believe mental attitude speeds up recovery?	Yes	196	66.2%	0.05
			No	40	13.5%	
			Don't know	50	16.8%	
	≥65		Yes	56	87.5%	
			No	1	0.02%	
			Don't know	7	0.1%	
Education level	Higher	Do you think that HPV vaccinations fully protect against cervical cancer?	Yes	15	28.8%	<0.001
			No	18	34.6%	
			Don't know	19	36.5%	
	Lower		Yes	44	14.3%	
			No	130	42.4%	
			Don't know	132	41.3%	
Marital status	Married	Do you think obesity leads to cancer?	Yes	43	50%	0.009
			No	18	20.9%	
			Don't know	25	29%	
	Single		Yes	156	68.1%	
			No	25	10.9%	
			Don't know	48	20.9%	

Discussion

The aim of the survey conducted among residents of the Podkarpackie and Lesser Poland was to determine the percentage of correct answers given by respondents to questions regarding cancer risk factors. The study aimed to analyze the level of awareness of these factors among the local community. The work presented here shows that a significant percentage of respondents who have ever had cancer are people of retirement age. While this may seem expected, the increasing number of cancer cases among those under 65 years of age deserves special attention. This may indicate changing risk patterns that include environmental factors, lifestyle, and early detection. Therefore, it is a need to tailor prevention and education programs to younger age groups to effectively reduce the increasing incidence of cancer in this population.

Our own study shows that only 61.2% consider obesity as a risk factor for the development of cancer, and equally half agree with the statement that excessive sugar consumption may increase the risk of cancer. According to the American Institute for Cancer Research, inadequate nutrition, as well as overweight, obesity and low physical activity, have a significant impact on cancer risk.[6] In nearly 30% of cancers, the influence of an incorrect diet on the onset and development of the disease has been proven, including colon, esophagus, mouth and stomach.[7]

Promoting a life without substances should become a basic element of cancer prevention. Tobacco smoking is the main cause of cancer, which has been confirmed in numerous studies. When asked whether smoking traditional cigarettes and electronic cigarettes increases the risk of developing cancer, a significant number of study participants agreed with this statement. In the study "Knowledge of high school students about smoking tobacco as a risk factor for lung cancer and its treatment" Both girls (53.8% - yes) and boys (40% - yes) definitely claim that smoking has a carcinogenic effect.[8] However, when asked about passive smoking, 86% of respondents answered affirmatively, while in the study "Knowledge of cancer and attitudes related to this issue among adolescents and young adults aged 15-30", as many as 95% of respondents agreed that passive smoking increases the risk of developing cancer.[9] A high percentage of respondents acknowledge that tobacco smoking increases the risk of developing cancer. This is a positive signal, suggesting that awareness of the link between smoking and cancer is quite widespread.

Tobacco smoke contains several thousand harmful substances, including substances with proven carcinogenicity.[5] The percentage of smokers among lung cancer patients is approximately 90%.[10] The influence of alcohol consumption on the development of cancers of the mouth, throat, larynx, esophagus, liver and breast has also been demonstrated.[11,12]

50% of respondents believe consuming a lot of sugar accelerates cancer development, while a significant 40% do not know. Misunderstanding of the relationship between sugar intake and cancer is probably the reason. Although sugar does not directly cause cancer, it contributes to obesity, which is a risk factor. The solution could be to clarify the role of sugar in obesity and its indirect link to cancer, rather than suggesting direct causality, through public health education and nutritional guidance.

More than half of the respondents (56%) believe that benign tumors do not become malignant, which highlights a common misconception. This misunderstanding probably stems from an oversimplification of the differences between benign and malignant tumors. While benign tumors are generally noncancerous, there is a small risk that some may transform into malignant ones over time. To address this knowledge gap, public health initiatives must focus on educating the public about the characteristics of both types of tumors and the potential, though rare, for benign tumors to become cancerous.

The vast majority of respondents consider early detection crucial for effective cancer treatment. This pos-

itive attitude may contribute to a greater willingness to undergo screening tests. Despite the positive approach to early detection, there is some misunderstanding about whether early diagnosis guarantees complete cure. This may require additional information on the cancer treatment process.

Although early diagnosis is a key factor in cancer prognosis, there are nuances and challenges associated with its outcomes. It does not always lead to higher cure rates, and there is also potential risk associated with over diagnosis and overtreatment. Furthermore, the concept of a "time window" from the moment ctDNA becomes detectable to when cancer becomes incurable emphasizes the existence of a period when early diagnosis may be most effective. However, the length of this time window can vary significantly depending on the type of cancer and individual cases.[14]

Less than half of the respondents believe that there are vaccines preventing or reducing the risk of certain types of cancer. This may indicate the need to increase awareness of available vaccinations.

Currently, all approved anticancer vaccines used in clinical practice focus on combating viruses-causing cancers, known as oncoviruses.[15] One of the most commonly used vaccines is the human papillomavirus (HPV) vaccine, known as Gardasil. Approved by the FDA for the first time in 2006, it is recommended for women aged 9 to 26 to prevent cervical cancer and other HPV-related cancers, such as vaginal, vulvar, anal, and oral cancers.[16] Results from phase III clinical trials indicate that the HPV vaccine effectively protects against more than 90% of infections caused by HPV 16 or 18 in females who received three doses of the vaccine.[17]

Another example of a prophylactic vaccine is the hepatitis B virus (HBV) vaccine, which provides protection against chronic HBV infection, significantly increasing the risk of hepatocellular carcinoma. Similar to the HPV vaccine, its mechanism of action relies on stimulating an antibody response to prevent initial HBV infection.[18-23]

The results of the study indicate the need to educate the public in the field of cancer prevention and diagnosis. We can compare them with the study conducted in 2017 in Lublin. According to the authors, knowledge on this subject is at different levels depending on the profile of the university. As expected, medical university students have the broadest knowledge in this field. The need for deeper education in secondary schools, as well as in higher education institutions, especially with technical and non-medical profiles is revealed.[24]

HPV vaccination is most effective when done before the onset of sexual intercourse, but adults who were not vaccinated as teenagers can also benefit from delayed vaccination. Even if someone already has an active HPV infection or disease, vaccination can still benefit by reducing the risk of further HPV-related illness. The US Advisory Committee on Immunization Practices (ACIP) recommends routine HPV vaccination by age 26. For those aged 27-45 years who have not been adequately vaccinated, shared clinical decision making is recommended, taking into account individual risks and benefits of vaccination. In particular, immunocompromised individuals such as people with HIV, transplant recipients, people undergoing immunotherapy, men who have sex with men (MSM), and transgender people may particularly benefit from supplemental HPV vaccination.

The international recommendations for HPV vaccination vary in some countries, vaccines are recommended for people between the ages of 9 and 45, while in others they are only recommended until age 45. In countries with limited resources, vaccination may be considered outside the standard indications, especially for those at increased risk. Vaccination of children in areas with limited screening capacity can significantly reduce the risk of cancer that would go undetected in the precancerous stage. In areas with low potential for post-treatment evaluation, follow-up vaccination may also reduce the risk of recurrence and cancer. The consideration of these aspects can provide important insights into the benefits and challenges of implementing vaccination in different settings.[25]

A significant 61% of the respondents felt that their cancer education during school was insufficient. This likely stems from a lack of emphasis on cancer awareness within the school curricula. To resolve this, schools should incorporate more comprehensive modules that cover cancer prevention, the importance of early detection, treatment options, and common myths surrounding the disease. By improving cancer education, students can be better equipped with the knowledge needed to make informed health decisions throughout their lives.

Conclusion

The results of the study provide important information on the state of knowledge of Polish society about cancer and also allow for the identification of areas requiring special attention and improvement. Although the overall level of awareness of cancer can be considered satisfactory in the context of basic information, such as the role of tobacco smoking in the development of cancer, many key areas remain poorly understood. The results of the study clearly indicate significant gaps in knowledge about prevention, in particular vaccinations and lifestyle factors such as obesity and lack of physical activity. The most important conclusion of the study is the need to expand education on primary cancer prevention. Only 36% of the respondents knew that there are vaccines to prevent some types of cancer, indicating a low level of awareness in this area. In particular, vac-

cinations against oncogenic viruses, such as HPV, are key to preventing cancer, and knowledge on this subject is still insufficient. Educational campaigns should therefore focus on spreading knowledge about available prevention methods, including vaccinations, which can significantly reduce the risk of developing cancers caused by viruses. Another significant problem is the lack of awareness of the link between obesity and cancer. Although 61% of the respondents were aware that obesity can lead to the development of cancer, up to 39% were unsure or did not know whether such a link existed. Considering that obesity is considered a significant risk factor for many types of cancer, these results show the urgent need to increase awareness in this area. Campaigns promoting a healthy lifestyle, including a healthy diet and regular physical activity, could significantly reduce the risk of cancer in society. Analysis of the results also showed that the level of knowledge about cancer is closely related to demographic factors, such as age, education, and a family history of cancer. Older people (over 65 years) and those with a lower level of education showed a significantly lower level of knowledge about cancer compared to younger and better educated respondents. Furthermore, people with a family history of cancer were more aware of risk factors, suggesting that direct experience of the disease may contribute to a better understanding of issues related to cancer prevention and treatment. These results suggest that educational activities should be aimed primarily at groups most at risk of low levels of knowledge, in particular the elderly and those with lower education. Educational programs could be implemented through public campaigns, but also within the education system, both at the school level and as part of continuing education for adults. Including cancer prevention in school curricula could contribute to raising awareness in the younger generation, as well as better preparing society for the health challenges associated with the increasing number of cancer cases. One of the key areas that requires further intervention is also understanding the differences between malignant and benign tumors. The results of the study show that 56% of respondents are unaware that benign tumors can, in some cases, develop into malignant tumors, which indicates insufficient knowledge of the mechanisms of cancer development. Education in this area should emphasize understanding the risks associated with benign tumors and the need for regular health checks. Although the results of the study indicate a relatively high awareness of the importance of early cancer detection – 93% of the respondents believe that early cancer detection increases the chances of the recovery – only 46% of respondents were convinced that it can lead to complete recovery. This shows that despite the general acceptance of the role of early diagnosis, there is a lack of complete understanding of the cancer treatment process and its potential outcomes. Public education in this area should focus on explaining the real options for cancer treatment at different stages of the disease, as well as what factors influence therapeutic success. The conclusions of the study clearly indicate the need to intensify educational activities on cancer prevention and early diagnosis. In particular, the focus should be on increasing awareness of available vaccines, the relationship between lifestyle and risk of developing cancer, and the importance of regular screening tests. To achieve positive results in the fight against cancer, the cooperation of the entire society is necessary, including health organizations, educational institutions, and the media. In summary, despite general awareness of some risk factors for cancer, there are still many areas that require improvement. It is particularly urgent to increase knowledge about vaccinations, obesity, and lifestyle, which can significantly reduce the number of cancer cases.

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Author contributions

Conceptualization, A.S. and B.S.K.; Methodology, S.S., M. J.; Software, P.S.; Validation, Kl.Z., Ka.Z. and A.L.; Formal Analysis, A.S.; Investigation, A.S.; Resources, J.K. and J.F.; Data Curation, A.S.; Writing – Original Draft Preparation, A.S.; Writing – Review & Editing, A.S.; Visualization, A.A.; Supervision, B.S.K.; Project Administration, K.W.; Funding Acquisition, C.L.

Conflicts of interest

Authors declare no conflicting interest.

Data availability

The data sets generated and/or necessary during the research are available from the authors.

Ethics approval

Our study obtained positive opinion of the Bioethics Committee at Rzeszow University (Resolution number: 2022/101 date; 07 December 2022).

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ORIGINAL PAPER

Evaluation of oxidative stress level and glutathione system in patients with psoriasis in Basrah Governorate, Iraq

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ABSTRACT

Introduction and aim. Psoriasis is a persistent chronic disease with no known cause or cure. This study aimed to estimate oxidative stress and glutathione systems, and their association with factors (age, gender, disease severity, and geographical location) in psoriasis patients.

Material and methods. The study was carried out in the Al-Fayhaa and the Basrah Teaching Hospitals. The number of patients was 45 with 45 in the control group. We quantified the amounts of malondialdehyde (MDA), protein carbonyl (PC), 8-hydroxyguanosine, glutathione, glutathione reductase (GR), glutathione peroxidase (GPx), and selenium.

Results. The results showed significant differences in all variables at multiple statistical levels ($p<0.05$, $p<0.01$, $p<0.001$). The study found significant differences between two groups within the allowed concentration range. Some inter-factor fluctuations were found, and these fluctuations were noticeable in age and sex, and not significant in disease severity or location. The patients did not experience oxidative stress due to the oxidation of lipids and proteins, but rather DNA oxidation.

Conclusion. Lipid peroxidation or protein oxidation do not correlate with psoriasis. While a marker for DNA oxidation exists, it yields different results in psoriasis patients compared to healthy individuals. We observed a correlation between MDA, GR and PC, GPx, PC, and selenium, which serves as the cofactor of the GPx enzyme.

Keywords. glutathione system, Iraq, oxidative stress, psoriasis

Introduction

Psoriasis is a persistent, chronic disease for which there is no known cause or treatment. It is believed that there is a defect in the immune system that attacks itself.[1] While some studies have suggested that skin infiltration is the cause, others have indicated that intestinal infiltration is involved in stimulating the immune system.[2-4] Psoriasis is characterized by red, inflamed spots topped with white scales, which frequently cause itching and appear on the elbows, knees, chest, and scalp. It also has multiple forms.[5] Psoriasis is considered a quite common skin disease, and its prevalence varies by age, sex, geographical area, and surroundings.[6] Psoriasis is prevalent in children (0–2.1%) with an incidence of 40.8 cases per 100,000 people; its prevalence in adults (0.91–8.5%) has an incidence of 78.9-230 cases per 100,000 people.[7] In Iraq, outbreaks of psoriasis range from 0.5% to 0.7%. Psoriasis affects patients' quality of life, as most suffer from feelings of depression and shyness due to their condition.[8] Research also indicates that psoriasis frequently coexists with cardiovascular disease, diabetes, and obesity, extending beyond the skin.[9] It is believed that multiple, mostly genetic, factors exacerbate psoriasis. One of these factors is stress, having co-developed with psoriasis.[10,11] However, non-genetic factors, such as infections, bac-

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terial imbalance in the skin and intestines, lipid metabolism disorders, sex hormone imbalances, and mental illness can stimulate the onset and recurrence of psoriasis in genetically predisposed individuals.[12,13] Other environmental factors such as skin trauma, unhealthy lifestyles, and medications, can also cause psoriasis.[14] Accordingly, many theories have attempted to explain the pathophysiology of psoriasis by investigating the role of oxidation and antioxidants in the exacerbation of psoriasis.[15,16] Oxidative stress refers to an imbalance between levels of reactive oxygen species (ROS) and nitrogen free radicals on the one hand and the antioxidant defense system on the other hand.[17] Since the skin is more exposed to environmental factors, being a source of free radicals, it counters microorganisms and differentiates cells when they are at low concentrations.[18-20] When free radicals increase in the body, they participate in lipid oxidation, cell protein degradation, DNA alteration, programmed cell death, and tissue injury. All these alterations jointly trigger the initiation and intensification of psoriasis.[21,22] Therefore, to evaluate the involvement of oxidative stress in the exacerbation of psoriasis, we examined the status of lipid oxidation, cell protein degradation, DNA oxidation, as well as the effect of the glutathione antioxidant system.[23-26] Moreover, studies have held that glutathione is crucial in supporting tissue repair and regeneration, which is essential for maintaining skin elasticity, and the investigation of the associations between oxidation balance and reduction following age, sex, disease severity, and geographical location in both patients and the control group.[27,28]

Aim

Estimate oxidative stress and glutathione systems, their association with factors (age, gender, disease severity, and geographical location), and their effects on psoriasis patients.

Material and methods

Study population

This is a case-control study that was conducted at Basrah College of Education for Pure Sciences, Department of Biochemistry, Basrah, Iraq, from March 2024 to July 2024, a ninety-participant sample was chosen; we randomly selected 45 psoriasis patients as cases and 45 healthy individuals as controls, with both groups being matched in age and sex. Psoriasis patients often visit the dermatology clinic at both Al-Faihaa Teaching Hospital and the Basrah Teaching Hospital for consultations or routine check-ups. We collected blood serum early in the morning after an eight-hour fast. All subjects gave their informed consent for inclusion before they participated in the study. The Ethics Committee approved the protocol on 7/1/2024, Issue 12, and we conducted the study in accordance with the Declaration of Helsinki.

Criteria of exclusion

Patients with liver disease, hypertension, diabetes, kidney disease, tumors, heart disease, and thyroid disease were excluded. Patients undergoing gastric bypass surgery were excluded. Patients who were younger than 13 years and older than 70 years were excluded. In addition, we excluded patients with any other type of skin disease, smoking, or other diseases. The control group also excluded any chronic disease. During morning hours in the hospital, the patients and the controls were requested to fill a questionnaire containing their demographical data., i.e., age, and gender.

Sample collection

Three milliliters of venous blood were drawn by syringe, and the blood samples were placed into tubes containing a clotting-inducing gel. Then, the tubes were left for half an hour and transferred to a 3000-rpm centrifuge for 10 minutes. The serum was divided into five sections, each placed in a Pendrov tube. The tubes were frozen at -20 °C pending analysis avoiding serum re-thawing.

Laboratory tests

We measured the concentrations of malondialdehyde ((MDA), REF:YBS-16322), protein carbonyl ((PC), REF:YBS-10911), 8-hydroxy-deoxyguanosine ((8-OHdG), LOT:202406), reduced glutathione ((GSH), REF:YBS-11265), oxidized glutathione ((GSSG), REF:YBS-12563), glutathione peroxidase ((GPx), LOT:202406), glutathione reductase ((GR), REF:YBS-11277), and SELENBP1 (REF:YBS-14855) using a human-adaptable ELISA kit provided by Shanghai Ideal Medical Technology Co., Ltd.[29] Furthermore, we measured absorbance at 450 nm. We used a BioTek (USA) 800TS microplate reader and constructed a standard curve of optical density versus concentration using dilutions specified in the flask of each kit. Then, we measured the concentrations of the obtained samples against the standard curve, which has an analysis-specific detection range.

Statistical analysis

The current study used SPSS version 25 (IBM, Armonk, NY, USA) for statistical analysis; we extracted the results using descriptive statistics such as mean, standard deviation (SD), and percentages, as well as one-way ANOVA analysis and the Kruskal-Wallis test. We applied Pearson's correlation coefficient to evaluate the correlation coefficient (r-value), and p values less than 0.05 were considered significant.

Study design

Study design is presented in Figure 1.

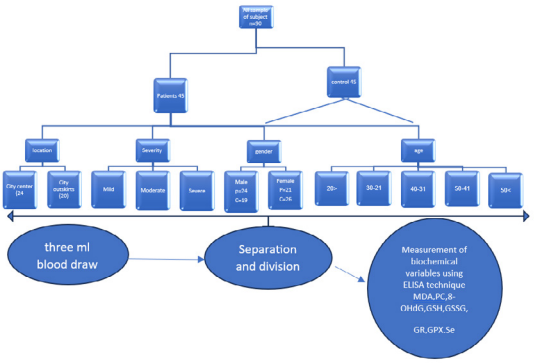

Fig. 1. Research design

Results

Table 1, which displays the demographic data and biochemical variables of the participants, indicates no statistically significant differences in the numbers of the patients and the controls, as male patients exceeded 50%. Also, the results showed more statistically significant differences in healthy people than in the patients for each variable, namely in MDA, GSSG, and GR. Moreover, the results indicated statistically significant differences in 8-OHdG and GPx in which these variables were higher in the patients than in the controls. However, these results showed no significant differences in PC, GSH, and Se levels.

Table 1. Demographic data and biochemical variables of study participants*

p	Mean±SD		Variables	
	Patients (n=45)	Controls (n=45)		
NS	33.71±13.507	32.91±12.236	Age (years)	
NS	24 (53.3%)	19 (42.2%)	Male	Gender
NS	21 (46.6%)	26 (57.7%)	Female	
0.002	1.297±3.035	1.190±3.84	MDA (0.5–8) nmol/mL	
NS	1.467±6.663	1.520±6.06	PC (0.5–8) nmol/mL	
0.001<	8.666±54.257	8.814±43.741	8-OHdG (6.25–100) ng/L	
NS	2.334±13.154	3.280±13.586	GSH (1.25–20) µmol/L	
0.001<	67.965±537.172	109.464±630.032	GSSG (150–2400) nmol/L	
0.001<	31.010±97.197	41.595±129.395	GR (10–160) U/mL	
0.013	53.661±224.706	52.282±194.785	GPx (10–160) U/L	
NS	0.683±3.725	0.861±3.679	Se (0.5–8) ng/ml	

* NS – not significant

To reveal the effect of age on serum variables, participants were divided into five age groups, as shown in Table 2. The study revealed fluctuations in MDA and PC levels, statistically significant at p<0.01, in patients and controls aged 31–40 years and different levels in 8-OHdG in most age groups shown in the table. Similarly, differences related to GSSG were observed in individuals aged 31–50 years with the same significance as the previous levels, and a decrease in GR level was observed in patients with a difference of p<0.01 at ages (31–40) and an increase in GPx level in patients with a level of p<0.01 at ages (21–30) years. No distinction was observed between the five age groups with regard to the remaining variable.

Table 2. Levels of variables according to age (Kruskal-Wallis test)*

Variables		Mean rank		p
		Controls (n=45)	Patients (n=45)	
MDA (nmol/mL)	<20	10.17	7.50	NS
	21–30	16.04	12.11	NS
	31–40	16.08	8.27	<0.01
	41–50	10.13	6.88	NS
	>50	6.20	5.83	NS
PC (nmol/mL)	<20	11.67	6.60	NS
	21–30	11.77	16.07	NS
	31–40	8.38	17.36	<0.01
	41–50	7.69	9.31	NS
	>50	6.00	6.00	NS
8-OHdG (ng/L)	<20	11.10	4.17	<0.01
	21–30	8.62	19.0	<0.001
	31–40	9.23	16.36	0.05
	41–50	6.50	10.50	NS
	>50	3.80	7.83	<0.05
GSH (µmol/L)	<20	8.00	8.80	NS
	21–30	12.62	15.29	NS
	31–40	11.31	13.91	NS
	41–50	8.88	8.13	NS
	>50	8.00	4.33	NS
GSSG (nmol/L)	<20	7.83	8.90	NS
	21–30	16.46	11.71	NS
	31–40	16.46	7.82	<0.01
	41–50	11.44	5.56	<0.05
	>50	8.20	4.17	<0.05
GR (U/mL)	<20	8.50	8.50	NS
	21–30	17.00	11.21	NS
	31–40	16.38	7.91	<0.01
	41–50	10.50	6.50	NS
	>50	8.10	4.25	NS
GPx (U/L)	<20	7.17	9.30	NS
	21–30	8.23	16.12	<0.01
	31–40	8.50	13.27	NS
	41–50	4.00	7.14	NS
	>50	6.80	5.33	NS
Se (ng/mL)	<20	7.50	9.10	NS
	21–30	12.42	15.46	NS
	31–40	14.62	10.00	NS
	41–50	8.75	8.25	NS
	>50	6.40	5.67	NS

* NS – not significant

The results in Table 3 show significant differences between males in the two groups (p<0.01) at MDA and (p<0.05) at PC. Differences appear at 8-OHdG at a significance level (p<0.01) and at GSSG with a significant difference in favor of controls at a level (p<0.001). For GPx, differences appeared in male patients at a statistical lev-

el ($p<0.05$). No significant differences were recorded in GSH and Se in males. As for females from the two groups, no significant differences appeared in MDA, PC, GSH, GSSG, and Se. While significant differences were found at 8-OHdG at a significance level ($p<0.001$). A significant increase in GR was observed in female controls ($p<0.05$) and a significant increase in GPx in female patients ($p<0.05$).

As Table 4 shows, no statistically significant differences in all variables between female and male patients have been detected.

Table 3. Level of variables in the study community according to gander (Kruskal-Wallis test)*

Variables		Mean rank		p
		Controls (n=45)	Patients (n=45)	
MDA (nmol/mL)	Male	30.47	19.61	<0.008
	Female	27.13	20.12	NS
PC (nmol/mL)	Male	18.55	27.70	<0.025
	Female	22.79	25.50	NS
8-OHdG (ng/L)	Male	16.89	28.82	<0.003
	Female	16.23	33.62	<0.001
GSH (µmol/L)	Male	25.18	23.20	NS
	Female	22.15	26.29	NS
GSSG (nmol/L)	Male	33.92	17.27	<0.000
	Female	27.12	20.14	NS
GR (U/mL)	Male	32.47	18.25	<0.001
	Female	27.60	19.55	<0.045
GPx (U/L)	Male	15.15	23.71	<0.033
	Female	16.41	27.66	<0.003
Se (ng/mL)	Male	22.79	24.82	NS
	Female	25.23	22.48	NS

* NS – not significant

Table 4. Levels of variables according to gender*

p	Mean±SD		Variables
	Female (n=21)	Male (n=24)	
NS	1.403±2.988	1.236±3.070	MDA (nmol/ml)
NS	1.483±6.726	1.480±6.616	PC (nmol/ml)
NS	10.875±55.246	6.677±53.515	8-OHdG (ng/L)
NS	2.403±13.659	2.250±12.776	GSH (µmol/l)
NS	56.695±555.510	73.302±523.418	GSSG (nmol/L)
NS	25.840±98.731	34.809±96.047	GR (U/ml)
NS	52.506±244.115	51.146±211.535	GPx (U/L)

* NS – not significant

In Table 5, the patient groups were divided according to how severe the disease is per the topical spread of psoriasis. Thus, the patients were subdivided into three groups; mild, moderate, and severe. No statistically significant differences have been noticed between the three groups in any variable.

In Table 6, the patient groups were subdivided into two groups following their places of residence (urban or suburban) and the questions answered by the patients. No significant differences regarding geographical location were noticed among the groups.

Table 5. Levels of variables according to disease severity*

Variables		Mean±SD	p	Variables		Mean±SD	p
MDA (nmol/mL)	Mild	0.707±2.494	NS	GSSG (nmol/L)	Mild	72.594±544.779	NS
	Moderate	1.349±2.874			Moderate	56.075±538.146	
	Severe	1.349±3.328			Severe	76.270±533.947	
PC (nmol/mL)	Mild	1.570±6.246	NS	GR (U/mL)	Mild	20.325±85.206	NS
	Moderate	1.313±7.250			Moderate	28.321±95.802	
	Severe	1.467±6.386			Severe	35.295±102.182	
8-OHdG (ng/L)	Mild	11.204±50.713	NS	GPx (U/L)	Mild	56.259±232.324	NS
	Moderate	7.446±52.713			Moderate	43.768±223.716	
	Severe	8.119±56.822			Severe	60.524±223.143	
GSH (µmol/L)	Mild	2.984±13.928	NS	Se (ng/mL)	Mild	0.417±3.926	NS
	Moderate	1.567±13.289			Moderate	0.885±3.655	
	Severe	2.576±12.801			Severe	0.599±3.708	

* NS – not significant

Table 6. Levels of variables according location*

p	Mean±SD		Variables
	City outskirts (n=20)	City center (n=24)	
NS	1.301±3.004	1.317±3.056	MDA (nmol/mL)
NS	1.311±6.786	1.582±6.578	PC (nmol/mL)
NS	10.593±53.984	7.247±54.444	8-OHdG (ng/L)
NS	2.446±12.849	2.274±13.365	GSH (µmol/L)
NS	64.366±530.545	71.094±541.742	GSSG (nmol/L)
NS	28.747±93.131	32.063 ±100.001	GR (U/mL)
NS	64.934±918.585	45.284±222.259	GPx (U/L)
NS	0.881±3.599	0.504±3.812	Se (ng/mL)

* NS – not significant

The study found a positive age-gender correlation at MDA, GSSG, and GR. Also, the study revealed a negative correlation at 8-OHdG and GPx with age and a positive correlation between 8-OHdG and disease severity. Table 7 shows significant differences in the correlation coefficient for other variables.

Table 7. Pearson correlation coefficient for biochemical variables and other relevant variables

Location		Severe		Gender		Age		Variables
p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	
NS	0.200-	NS	0.258	0.012	0.258	0.002	0.311	MDA (nmol/mL)
NS	0.071	NS	0.063-	NS	0.155-	NS	0.199-	PC (nmol/mL)
NS	0.026-	0.044	0.289	0.000	0.522-	0.000	0.519-	8-OHdG (ng/L)
NS	0.110-	NS	0.175-	NS	0.100	NS	0.077	GSH (µmol/L)
NS	0.082-	NS	0.057-	0.000	0.360	0.000	0.461	GSSG (nmol/L)
NS	0.110-	NS	0.194	0.002	0.316	0.000	0.407	GR (U/mL)
NS	0.095-	NS	0.050-	NS	0.157-	0.013	0.272-	GPx (U/L)
NS	0.155-	NS	0.082-	NS	0.065	NS	0.030-	Se (ng/mL)

* NS – not significant

Table 8 indicates a direct correlation between MDA-GR, PC-GPx, and PC-Se. Additionally, the correlation between (8-OHdG) and antioxidants from the glutathione system showed no significant differences.

Table 8. Pearson correlation coefficient for oxidative stress and antioxidant levels of the glutathione system*

Se		GPX		GR		GSSG		GSH		Variables
p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	
NS	0.151	NS	0.043-	0.045	0.208	NS	0.178	NS	0.062	MDA
0.042	0.210	0.026	0.246	NS	0.143-	NS	0.023-	NS	0.055	PC
NS	0.120	NS	0.165	NS	0.021-	NS	0.015	NS	0.017	8-OHdG

* NS – not significant

Figures 2 to 4 show us that there is a positive relationship in the glutathione system.

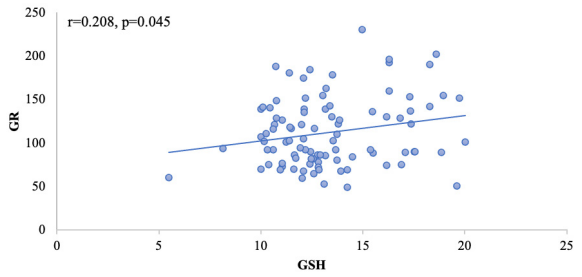

Fig. 2. Correlation between GSH and GR in psoriasis patients

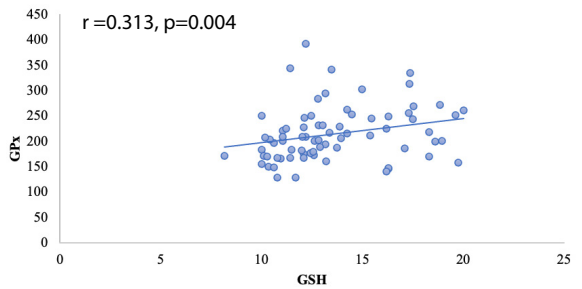

Fig. 3. Correlation between GSH and GPx in psoriasis patients

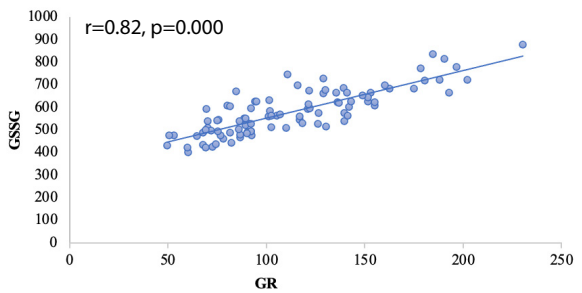

Fig. 4. Correlation between GSSG and GR in psoriasis patients

Discussion

MDA is one of the final oxidation products of unsaturated fats in cells. As free radicals trigger MDA production, it is a sign of oxidative stress.[30] The apparent results showed that psoriasis patients do not suffer from high fat oxidation, and these results were consistent with another study.[31] A positive association was observed between (MDA-GR) and age. The GR enzyme is important in renewing reduced glutathione in the detoxification of peroxides. Obviously there is a balance between antioxidants and oxidation as patients were found to have not suffered from fat oxidation. Hence, this study is consistent with another study.[32] Also, there is a gradual increase in PC in patients as it increases at 31–40 year-old patients, which indicates statistically significant differences. In other words, psoriasis patients suffer from protein oxidation. A previous study conducted in Basrah, Iraq reported similar findings with this study, having indicated a positive association between (PC-PX) and (PC-Se).[33] Likewise, other studies have confirmed that the presence of GPx reduces the amount of protein carbonyl compounds treated with oxidative stress-generating factors.[34] There are four main forms of GPx, with GPx1 depends mainly on selenium.[35] In the same view, results showed that PC was positively associated with both GPX and Se. Additionally, the results revealed significant differences as an increase in 8-OHdG in patients. This is one of the dominant forms of DNA in mitochondria and is considered a sign of oxidative stress that causes DNA damage.[36] It is also a good biomarker for assessing the risk of developing various types of cancer.[37] A previous study that agrees with the results of the current study that showed that the level of 8-OHdG can be considered a useful biomarker for early detection of psoriasis.[38] Another study conducted at the University of Basra evaluated 8-OHdG in saliva and found it to be a suitable sample for diagnosing and identifying many diseases.[39] The results showed that the level of 8-OHdG in female patients is higher than in males. The control group included more males than females, prompting us to consider the impact of female hormones on the level of oxidation, as noted in a study.[40] It also found no correlation between 8-OHdG and glutathione antioxidants, supporting another study.[41] A meta-analysis of 298 original articles found that several polymorphisms in genes encoding markers or enzymes related to redox homeostasis influence the interaction between psoriasis and oxidative stress.[31] The study demonstrated increased levels of oxidized DNA/RNA molecules in the serum of patients with exacerbated psoriasis vulgaris. Sex, the presence of metabolic syndrome, or cigarette smoking minimally influenced the results. In the psoriatic blood cells' DNA, the authors observed longer telomeres compared to healthy controls, particularly in females. The psoriasis cases exhibited marginal clinical importance due to the marginally higher global DNA methylation in their DNA compared to the controls.[42] UV radiation also leads to DNA damage, generating immune-stimulatory DNA motifs, such as 8-hydroxyguanosine.[43] Furthermore, we noticed a positive correlation between GSH-GR and GSH-GPx, and according to the

mechanism of glutathione's action in the body, it is certain that there is a positive correlation between them.[44] The results show that the glutathione system is effective in psoriasis patients, despite the apparently significant differences. The results agree with a study of the glutathione system on psoriasis patients, but they are within the limits of measurement, i.e., they do not threaten the patient with glutathione system dysfunction-associated diseases.[45,46] What is questionable is that in most chronic diseases, oxidative stress values increase with increasing severity of the disease.[47] However, in psoriasis, we observed stability and relatively negligible fluctuations in the level of the variables in relation to the severity of the disease, which prompts us to expect stability in the oxidation system and antioxidants in psoriasis patients. The study also found that the participants' geographical locations had no significant effect on the relevant variables, which could be attributed to the lack of different eating styles in both groups and that most rural residents living near urban communities have embraced city-like norms. We recommend continuing this research to confirm the importance of the studied variables, as 8-OHdG may be a risk indicator for psoriasis patients, and the results may appear different depending on the measurement method, as the accuracy of the results cannot be completely confirmed.

Study limitations

Even though we eliminated numerous samples due to their unsuitability for analysis or delays in storage, we cannot ensure the validity of all the samples under study. This is because data from the patient and control groups, as well as from the researcher, play a crucial role. We also employed several research measurements specific to ELISA, and given exposure to poor storage and transportation conditions, may have yielded varying results.

Conclusion

To some extent, psoriasis patients suffer from oxidative stress. Moreover, psoriasis is not related to lipid peroxidation or protein oxidation. While there is a marker for DNA oxidation, it has different results in psoriasis patients than in healthy individuals. We observed a correlation between MDA-GR and PC-GPx, as well as a correlation between PC and selenium, which serves as the cofactor of the GPx enzyme. However, we found no correlation between the glutathione system and DNA oxidation, indicating that the glutathione system does not influence the latter. The results of the analyses showed no significant differences as far as disease severity and geographical location are concerned.

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Declarations

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Author contributions

Conceptualization, A.A. and S.S.; Methodology, A.A.; Software, A.A.; Validation, A.A. and S.S.; Formal Analysis, A.A.; Investigation, S.S.; Data Curation, A.A.; Writing – Original Draft Preparation, S.S.; Writing – Review & Editing, A.A.; Visualization, A.A.; Supervision, S.S.; Project Administration, A.A.; Funding Acquisition, S.S.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The data that support the findings of this study are available from Hassan A.A. but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Hassan A.A.

Ethics approval

The Ethics Committee approved the protocol on 7/1/2024, Issue 12, and we conducted the study in accordance with the Declaration of Helsinki.

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ORIGINAL PAPER

Identification of bacterial isolates in urinary tract infections patients of Basrah province

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ABSTRACT

Introduction and aim. Urinary tract infections (UTIs) are among the most common bacterial diseases worldwide that are caused primarily by members of the Enterobacteriaceae family. This study aimed to identify the most frequent bacterial agents associated with UTIs and analyze their patterns of antibiotic resistance using the Vitek®2 system.

Material and methods. The study included 200 urine samples collected from adult UTI patients of both sexes.

Results. The characterization of bacterial isolates revealed the following distribution: *Escherichia coli* (35 isolates, 50%), *Staphylococcus aureus* (18 isolates, 25.7%), *Klebsiella pneumoniae* (5 isolates, 7.14%), *Staphylococcus* spp. (4 isolates, 5.7%), *Streptococcus* spp. (3 isolates, 4.2%), *Pseudomonas* spp. (3 isolates, 4.2%), and *Proteus mirabilis* (2 isolates, 2.86%).

Antibiotic resistance testing showed that ceftazidime had the highest resistance rate (88.57%), while amikacin had the lowest (17.14%). Additionally, extended-spectrum β-lactamase (ESBL) production was detected in 35 *E. coli* isolates. Of these, 22 isolates (62.86%) tested positive for ESBL production, while 13 isolates (37.14%) were negative.

Conclusion. This study concluded that *E. coli* is the most prevalent bacterial species causing UTIs. Furthermore, the *E. coli* isolates demonstrated a high capacity for ESBL production, highlighting the need for effective antimicrobial management and monitoring.

Keywords. antibiotic susceptibility, *E. coli*, ESBLs

Introduction

Urinary tract infections (UTIs) are inflammation of the urinary tract epithelium resulting from invasion by microorganisms.[1] It is an important and common disease affecting men and women of all ages.[2,3] UTIs are among the most common bacterial infections, affecting more than 150 million people annually.[4] *Escherichia coli*, a natural gut flora in humans, causes 70–95% of UTIs. Germs enter the urinary tract.[5] Uropathogenic Escherichia coli (UPEC) has the ability to penetrate the deeper layers, develop quiescent intracellular reservoirs, and remain there for months at a time before recurring infections.[6] UTIs can be caused by obstructions or defensive factors, such as urinary incontinence, retention, immunosuppression, renal failure, pregnancy, or the use of indwelling catheters.[7] Identifying UPEC strains based on their virulence factor-encoding genes and their function in illness progression is possible.[1] UPEC is caused by a variety of components, including secreted proteins, hemolysins, capsules, lipopolysaccharides, biofilm, fimbriae adhesions, and iron acquisition mechanisms.

The best type of medication for treating pathogenic bacteria is an antibiotic. When treating infections caused by Gram-negative bacteria, fluoroquinolones, cephalosporins, β-lactams, and β-lactamase inhibitors are often prescribed, alone or in combination.[8] One ma-

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jor problem threatening the global healthcare system is multidrug resistance (MDR). Usually, the widespread Enterobacteriaceae family is linked to the problem. Extended-spectrum β-lactamases (ESBLs) are the main antimicrobial resistance mechanism in this family of bacteria that renders β-lactam antibiotics ineffective.[9] Gram-negative bacteria can generate enzymes that hydrolyze β-lactam rings, making them resistant to one of the most effective medications.[10] ESBLs, which hydrolyze penicillins, monobactams, and cephalosporins, were first discovered in Germany in 1983. Since then, the number of diseases caused by bacteria with this resistance mechanism has grown. Furthermore, *E. coli* has evolved resistance to several antibiotics, leading to a major cause of illness. Multidrug-resistant *E. coli* bacteria, especially those producing ESBLs, pose a significant hazard to public health.

Aim

The current study aimed to detect the most frequent bacterial agents associated with UTIs and to analyze the pattern of antibiotic resistance using the Vitek®2 system test.

Material and methods

Urine samples were collected from Al Basrah General Teaching Hospital, Al Mawani General Teaching Hospital, and Al Sadir Teaching Hospital in Basrah Province between October 2023 and March 2024. A total of 250 individuals suspected of having UTIs, aged between 20 and 60 years, provided clinical samples. After collection, urine samples were centrifuged and cultured on blood agar, MacConkey agar mannitol salt agar, and eosin methylene blue agar. Biochemical tests were then performed for the identification of Gram-positive bacteria. This study was conducted in accordance with the Declaration of Helsinki and received ethical approval No. (773) dated (10/3/2024) from the Basra General Health Directorate.

Identification by Vitek®2 system

The Vitek®2 system (bioMérieux, France) was used to identify bacterial species with high accuracy. The system includes 64 biochemical tests for bacterial diagnosis.[11]

Bacterial DNA extraction

Genomic DNA was isolated from bacterial isolates using the Wizard® Genomic DNA Purification Kit (Promega, USA).

Detection of 16S rDNA

The extracted bacterial DNA was amplified using PCR to target the 16S rDNA gene with a specific primer approximately 585 bp in length (Table 1). The primer sequence for 16S rDNA was used as described previously.[12] A standard molecular DNA ladder (2000 bp) was used to compare the PCR results.

Table 1. The bacterial 16S rDNA gene was amplified by PCR using specific primers

Primer		Sequence of primer	Length (bp)	Product (bp)
16SrDNA	Forward	5-GAC CTC GGT TTA CTT CAC AGA-3	21	585
16SrDNA	Revers	5-CAC ACG CTG ACG CTG ACC-3	18	

Reagents

The reagents and their volumes used in PCR amplification are described in Table 2.

Table 2. Reagents used in PCR amplification of 16SrDNA

No.	Reagent	Volume
1	Genomic DNA	1 µL
2	Forward primer	1 µL
3	Reverse primer	1 µL
4	Master Mix	12.5 µL
5	Nuclease-free	water 9.5 µL
Total volumes 25 µL		

Thermal cycling condition: The program is described in Table 3.

Table 3. Program used in PCR amplification

Steps	Temperature	Time	No. of cycles
Initial denaturation	94°C	4 min	1
Denaturation	94°C	90 sec	30
Annealing	62°C	90 sec	
Extension	72°C	2 min	
Final extension	2°C	7 min	1

Procedure

Approximately 5 µL of a 2000 bp DNA ladder and 5 µL of 16S rDNA amplicons were subjected to gel electrophoresis for 45 minutes at 70 V using a casting tray with 1.5% agarose gel prepared in 1×TBE buffer containing 0.2 µL of ethidium bromide. The products were visualized under a UV light system.

Antibiotic susceptibility test by Vitek®2 system

The antibiotic susceptibility test was performed using the Antibiotic Susceptibility Kit Card (Vitek®2 AST, reference number 413083, bioMérieux, France). This kit includes a comprehensive range of antimicrobial tests.

Detection of extended spectrum β-lactamase (ESBL)

Double disk approximation method (DAM)

Bacterial isolates, prepared as previously described, were spread onto Mueller-Hinton agar plates. A disk of Augmentin (amoxicillin-clavulanic acid) was placed at the center of the agar surface. Disks of ceftazidime (30 µg), cefotaxime (30 µg), amikacin (30 µg), and ceftri-

axone (30 µg) were positioned around it at a distance of approximately 20 mm (center to center). After incubation at 37°C for 24 hours, bacteria were considered ESBL producers if the zone of inhibition around aztreonam or any of the antibiotic disks showed a clear-cut increase directed toward the Augmentin disk.[13]

Results

The present study included 100 patients with UTIs. The distribution of UTIs patients according to sex is shown in Figure 1. In this study, the proportion of female patients with UTIs was significantly higher than that of male patients, with 78 females and 22 males.

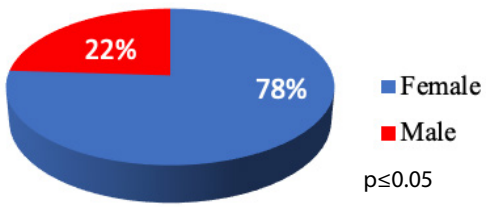

Fig. 1. Distribution of patients according to sex

The current data on the distribution of UTIs patients based on marital status showed that 28% were single and 72% were married, with a statistically significant difference (p≤0.05) (Fig. 2).

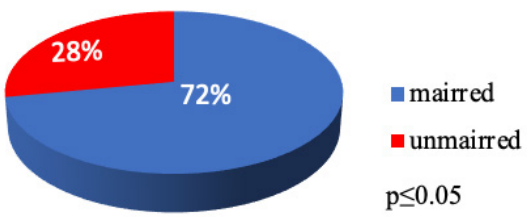

Fig. 2. Distribution of UTIs patients according to the marital status

The present work detected uropathogenic *E. coli* (35 isolates, 50%), *S. aureus* (18 isolates, 25.71%), *K. pneumoniae* (5 isolates, 7.14%), *Staphylococcus* spp. (4 isolates, 5.71%), *Streptococcus* spp. (3 isolates, 4.29%), *Pseudomonas* (3 isolates, 4.29%), and *Proteus mirabilis* (2 isolates, 2.86%), as shown in Figure 3, with significant differences (p ≤ 0.05).

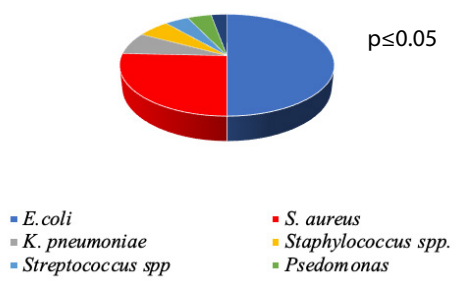

Fig. 3. Frequency of bacterial isolates in UTIs patients

The current investigation utilized the coagulase test, catalase test, mannitol salt agar, blood agar, and Gram's stain to identify Gram-positive bacteria (Table 4).

Table 4. Gram-positive bacteria are identified by biochemical tests

Type of isolate	Type of Test				
	Gram's stain	Catalase test	Fermentation of mannitol	Coagulase test	Isolates number
Staphylococcus aureus	+	+	+	+	18
Staphylococcus spp.	+	+	-	-	4
Streptococcus spp.	+	-	*N	N	3
Total			25		

* N – not detectable

The present work indicated that the percentages of Gram-negative bacteria identified by the Vitek®2 system were the following: *E. coli* (35 isolates, 50%), *K. pneumoniae* (5 isolates, 7.14%), *Pseudomonas* (3 isolates, 4.29%), and *Proteus mirabilis* (2 isolates, 2.86%).

The 35 *E. coli* isolates were examined, and genomic DNA was extracted using the kit protocol method. The presence of genomic DNA was confirmed by gel electrophoresis on a 0.8% agarose gel, with the results visualized under UV light (Fig. 4).

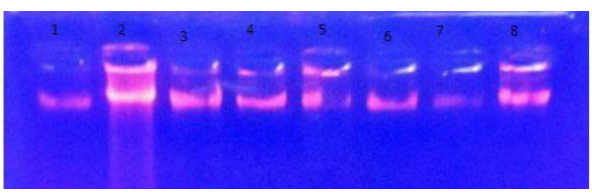

Fig. 4. Total genomic DNA extracted from *E.coli* isolates, using 0.8% agarose gel, 70V, 45 min

PCR was used to amplify the 16S rDNA from the extracted DNA. The individual 16S rDNA band (585 bp) was compared to the standard molecular DNA ladder (2000 bp) (Fig. 5).

In this study, the Vitek®2 system was used to detect and confirm antibiotic susceptibility tests, as shown in Table 5 and Figure 4.

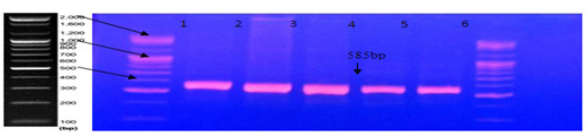

Fig. 5. PCR amplified products of 16S rDNA are seen in agarose electrophoresis patterns. Lane 1: 2000 base pair DNA ladder; Lane 2: 16S rDNA band of *E. coli* isolates,. using 70V, 45 min, 1.5% agarose gel

The Vitek®2 system was used to detect the ability to produce extended-spectrum β-lactamases (ESBLs) in 35 *E. coli* isolates. The results showed that 22 (62.86%)

E. coli isolates tested positive for the ability to produce ESBLs, while 13 (37.14%) *E. coli* isolates tested negative (Fig. 7).

Table 5. Sensitive testing against different species of gram-negative bacteria and detection of ESBLs

Antibiotic group	Antimicrobial agent	*E. coli*			*K. pnemoa*		*P. mirabilis*		*P. aerogiosa*	
		R	S	I	R	S	R	S	R	S
β-lactam combinations	Ampicilli/ sulbactam (SAM)	19 54.29%	10 28.57%	6 17.14%	4 80%	1 20%	2 100%	-	-	-
	Piperacillin/ Tazobactam(PIT)	18 51.43%	11 31.43%	6 17.14%	5 100%	-	2 100%	-	2 66.7%	1 33.3%
Cephalosporins (II, III, IV)	Cefuroxime (CFX)	21 60%	5 14.29%	9 25.71%	4 80%	1 20%	--	2 100%	2 66.7%	1 33.3%
	Cefuroximeaxetil (CFA)	21 60%	14 40%	-	4 80%	1 20%	-	2 100%	20%	80%
	Cefoxitin (FOX)	26 74.29%	9 25.71%	-	-	5 100%	-	2 100%	-	100%
	Cefixme (CXM)	23 65.71%	12 34.29%	-	-	5 100%	-	2 100%	1 33.3%	2 66.7%
	Ceftazidime (CAZ)	31 88.57%	4 11.43%	-	1 20%	4 80%	-	2 100%	1 33.3%	2 66.7%
	Ceftriaxone (CRO)	21 60%	14 40%	-	1 20%	4 80%	-	2 100%	1 33.3%	2 66.7%
	Cefepime (CPM)	28 80%	7 20%	-	-	5 100%	-	2 100%	1 33.3%	2 66.7%
β-lactam carabapenem	Ertapenem (ETP)	12 34.29%	23 65.71%	-	4 80%	1 20%	-	2 100%	3 100%	-
	Meropenem (MEM)	11 31.43%	24 68.57%	-	1 20%	4 80%	-	2 100%	1 33.3%	2 66.7%
Aminoglycoside	Amikacin (AMK)	6 17.14%	29 82.86%	-	4 80%	1 20%	-	2 100%	2 66.7%	1 33.3%
	Gentamicin (GEN)	7 20%	28 80%	-	2 40%	3 60%	2 100%	-	-	3 100%
Fluroquinolones	Ciprofloxacin (CIP)	24 68.57%	11 31.43%	-	-	5 100%	-	2 100%	3 100%	-
Nitrofuran	Nitrofurantoin (NIT)	14 40%	21 60%	-	-	5 100%	-	2 100%	-	3 100%
Sulfonamides	Trimethoprim sulfamethoxazole (TRI-S)	20 57.14%	10 28.57%	5 14.29%	-	5 100%	-	2 100%	2 66.7%	1 33,3%

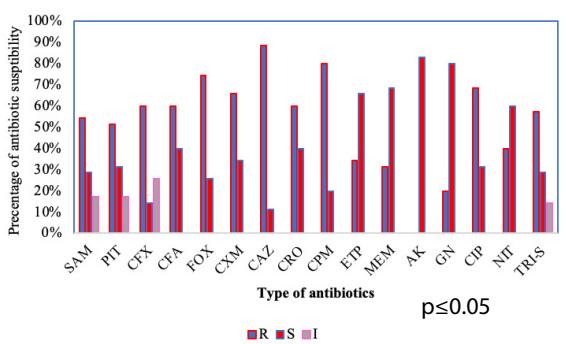

Fig. 6. Antibiotic susceptibility of *E. coli*

Of the 35 *E. coli* isolates, 19 (54.29%) developed ESBLs with positive results, while 16 (45.71%) isolates showed negative results for ESBL production, as determined by the DAM method (Fig. 8).

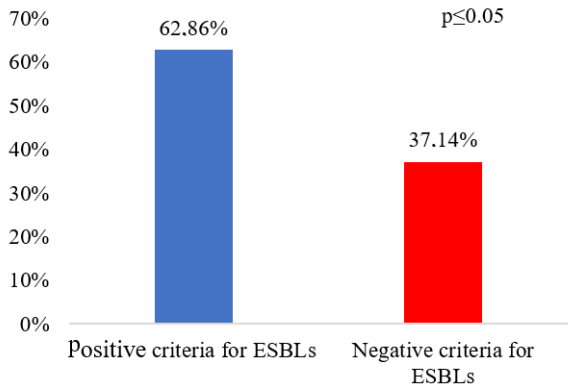

Fig. 7. Demonstrated that positive and negative results of ESBL by *E.coli*

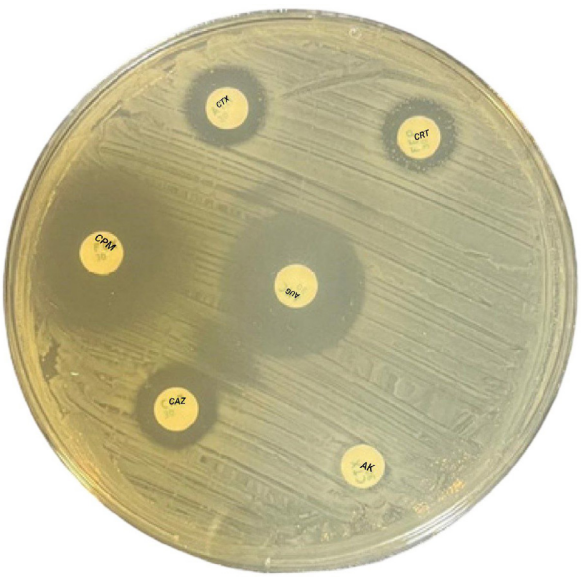

Fig. 8. DAM test for ESBL detection using *E. coli* isolates

Discussion

E. coli spreads in the urinary tract due to its natural habitat in the digestive tract, its proximity to the anus opening near the urinary tract opening, and its virulence factors, which allow it to adhere to surfaces, withstand urine-induced flow, and cause urinary tract infections. The current study revealed that women constituted the majority of cases compared to males, a finding that is consistent with most previous studies. A study by Kamel et al. showed that women represented the highest percentage of UTIs, with 77% of cases in women and 23% in males.[14] This finding is supported by most research due to the anatomical structure of females, where the urethra is shorter than that of males, thus reducing the distance bacteria must travel to reach the bladder.[15]

Current data on the distribution of UTI patients based on marital status showed that 28% were single and 72% were married, which is consistent with the study by Al-Musawi and Al-Husseini.[16] According to Alhamdy and Shani, married women had a higher prevalence and incidence of UTIs, which may be due to hormonal impacts and behavioral habits.[17]

The present study employed several techniques for identifying Gram-positive bacteria, including Gram's stain, microscopic examination, blood agar culture, mannitol salt agar, coagulase test, and catalase test. *Streptococcus* species were catalase-negative, Gram-positive, and arranged in chains, while *Staphylococcus* species appeared as purple clusters resembling grapes. The colony size of *S. aureus* ranged from 1 to 4 mm, spherical, convex, and with sharp boundaries. It was golden yellow in color, Gram-positive when stained, catalase-positive, and surrounded by areas of β-hemolysis on blood agar. Nonmotile clusters of *S. aureus* resembled grapes.[18] Furthermore, *S. aureus* may ferment mannitol when grown on mannitol salt agar, resulting in a positive coagulase production test and changes the indicator's color from red to yellow by producing acid. However, some species of *Staphylococcus* do not change color on mannitol agar and show negative results from the coagulase test.[19] Since Gram-positive bacteria received limited attention in this study, simple methods, such as the color change from red to yellow due to acid production and a positive coagulase test, were sufficient for their identification. Other *species of Staphylococcus,* such as *S. alhilua* and *S. al-shujairib*, grow on mannitol agar without changing color and provide negative coagulase results.[19]

In this study, different bacterial species were identified through biochemical analysis, with *E. coli* accounting for 35 isolates, *S. aureus* for 18 isolates, *K. pneumoniae* for 5 isolates, *Staphylococcus* spp. for 4 isolates, *Streptococcus* spp. for 3 isolates, *Pseudomonas* for 3 isolates, and *P. mirabilis* for 2 isolates. These were the most frequently occurring bacteria in UTIs, which are a prevalent issue affecting both men and women and are typically caused by bacterial infections.[20,22] The current study found that *E. coli* was the most common bacteria isolated from UTIs, accounting for 50% of cases. This is consistent with previous studies from Baghdad, Iraq, which reported an *E. coli* rate of 50%, and from Basrah, which reported a rate of 60%.[20,21] *E. coli* is dominant in UTIs due to its presence in the intestines and its ability to enter the urinary tract. It has virulence factors such as biofilm formation, fimbriae, alpha-hemolysin, cytotoxic necrotizing factor, adhesins, and iron acquisition systems, as well as antibiotic resistance genes. The majority of *E. coli* strains are resistant to β-lactam antibiotics.[22,23] *UPEC* has the ability to penetrate the urothelium's deeper layers, develop quiescent intracellular reservoirs, and remain there for months before causing recurrent infections.[6]

The results of the biochemical identification test, used to identify Gram-negative bacteria in the current investigation, were validated and supported by the Gram-negative identification card of the Vitek®2 System test. Based on the findings from the Vitek®2 System, 35 isolates were identified as *E. coli*, 5 isolates as *K. pneumoniae*, 4 isolates as *Pseudomonas*, and 2 isolates as *P. mirabilis*. Crowley et al. demonstrated that the Vitek®2 system accurately identifies bacteria down to the species level. The study evaluated the technique and concluded that the Vitek®2 GN identification method is a suitable automated method for the rapid identification of Gram-negative bacteria. Additionally, the results of Salumi and Abood demonstrate the high quality and reliability of the Vitek®2 GN technology.[24,25] A study by Pincus also highlighted that Vitek®2 offers an efficient timeframe for detecting isolates without mutations, boasting a high degree of accuracy (99%) and an extremely narrow margin of error. Another study by Ossman et al. confirmed the utility of the Vitek®2 Compact method for identifying bacteria in UTIs samples, combining conventional and biochemical methods.[26] In the current study, the results of Gram-negative bacterial identification by the Vitek®2 system were completely consistent with the primary identification, in addition to saving time and effort, avoiding laboratory errors, and providing more accurate results.

16S rDNA was amplified using PCR with the extracted DNA. A single 16S rDNA band (585 bp) was identified by comparing it to the conventional molecular DNA ladder (2000 bp). The diagnostic gene, 16S rDNA, which is stable and exhibits some heterogeneity over time within bacterial species, was used for molecular diagnostics on all *E. coli* isolates using PCR technology. The results showed that the 16S rDNA gene sequence was present in all isolates at a 100% rate, and the molecular weight of the band was 585 bp when compared to the DNA ladder. The findings of this study are consistent with those of Maleki et al. in Iran and Lai et al.[27,28] The results align with the study conducted by Jenkins et al. in Malaysia.[29] This gene exhibits slight heterogeneity but contains conserved regions that overlap with variable portions, which are used to identify the bacterial genus and species. This makes it one of the most rapid, accurate, and sensitive methods for diagnosing bacteria, with minimal random variation in the genomic sequence over time.[30,31]

This study aims to determine the antibiotic resistance patterns of predominant uropathogens and confirm *E. coli*, the most common Gram-negative bacterium, as a frequent cause of urinary tract infections.[32] Appropriate treatment heavily depends on the proper prescription of both effective and efficacious antibiotics. The development of resistance by pathogenic bacteria has become synonymous with antibiotic use. Antibiotic resistance is a major global threat, particularly rec-

ognized in *E. coli* and other Gram-negative bacteria, making treatment difficult. Determining the resistance patterns of *E. coli* is crucial for guiding empirical and targeted therapy.[34]

The study revealed low resistance rates to aminoglycosides, specifically amikacin and gentamicin. These findings were consistent with prior studies, such as those by Mohamed and Aljanaby and Ayatollahi et al., which demonstrated high sensitivity to these antibiotics.[35,36] Similar observations were noted in research by Al-Khikani et al. regarding gentamicin. Aminoglycosides exert their action by binding to the 30S ribosomal subunit of bacteria, disrupting protein synthesis, and either terminating protein synthesis prematurely or incorporating the wrong amino acids.[37] Aminoglycosides are bactericidal, displaying concentration-dependent bacterial killing. However, it is still not fully understood whether there is an additional mechanism of action involved.

Carbapenem resistance was observed at varying levels across studies. The current study found a resistance rate of 34.8%, which is in agreement with Mujahid et al.[38] However, lower resistance rates of 2.4% were reported by Jalil and Atbee.[39] These variations could be attributed to regional differences in antibiotic usage and resistance monitoring practices.

The resistance to trimethoprim-sulfamethoxazole was 40%, as also observed by Critchley et al.[40] The rise in resistance is fueled by the easy access to antibiotics without prescriptions, combined with a lack of knowledge, particularly in communities with low incomes. Educational programs focusing on accurate disease diagnosis, selecting the right antibiotics, and promoting responsible antibiotic use can greatly help reduce resistance. Ahmed and Andaleeb et al. also found high resistance rates against β-lactam and second- to fourth-generation cephalosporin antibiotics.[41,42] The development of resistance mechanisms involves the production of β-lactamases, such as cephalosporinase and penicillinase enzymes, which break down the β-lactam ring of antibiotics, rendering them ineffective, as mentioned in the studies by Paltansing and Al-Shoyaikh.[43,44]

The bacteria developed resistance to ciprofloxacin by producing biofilms, which prevented antibiotics from penetrating and killing them, allowing them to survive. According to Paltansing, resistance to quinolones is attributable to target site alterations or changes in outer membrane permeability.[43] The major mechanism involves DNA gyrase mutations, as described by Zaman et al.[45] Variations in antibiotic resistance and sensitivity among *E. coli* strains highlight the importance of antimicrobial susceptibility testing as a fundamental step in guiding effective treatment.

The results showed that out of 35 *E. coli* isolates, 22 (62.8%) were ESBL producers, while 13 isolates (37.14%) did not produce the enzyme. These results align with Veve et al., who reported that 83.7% of *E. coli* isolates were able to produce ESBL.[46] However, the present study disagrees with Talan et al., which found that only 14.8% of 453 *E. coli* isolates produced ESBL.[47] ESBL and carbapenemase infections have been on the rise in recent years and are leading causes of hospital- and community-acquired infections. As a result, a rapid and reliable test is required to determine the appropriate antibiotic. The Vitek®2 system demonstrated rapid and accurate detection, saving time and effort. The Vitek®2 compact system is a fully automated, standardized microbiological system that performs both drug susceptibility testing and bacterial identification simultaneously. The Vitek®2 ESBL test panel is used for the quick detection of ESBL production, in addition to accurate identification and susceptibility testing, by evaluating the inhibitory effects of cefepime, cefotaxime, and ceftazidime both alone and in combination with clavulanic acid. Both molecular methodology and the Vitek®2 ESBL test panel successfully detected the formation of ESBL in Enterobacteriaceae.[48]

The isolates were also tested for ESBL enzyme production using the Double Disk Approximation Method (DAM). Results showed that out of 35 *E. coli* isolates, 19 (54.29%) were ESBL producers, while 16 (45.71%) did not produce the enzyme. The results of this study agree with Samiyah et al., who found that 67% of their isolates were ESBL producers in Saudi Arabia, and with Mohammed et al. in Iraq.[49,50] A study in India by Sudharani et al. found that 53% of their *E. coli* isolates had the ability to produce ESBL.[51] However, the present study disagreed with Riaz et al., in Pakistan.

β-lactamase is a major virulence factor that destroys the β-lactam ring in some antibiotics, enhancing antibiotic resistance and pathogenicity in *E. coli*. ESBLs have emerged as a significant mechanism of β-lactam and other antibiotic resistance in Enterobacteriaceae. ESBL enzymes provide resistance to penicillins, cephalosporins, monobactams, and other antibiotics.[46] The World Health Organization has identified bacterial antibiotic resistance as one of its top health concerns. The extensive use of antibiotics without susceptibility testing is a major cause of the evolution of multidrug-resistant bacteria, which significantly hampers treatment efforts and may compromise the effectiveness of other treatments.[54]

Study limitations

Despite the significance of this study's findings on the isolation of *E. coli* from individuals suffering from UTIs and its resistance to antibiotics, several limitations exist. The study did not focus on the virulence factors that exacerbate infections or contribute to antibiotic resistance. This limitation can be addressed in future studies by employing advanced analyses, such as PCR for identifying genes responsible for virulence factors and whole-genome sequencing.

Whole-genome sequencing of the bacterial strains was not conducted due to financial and time constraints. Consequently, the diversity of *E. coli* strains and the mechanisms underlying antibiotic resistance development were not explored. Future studies should include such analyses to provide a comprehensive understanding of the genetics of these bacteria and their potential impact on therapy.

Although the number of participants in this study was statistically adequate, it may not have fully captured the variations in genetic patterns and resistance mechanisms prevalent in other regions or communities worldwide. Previous studies addressing these factors utilized larger sample sizes representative of diverse geographical areas. Future research should consider a broader and more diverse participant base to enhance the generalizability of the findings.

Conclusion and future scope

The current study identified *E. coli* as the primary causative bacterial species responsible for UTIs among hospitalized patients in Basrah. Antibiotic resistance patterns of the causal microorganisms revealed that *E. coli* exhibited the highest prevalence of resistance to ceftazidime, whereas amikacin showed the lowest resistance. Furthermore, 62.86% of the samples tested positive for ESBL production, resulting in resistance to third-generation cephalosporins.

These findings have significant practical implications. The high resistance to ceftazidime underscores the urgent need to reconsider its use as a first-line treatment for UTIs, particularly among patients in Basrah's hospitals. Amikacin may serve as a suitable alternative, provided its use aligns with local antibiotic stewardship guidelines and individual patient needs.

The prevalence of *E. coli* strains producing ESBL highlights the necessity of routine screening for these strains in clinical laboratories and diagnostic centers. Such measures would enable physicians to select appropriate medications, avoid ineffective treatments, and mitigate complications through the timely identification of ESBL production.

These findings also emphasize the importance of antibiotic stewardship programs that prioritize local resistance patterns in Basrah. Implementing such programs can help regulate antibiotic prescriptions, reduce the overuse of cephalosporins, and curb the spread of resistant bacterial strains.

Conclusion

The current study identified *E. coli* as the primary bacterial species responsible for UTIs among hospitalized patients in Basrah. Antibiotic resistance patterns of the causative microorganisms revealed that *E. coli* exhibited the highest prevalence of resistance to ceftazidime, while amikacin showed the least resistance. Additionally, 62.86% of the isolates tested positive for extended-spectrum β-lactamase (ESBL) production, resulting in resistance to third-generation cephalosporins.

These findings have significant practical implications. The notable resistance to ceftazidime underscores the urgent need to reconsider its use as a first-line treatment for UTIs, especially in Basrah's hospitals. Given that amikacin demonstrated lower resistance, it may serve as a viable alternative, provided its use aligns with local antibiotic guidelines and patient needs. The high prevalence of *E. coli* strains that produce ESBL highlights the necessity for routine screening of these strains in clinical laboratories and diagnostic centers. This would enable healthcare providers to select appropriate treatments, avoid ineffective therapies, and promptly identify ESBL production to prevent complications.

Furthermore, these findings emphasize the importance of antibiotic stewardship programs that focus on local resistance trends in Basrah. Such programs can help regulate antibiotic prescriptions, minimize the overuse of cephalosporins, and curb the spread of resistant strains, ultimately improving patient outcomes and reducing the public health burden of antimicrobial resistance.

Declarations

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Author contributions

Conceptualization, Z.A.E. and W.S.S.; Methodology, Z.A.E.; Software, W.S.S.; Validation, Z.A.E., W.S.S. and M.A.I.A.; Formal Analysis, W.S.S.; Investigation, M.A.I.A.; Resources, M.A.I.A.; Data Curation, Z.A.E.; Writing – Original Draft Preparation, Z.A.E.; Writing – Review & Editing, Z.A.E.; Visualization, M.A.I.A.; Supervision, Z.A.E.; Project Administration, Z.A.E.; Funding Acquisition, W.S.S.

Conflicts of interest

The authors have disclosed no conflicts of interest.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki, and based on ethical approval No. (773) dated (10/3/2024) from the Basra General Health Directorate.

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ORIGINAL PAPER

Design and testing of breathing retraining device a multiphasic exploratory study in healthy subjects

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ABSTRACT

Introduction and aim. Traditional spirometers are limited by bulkiness and lack of biofeedback, which can hinder their effectiveness in pulmonary rehabilitation. This study aimed to evaluate the accuracy of an innovative breathing retraining device in measuring inhaled volume and assess user satisfaction compared to standard spirometers.

Material and methods. A multiphasic exploratory study was conducted with 102 healthy adults (aged 18–60 years). The study included three phases: need analysis through focus group discussions, prototype development using polycarbonate materials and 3D printing, and effectiveness testing. Inhalation exercises were performed with both the new device and a standard spirometer. Primary outcomes were inhaled volume and marker displacement, with user satisfaction assessed via the Quebec User Evaluation of Satisfaction with Assistive Technology (QUEST 2.0) questionnaire.

Results. The new device showed a strong correlation between inhaled volume and marker displacement ($r=0.842$, $p<0.001$). The mean inhaled volume was 2.07±0.61 liters, with a mean marker displacement of 5.19±0.59 cm. The mean QUEST 2.0 satisfaction score was 3.54, indicating high user satisfaction.

Conclusion. The redesigned breathing retraining device not only addresses critical gaps in existing technologies but also offers a practical, user-friendly solution for pulmonary rehabilitation. By combining accuracy, real-time feedback, and portability, this innovation has the potential to redefine respiratory therapy standards in both clinical and home-based settings, paving the way for broader applications and improved patient outcomes.

Keywords. breathing retraining device, incentive spirometer, inhalation exercises, pulmonary rehabilitation, respiratory therapy, volumetric measurement

Introduction

Breath is Life. The act of breathing is fundamental to existence, and its significance became starkly evident during the COVID-19 pandemic, where ventilation represented the thin line between survival and mortality. The respiratory system, constantly interfacing with the external environment, not only sustains life but also serves as a gateway for infections. This duality underscores the importance of preserving and enhancing pulmonary function, particularly through chest physiotherapy. Lung expansion exercises, a cornerstone of chest physiotherapy, are pivotal in optimizing ventilation, perfusion, and diffusion, making them indispensable in respiratory care.

Among the therapeutic interventions, volume-oriented incentive spirometers have proven superior to their

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flow-oriented counterparts in improving lung function, promoting better thoracic expansion, and enhancing diaphragmatic activity.[1] These devices have evolved since 1973, when Bartlett first conceptualized the incentive spirometer. He recognized the pulmonary benefits of yawning in postoperative patients and developed a device that allowed individuals to inhale deeply, achieving volumes between 200 and 2500 mL. This device included innovative features such as a volume indicator, an incidence counter, and a light bulb that activated upon reaching the desired volume, encouraging sustained inhalation.[2]

Over the decades, advancements in spirometer technology have sought to improve usability and therapeutic efficacy. In 1983, Edward introduced a refined model, followed by Kenneth's turbine-based incentive spirometer in 1992. Further innovations included Lawrence's goal-recording mechanism in 2001, Terry's verbal stimulation features in 2012, and Yu-Fu Wu's electronic spirometer in 2018.[3–8] Despite these technological strides, the clinical practice guidelines from the American Association for Respiratory Care (AARC) in 2011 highlighted limitations, stating that incentive spirometers alone are inadequate for preventing or treating postoperative pulmonary complications.[9]

Traditional bedside incentive spirometers, while prevalent, face notable limitations. These include difficulty in one-handed operation, the need for assistance from healthcare professionals, and inconsistencies in the performance of flow-oriented devices. Additionally, these devices often lack adequate biofeedback mechanisms to engage patients effectively, resulting in suboptimal adherence and outcomes. Such challenges underscore the necessity for innovation to address these gaps.

While the background has established the importance of pulmonary rehabilitation and the limitations of existing spirometers, the innovative breathing retraining device provides distinct advantages that go beyond traditional usage. Unlike standard incentive spirometers, this device combines precise volumetric measurement with real-time visual feedback, enabling users to monitor their progress with unparalleled accuracy. Such features make it not only a diagnostic and therapeutic tool but also a highly effective exercise device for improving respiratory muscle strength and lung capacity.

The device's ability to provide adjustable resistance and user-friendly feedback transforms it into a versatile tool for various clinical and home-based applications. As a result, it is particularly beneficial for diverse populations, including post-surgical patients, individuals with chronic respiratory conditions, and even healthy individuals seeking to enhance their pulmonary function as part of a fitness regimen. This dual-purpose design bridges the gap between measurement and active engagement, offering a holistic approach to respiratory therapy.

By addressing these critical gaps and offering unique functionalities, the breathing retraining device sets a new standard for pulmonary rehabilitation, making it both accessible and impactful in a variety of scenarios. This study aims to validate these advantages and establish the device as an essential tool for comprehensive respiratory care.

This study presents the design and testing of an innovative breathing retraining device aimed at overcoming these challenges. By integrating features such as portable design, real-time visual biofeedback, and precise volumetric measurement, this device addresses the critical shortcomings of existing spirometers. Its user-centric approach not only improves accessibility and ease of use but also enhances engagement, thereby fostering better therapeutic outcomes. Through this effort, the study aims to redefine the standard of care in pulmonary rehabilitation, contributing a significant advancement to the field of respiratory therapy.

Aim

The aim of the study is to design and test an innovative breathing retraining device.

Material and methods

This study follows a multiphase exploratory design with an interdisciplinary approach to design and develop a breathing retraining device. The study is conducted in three phases:

Phase 1: need analysis

Objective

To identify the specific limitations of existing bedside incentive spirometers and gather expert insights to guide the design of a new breathing retraining device.

Study population

Healthcare professionals with expertise in respiratory therapy, surgery, and medical rehabilitation were invited to participate. The panel consisted of cardiothoracic vascular surgeon, specialists in respiratory medicine, gynecologist, anesthetist and physiotherapists with expertise in cardiopulmonary rehabilitation (MPT in cardio-respiratory). The diversity of the panel ensured a comprehensive understanding of clinical needs across various domains.

Study design

A qualitative focus group discussion (FGD) was conducted to gather detailed insights into the limitations of existing spirometers and potential areas for improvement. The study utilized an exploratory design to capture a wide range of expert opinions and thematic feedback.

Data collection: date and setting

The FGD was conducted on 27th December 2023 at RK University School of Physiotherapy. The discussion was held online via Zoom to accommodate experts from different cities (Chennai, Ahmedabad, Baroda, and Rajkot). Recruitment of Participants: Experts were identified and approached through professional networks, academic institutions, and recommendations from medical associations. A total of 15 experts were selected based on their clinical experience, academic background, and familiarity with incentive spirometers. Discussion framework: The FGD was moderated by a senior physiotherapist with expertise in qualitative research to ensure structured and unbiased discussions. The key discussion points included: A. Experience with incentive spirometers: Participants were asked about their clinical experiences with existing spirometers, including their advantages and limitations. B. Initial Impressions: For participants unfamiliar with spirometers, their perspectives on its potential utility and design were explored. C. Target populations: Experts were encouraged to identify patient populations (e.g., post-surgical, ICU, COPD patients) that could benefit most from a redesigned device. D. Improvement areas: Participants shared specific design features they believed would enhance usability and therapeutic outcomes. Examples included: Portability and ease of handling, Resistance settings and volumetric accuracy, Feedback mechanisms for patient motivation. Role of technology: The potential for integrating advanced features (e.g., digital biofeedback, wireless connectivity) was discussed, considering patient and clinician needs. Recording and documentation: The session lasted approximately 90 minutes and was recorded with participant consent. A note-taker documented key points, ensuring redundancy in data collection for accuracy.

Data analysis: Thematic analysis: The recorded discussion was transcribed verbatim. A coding framework was developed to identify recurring themes, such as device portability, accuracy, and patient engagement. Thematic analysis was conducted using NVivo software to systematically organize and interpret the data. Validation: To ensure reliability, the findings were reviewed by two independent researchers. Discrepancies in theme identification were resolved through consensus.

Findings and outcomes: Identified limitations: The analysis revealed the following key limitations in existing devices: difficulty in one-handed operation, lack of adjustable resistance settings, insufficient feedback mechanisms for user motivation, bulky and non-portable design. Design recommendations: Incorporation of real-time visual feedback to engage patients, reduction in size and weight for portability, use of durable yet lightweight materials to improve accessibility, and integration of adjustable resistance and volumetric measurement for precision. Consensus on device need: Experts unanimously agreed on the necessity of redesigning the spirometer to address these limitations and improve patient compliance.

Phase 2: design and development

Objective

To conceptualize, design, and develop an innovative breathing retraining device based on findings from Phase 1.

Design conceptualization

The insights from Phase 1 guided the design requirements. Specific needs identified included: Portability and ease of handling, adjustable resistance for varied therapeutic needs, real-time feedback through visual or mechanical means, accurate volumetric measurement to enhance therapeutic precision.

The following steps were undertaken, A. Material selection: Lightweight and durable materials, such as polycarbonate, were selected for their transparency, impact resistance, and cost-effectiveness. These materials ensured usability and compliance with medical device safety standards. B. Dimensional specifications: The mouthpiece was designed to be 20 cm in length with an internal diameter of 2 cm, ensuring comfort and compatibility with diverse patient anatomies. The cylinder height was set at 17 cm with volumetric gradations marked for precise measurement. The vertical chamber was designed with a 0.5 cm diameter to minimize airflow resistance.

Prototype development: The design was translated into a physical prototype through the following steps. A. 2D and 3D modelling: Initial sketches were created using 2D drawings to conceptualize the basic structure and functionality. AutoCAD software was employed for creating detailed 3D models, ensuring precision and alignment with the desired specifications. B. Material sourcing: Everyday household items, such as bubble maker sticks, plastic jars, and glue guns, were repurposed to create a cost-effective prototype for early iterations. Custom parts were fabricated as needed to meet the design's specific requirements. C. Assembly: The prototype was assembled in the laboratory, where components were systematically integrated. A floating disc mechanism was developed using foam material calibrated to fit snugly within the cylinder. The disc provided real-time visual feedback by rising with inhalation.

Iterative Refinement: Several iterations were conducted to optimize the prototype, feedback integration: Experts from the focus group were re-engaged to evaluate the prototype's usability, accuracy, and functionality. Adjustments were made based on feedback, such as improving the disc's stability and ensuring smooth airflow through the vent system. Performance testing: Pre-

liminary tests were conducted to validate the prototype's accuracy in measuring inspiratory volume. The device was compared against a standard spirometer to ensure its performance met clinical standards.

Design features: The final prototype incorporated the following features: A. Horizontal mouthpiece Chamber: Facilitates comfortable inhalation and exhalation, accommodating users with diverse respiratory capacities. B. Transparent cylinder with volume scale: Allows users and clinicians to monitor inspiratory volumes accurately in real time. C. Mechanical floating disc: Serves as a biofeedback mechanism, visually indicating lung expansion during inhalation. D. Vertical chamber and vent system: Ensures smooth airflow and minimizes resistance, particularly beneficial for patients with limited lung capacity. E. Vent inlet: Regulates external air intake to maintain pressure stability and ensure accurate volumetric readings.

Interdisciplinary collaboration: The design and development process involved a multidisciplinary team which are, mechanical engineers provided expertise in structural integrity, airflow mechanics, and material properties. Physiotherapists ensured the prototype met clinical requirements for therapeutic application.

Challenges and solutions: A. Cost-effectiveness: Sourcing affordable materials without compromising quality was a significant challenge. This was addressed by repurposing everyday materials for initial iterations. B. Technical precision: Achieving accurate measurements required several calibrations and adjustments, which were refined through iterative prototyping. C. User comfort: The prototype underwent ergonomic testing to ensure ease of use for patients with limited mobility or strength.

Outcome: The final prototype successfully met the identified design goals: Portable and user-friendly for a wide range of patients. Accurate in measuring inspiratory volumes, comparable to standard spirometers. Engaging for patients through real-time visual feedback, enhancing adherence to therapy.

Ethical considerations: The prototype adhered to ethical guidelines for device safety and usability. Feedback from participants was anonymized and used solely for the purpose of refining the device.

Phase 3: effectiveness and feasibility testing

The third phase aimed to validate the efficacy and accuracy of the newly developed breathing retraining device in comparison to a conventional spirometer. This multiphase exploratory study focused on assessing the device's functionality, user engagement, and clinical utility. A total of 102 healthy adults were enrolled using a convenient sampling technique, ensuring a diverse sample within the predefined inclusion criteria. The study spanned six months and was conducted in both community and institutional settings in Rajkot, Gujarat, with ethical approval obtained prior to initiation.

Study population and recruitment

Participants were recruited through community outreach and institutional announcements. Recruitment materials, including posters and direct communications, outlined the study objectives and eligibility criteria. Individuals aged 18 to 60 years, of both genders, with no known respiratory or cardiovascular pathologies, were eligible. Exclusion criteria included diagnosed lung diseases, chronic obstructive pulmonary disease (COPD), cardiovascular disorders, pregnancy, or reliance on respiratory medications. Written informed consent was obtained from all participants after a thorough explanation of the study objectives, procedures, risks, and benefits.

Device calibration and testing protocol

To ensure precision, the prototype device underwent calibration using the RMS Helios 401 Spirometer, a gold-standard pulmonary function testing machine. The calibration process involved securely connecting the device to the spirometer probe to maintain a leakproof interface. Each participant performed three calibration trials of inspiratory capacity (IC) maneuvers, and the best trial, defined by the highest inspiratory volume, was recorded for analysis. Calibration ensured that the device consistently measured inspiratory volume with accuracy comparable to the spirometer, meeting the required standards for data collection.

Validation against spirometer

Following calibration, participants were instructed to perform deep breathing exercises using both the conventional spirometer and the prototype device. To standardize testing conditions, posture and inhalation technique guidelines were provided. Participants were seated in an upright position to maximize thoracic expansion and diaphragmatic movement. The inhalation protocol emphasized slow and controlled breaths through the device mouthpiece to achieve full lung expansion. Three trials were conducted with each device, and the best trial results were used for analysis to minimize variability.

Data collection and analysis

Inspiratory capacity, measured as the maximum volume of air inhaled in liters, served as the primary respiratory parameter. Real-time feedback provided by the mechanical floating disc in the prototype device was recorded to evaluate its efficacy as a biofeedback mechanism. Data were collected systematically, ensuring accuracy and completeness. Descriptive and inferential statistical analyses were performed using SPSS version 21.0 (IBM, Armonk, NY, USA). Normality of data dis-

tribution was assessed through skewness, kurtosis, and the Shapiro-Wilk test. Parametric tests were applied given the normal distribution of data. Correlation analyses evaluated the relationship between inspiratory capacity, marker displacement, and user satisfaction scores. Statistical significance was determined at $p<0.05$.

User satisfaction assessment

The Quebec User Evaluation of Satisfaction with Assistive Technology (QUEST 2.0) questionnaire was administered to all participants to measure satisfaction with the prototype device. The questionnaire assessed dimensions such as ease of use, comfort, durability, and overall effectiveness. Responses were scored on a 5-point Likert scale, with higher scores indicating greater satisfaction. The results were analyzed to identify factors contributing to user satisfaction and areas for potential improvement in device design.

Ethical and safety considerations

Ethical approval was granted by the Institutional Ethical Committee at RK University (ECR/259/Indt/GJ/2016/RR-21), and the study was registered with the Clinical Trials Registry-India (CTRI) to ensure adherence to national guidelines. Participant safety was prioritized throughout the study, with clear protocols in place to address any adverse events or discomfort experienced during testing. Data confidentiality was maintained, and participants retained the right to withdraw from the study at any time without penalty.

Statistical methods

Data analysis was performed using SPSS version 21.0 (IBM, Armonk, NY, USA), with a level of significance set at 0.05 and a confidence interval of 95%. Normality was assessed using skewness, kurtosis, histograms, and the Shapiro-Wilk test. Skewness and kurtosis values between -1.96 and +1.96, along with a Shapiro-Wilk test p-value greater than 0.05, indicated normal distribution. As the data were normally distributed, parametric tests were employed.

Results

Phase-1 need analysis

Based on the literature review and insights from the focused group discussion, it was determined that there is a significant need to redesign the breathing retraining device. Experts highlighted several areas for improvement, including: size of the device, resistance during breathing, volumetric measurement, weight of the device, and length of the mouthpiece.

In response to these suggestions, a new design for the breathing retraining device was conceptualized, addressing the identified needs. The new design focused on making the device more user-friendly, accessible, and effective in pulmonary rehabilitation.

Phase 2: design and development

Based on the considerations identified in Phase 1 (need analysis), and through the iterative development process in Phase 2, the final prototype of the breathing retraining device was successfully designed and developed. The key challenges identified – such as size, resistance during breathing, volumetric measurement, weight, and mouthpiece length – were systematically addressed and integrated into the device's design.

The final prototype was developed with several key design components, reflecting the suggestions from experts and addressing the clinical needs identified throughout the study. The device features a horizontal mouthpiece chamber, designed to allow users to inhale and exhale comfortably and effectively, with an optimal length and diameter suited for a wide range of patients. This design resolved concerns related to the usability of the mouthpiece. The transparent cylinder of the device is enclosed and marked with a volume scale, providing clear visual feedback on the volume of air inhaled. This feature is crucial for accurate volumetric measurement, making it easier for both patients and therapists to monitor progress. Inside the cylinder, a mechanical floating disc rises with each inhalation, indicating the volume of air inhaled. The disc moves in accordance with the volume scale, offering users real-time insights into their lung capacity, which improves engagement and motivation during therapy. Additionally, a vertical chamber is connected to the horizontal mouthpiece chamber, ensuring smooth airflow and facilitating consistent readings during both inhalation and exhalation. A vent connecting the vertical chamber provides an open link between the cylinder and vertical chamber, allowing seamless airflow and reducing resistance or pressure build-up, ensuring patient comfort. Finally, the device includes a vent inlet located at the lower part of the cylinder, which introduces external air into the device, regulating pressure and ensuring that the floating disc and other mechanical components function accurately throughout each breathing cycle.

Considering all the expert feedback gathered during Phase 1, the development of this breathing retraining device in Phase 2 led to a prototype that is more compact, lightweight, and easy to use, while also ensuring accurate volumetric measurement. The final design meets the identified needs for patients requiring pulmonary rehabilitation, and future testing will confirm its clinical efficacy.

Phase 3: effectiveness and feasibility testing

Participant demographics

A total of 102 healthy subjects participated in the study, comprising 53 males and 49 females. The age range was broad, ensuring a diverse sample for analysis.

Normality testing

Normality assessments for key variables showed that all data were normally distributed. Table 1 presents the Shapiro-Wilk normality test results for variables such as age, volume inhaled, displacement of markers, and Quebec User Evaluation of Satisfaction with Assistive Technology Version 2.0 (QUEST 2.0) total score average.

Table 1. Shapiro-Wilk normality test results

Variable	W Statistic	p	Normality conclusion
Age	0.980	0.158	Normally distributed
Volume inhaled (L)	0.985	0.348	Normally distributed
Displacement of markers (cm)	0.987	0.462	Normally distributed
QUEST 2.0 total score average	0.976	0.093	Normally distributed

Descriptive statistics

The mean volume inhaled during maximal deep inspiration was 2.07±0.61 liters, and the mean displacement of the markers in the cylinder was 5.19±0.59 centimeters. Detailed values are provided in Table 2.

Table 2. Summary of volume inhaled and marker displacement

Parameter	Value	Standard Deviation
Mean volume inhaled (L)	2.07	±0.61
Mean displacement of markers (cm)	5.19	±0.59

Outliers were identified in the distribution of inspiratory capacity and marker displacement data, particularly at the higher ends of the ranges. To assess their impact, a sensitivity analysis was conducted by excluding the top and bottom 5% of data points. After exclusion, the mean inspiratory capacity slightly reduced from 2.07±0.61 L to 2.04±0.55 L, while the correlation between inspiratory capacity and marker displacement remained strong (r=0.837, p<0.001). These results indicate that the outliers did not significantly alter the overall findings, affirming the robustness of the dataset (Table 3).

Table 3. Independent samples t-test results comparing males and females

Variable	Gender	Mean±SD	t	p
Age (years)	Male	27.6±9.4	0.386	0.700
	Female	26.9±12.2		
Volume inhaled (L)	Male	2.20±0.62	1.730	0.087
	Female	2.03±0.50		
Displacement of markers (cm)	Male	5.32±0.67	0.860	0.392
	Female	5.24±0.62		
QUEST 2.0 total score average	Male	3.56±0.17	0.744	0.459
	Female	3.54±0.18		

An independent samples t-test was conducted to compare males and females across key variables. No significant difference was found in age between males (27.6±9.4 years) and females (26.9±12.2 years) (p=0.700). Similarly, the volume inhaled showed no significant difference between males (2.20±0.62 L) and females (2.03±0.50 L) (p=0.087). Table 3 states that Displacement of markers yielded comparable results for males (5.32±0.67 cm) and females (5.24±0.62 cm) (p=0.392). The QUEST 2.0 total score also indicated no significant difference in satisfaction between males (3.56±0.17) and females (3.54±0.18) (p=0.459). Correlation analyses using Pearson correlation coefficients were performed as given in Table 4 to assess the relationships between variables. A strong positive correlation was found between volume inhaled and displacement of markers (r=0.842, p<0.001), suggesting that higher inhaled volumes were associated with greater marker displacement. Additionally, a weak positive correlation was observed between volume inhaled and the QUEST 2.0 total score (r=0.274, p=0.014), indicating that participants who inhaled larger volumes reported slightly higher satisfaction. A weak positive correlation was also found between displacement of markers and the QUEST 2.0 total score (r=0.252, p=0.024), suggesting a slight association between greater marker displacement and higher usability and satisfaction scores. No significant correlations were found between volume inhaled and age, or displacement and age (p>0.05), indicating that age did not significantly affect these variables (Table 4).

Table 4. Pearson correlation coefficients between variables

Variables	Correlation Coefficient (r)	p	Interpretation
Volume inhaled vs. displacement	0.842	<0.001	Strong positive correlation
Volume inhaled vs. age	0.112	0.313	No significant correlation
Displacement vs. age	0.098	0.378	No significant correlation
Volume inhaled vs. QUEST total score	0.274	0.014	Weak positive correlation
Displacement vs. QUEST Total Score	0.252	0.024	Weak positive correlation

QUEST 2.0 satisfaction survey results

Participants completed the QUEST 2.0 satisfaction survey, which includes 12 items related to assistive device satisfaction, focusing on eight aspects mentioned in Table-5 of the new breathing retraining device.

The Quebec User Evaluation of Satisfaction with Assistive Technology (QUEST 2.0) satisfaction survey revealed an overall mean score of 3.54 out of 5, indicating high user satisfaction with the prototype device. Among the eight dimensions evaluated, the highest mean score was observed in "Ease of Use" (3.58±0.12), followed closely by "Durability" (3.62±0.15) and "Safety and Security" (3.57±0.13).

Subgroup analysis based on participant demographics showed no significant differences in overall satis-

faction scores between males (3.56±0.17) and females (3.54±0.18) (p=0.459, CI: -0.06 to 0.10). Similarly, age groups (18–30, 31–45, 46–60 years) did not exhibit significant variations in satisfaction scores (ANOVA, F(2, 99)=1.21, p=0.303). These findings suggest that the device's design and functionality meet diverse user needs, irrespective of gender or age (Table 5).

Table 5. Contains mean scores for each satisfaction item, with a total mean score of 3.54 out of 5, indicating high overall satisfaction.

QUEST 2.0	Mean score
Dimension	3.490196
Weight	3.578431
Ease in adjustment	3.558824
Safety and security	3.45098
Durability	3.617647
Ease in use	3.519608
Comfort	3.480392
Effectiveness	3.588235

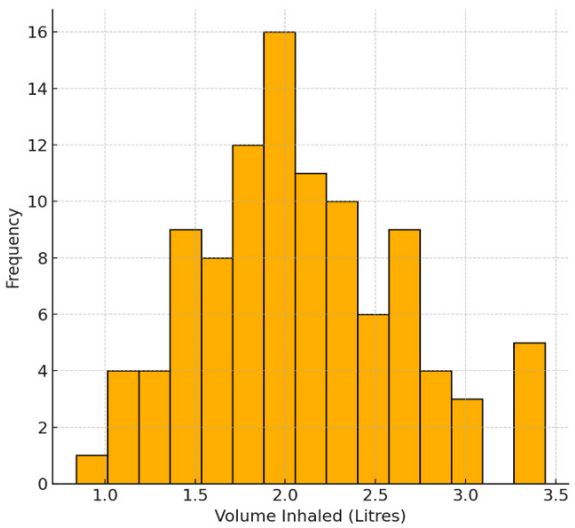

Fig. 1. The histogram of volume inhaled (L) with the frequency

Interpretation: Figure 1 displays, Distribution Shape: The histogram shows a somewhat normal distribution with a slight right skew, meaning the majority of participants inhaled between 1.5 and 2.5 liters. Peaks: The highest frequency is around 2 liters, indicating that this is the most common volume inhaled among the participants. Skewness: There are a few participants who inhaled either significantly less (around 1 liter) or more (above 3 liters), which are likely influencing the rightward skew of the distribution.

Interpretation: Linearity: Most of the data points lie close to the red reference line, which suggests that the "Volume inhaled (L)" follows a normal distribution overall. Outliers: A few data points at the higher end of the plot (top right corner) deviate from the line, indicating that there are some individuals with higher-than-normal inhaled volumes. As provided in Figure 2, the plot supports that the data is mostly normal, making parametric tests feasible, though there are a few outliers that may require further investigation or treatment depending on the analysis.

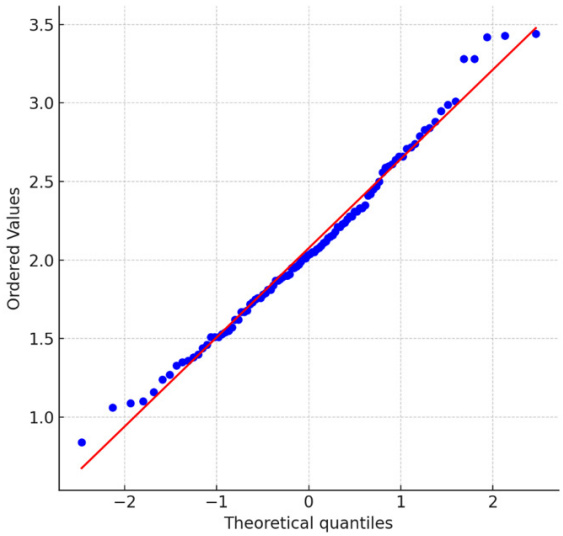

Fig. 2. Presents the Q-Q plot for volume inhaled, further confirming the normality of the data distribution

The findings from the validation phase demonstrate the prototype device's strong alignment with standard spirometer measurements, as evidenced by the significant correlation between inspiratory capacity and marker displacement (r=0.842, p<0.001). These results corroborate prior studies on the importance of feedback mechanisms in pulmonary rehabilitation devices, which enhance user engagement and therapeutic efficacy.[11] Additionally, the high satisfaction scores across all dimensions underscore the usability and practicality of the device in both clinical and home settings.

However, the absence of a control group and long-term efficacy assessments limits the generalizability of these findings. Future studies should address these gaps by including diverse patient populations and extended testing periods. Despite these limitations, the results highlight the device's potential to transform pulmonary rehabilitation by offering an innovative, user-friendly, and effective solution for improving respiratory health.

Discussion

The Phase 1: need analysis successfully identified critical areas for improvement in existing respiratory devices, including size, resistance during breathing, volumetric measurement, weight, and mouthpiece length. These findings align with prior research that emphasizes the role of patient-centered design in improving compliance and therapeutic outcomes. For instance, Chrystyn et al. highlighted that ease of handling and clear instruc-

tions are essential for ensuring proper usage and adherence to therapy in inhaler devices for COPD patients.[10] These principles directly support our efforts to redesign a breathing retraining device that prioritizes user comfort, durability, and functionality to enhance patient engagement.

The expert panel's recommendations closely reflect existing evidence on the efficacy of feedback mechanisms in respiratory devices. Studies, such as those by Kyoung Kim et al. (2011), have underscored the importance of accurate and timely feedback in improving chest expansion and pulmonary function in stroke patients.[11] This evidence validates the inclusion of volumetric measurement and real-time biofeedback in our device, which are anticipated to not only motivate patients but also foster better self-monitoring and adherence. In pulmonary rehabilitation, where sustained engagement is critical, such features are integral to achieving improved clinical outcomes.

The diverse composition of the expert panel further enriched the redesign process by addressing practical challenges commonly encountered in clinical practice. Specific recommendations – such as improving portability, enhancing usability with a one-handed design, and incorporating intuitive feedback mechanisms – were directly integrated into the prototype. For example, the mechanical floating disc was included to provide real-time visual biofeedback, while the device's lightweight construction addressed the need for portability. These innovations cater to both patient and clinician needs, making the device versatile for use in clinical settings, home-based rehabilitation, and resource-limited environments.

Moreover, the Phase 1 findings highlight a significant advancement in addressing psychological barriers to therapy. By offering immediate feedback on inhalation volume, the device empowers patients with a better understanding of their lung capacity, fostering motivation and long-term adherence. This dual focus on functionality and patient engagement not only improves therapeutic outcomes but also broadens the device's applicability to areas such as post-surgical recovery and ICU settings, where portability and ease of use are particularly valuable.

Incorporating these insights into the design framework provides a strong foundation for the next phase of development. By integrating both clinical expertise and user-focused design principles, the redesigned breathing retraining device has the potential to redefine pulmonary rehabilitation standards and improve overall patient outcomes.

The design and development of the breathing retraining device in Phase 2 directly responded to the functional and psychological needs identified in Phase 1. By incorporating patient-centered features, the device was conceptualized to optimize usability and therapeutic effectiveness. Each design element was meticulously planned, grounded in evidence-based principles, and supported by insights from prior research.

The Horizontal Mouthpiece Chamber was designed to accommodate diverse user anatomies, ensuring comfortable inhalation and exhalation. This focus on ergonomics is essential, as patient comfort significantly impacts adherence to therapy. Eltorai et al. demonstrated that the ergonomic design of spirometry devices enhances patient performance and engagement by reducing discomfort and making therapy more accessible.[12] By prioritizing user comfort, this feature aligns with established principles in respiratory device design, ensuring that patients with varying anatomical needs can use the device effectively.

The Cylinder with Volume Scale provides real-time visual feedback on inhalation volumes, a critical element for fostering patient motivation and self-monitoring. Feedback mechanisms have been extensively validated in respiratory therapy literature, as evidenced by Kim et al., who highlighted the efficacy of visual feedback in improving chest expansion and pulmonary function in stroke patients.[11] By enabling users to track their progress in real time, the volume scale integrates a simple yet powerful motivational tool, empowering patients to actively engage with their rehabilitation.

Another innovative feature is the Mechanical Floating Disc, which acts as a biofeedback mechanism, rising during inhalation to indicate lung expansion. This dynamic visual cue builds upon the principles of feedback-based respiratory training, which has been shown to enhance patient engagement and adherence to therapy. The floating disc's design emphasizes accuracy and responsiveness, ensuring that patients receive meaningful feedback throughout their breathing exercises.

The Vertical Chamber and Connecting Vent were incorporated to optimize airflow and minimize resistance, addressing the needs of patients with limited respiratory capacity. Curran et al. explored airflow dynamics in respiratory devices and demonstrated that modifications to airflow pathways can improve device performance while maintaining low resistance.[13] This principle guided the design of the vertical chamber and vent, ensuring smooth airflow to reduce strain during therapy. By minimizing resistance, the device becomes accessible for individuals with compromised lung function, supporting its applicability in pulmonary rehabilitation settings.

The inclusion of a Vent Inlet further optimizes air entry, reducing the effort required for breathing exercises. This design choice directly addresses the needs of patients with lower inspiratory capacity, making the device intuitive and effortless to use. By reducing physical strain, the vent inlet ensures that therapy remains feasi-

ble and engaging for users across a wide range of respiratory conditions.

The design principles underlying these features were rooted in enhancing patient compliance by addressing common barriers to effective use, such as weight, size, and complexity. By creating a portable, lightweight, and user-friendly device, the design prioritizes accessibility and daily usability. These improvements are expected to increase adherence to therapy and improve clinical outcomes, as supported by the literature on respiratory device design.

In briefing, the Phase 2 design and development process translated the insights from Phase 1 into a tangible, innovative breathing retraining device. By incorporating features that address both the functional and psychological needs of patients, the device represents a significant advancement in pulmonary rehabilitation technology. This foundation sets the stage for Phase 3, where the device's effectiveness and feasibility will be rigorously tested in a clinical setting.

The findings from the Effectiveness and Feasibility Testing phase underscore the success of the redesigned breathing retraining device in meeting its intended goals of enhancing usability, effectiveness, and patient engagement. By evaluating the device among 102 healthy participants across a broad demographic, the study ensured the robustness of its conclusions and laid the groundwork for future applications in clinical practice.

The results demonstrated that the device effectively facilitates significant lung expansion, as evidenced by the strong positive correlation between the volume of air inhaled and the displacement of markers in the cylinder ($r=0.842$, $p<0.001$). This validates the mechanical design of the device, where the floating disc provides accurate biofeedback that reflects inhalation volume. These findings align with prior research, such as Kim et al., which highlighted that feedback mechanisms during respiratory training improve both chest expansion and pulmonary function in stroke patients.[11] Similarly, the incorporation of real-time visual feedback through the volume scale enhances patient motivation and self-monitoring, a feature shown to foster adherence and improve outcomes in pulmonary rehabilitation.

The gender-neutral performance of the device is particularly noteworthy. No significant differences were observed between male and female participants in terms of volume inhaled, marker displacement, or user satisfaction. This finding is consistent with studies by Lo-Mauro and Aliverti and Sheel et al., which demonstrated that while anatomical differences exist between genders, they do not significantly influence outcomes in respiratory therapies when ergonomic and functional diversity are considered in device design.[15,16] This reinforces the device's versatility and its broad applicability across diverse patient populations, irrespective of gender.

User satisfaction was another key outcome, with participants reporting high levels of satisfaction on the QUEST 2.0 instrument, particularly in dimensions such as ease of use and comfort (mean satisfaction score: 3.54 out of 5). While a weak positive correlation was observed between performance measures (e.g., volume inhaled) and satisfaction scores ($r=0.274$, $p=0.014$), these findings suggest that factors beyond functionality, such as ergonomic design and user experience, significantly contribute to overall satisfaction. This is consistent with findings by Guerreiro et al. (2022), which emphasized that subjective aspects like comfort and ease of handling are critical determinants of satisfaction with assistive devices.[17]

While the study demonstrated a strong positive correlation ($r=0.842$, $p<0.001$) between inspiratory volume and marker displacement, other parameters exhibited weaker or non-significant correlations, such as the relationship between performance measures (e.g., volume inhaled) and satisfaction scores ($r=0.274$, $p=0.014$). These findings warrant further discussion in the context of physiological mechanisms and user behavior. Satisfaction and Performance: The weak positive correlation between satisfaction scores and performance metrics, such as volume inhaled, suggests that user satisfaction with the device is influenced by factors beyond measurable performance outcomes. Satisfaction is likely affected by subjective elements, including perceived comfort, ease of use, and the psychological impact of visual feedback mechanisms. Studies have shown that user experience with assistive devices often relies more on ergonomic and design features than on direct performance outcomes (Guerreiro et al.).[17] For example, a patient may find the device easy to handle and visually engaging, leading to high satisfaction even if their inspiratory volume improvement is modest. Physiological Factors: From a physiological perspective, individual variations in respiratory mechanics, such as lung compliance, airway resistance, and inspiratory muscle strength, may influence the relationship between performance and satisfaction. For instance: Patients with better baseline inspiratory capacity may derive less psychological benefit from visual feedback compared to those with lower baseline function, reducing the strength of the correlation. Variability in airway resistance across participants could result in different levels of effort for the same volume inhaled, influencing perceived comfort and satisfaction differently.

The inclusion of visual and mechanical feedback mechanisms, coupled with user-friendly features like portability and ease of handling, positions the device as a significant advancement in respiratory therapy. These features address both the functional and psychological aspects of pulmonary rehabilitation, empowering users with the tools to monitor and improve their lung func-

tion actively. The positive user feedback highlights the success of the design process, as it effectively bridges the gap between clinical needs and patient-centric design principles.

While the findings from this phase provide strong evidence for the device's effectiveness and feasibility, future research should focus on expanding the sample to include clinical populations, such as patients with COPD, post-surgical conditions, or other respiratory pathologies. Longitudinal studies assessing sustained engagement and long-term outcomes would further validate the device's clinical utility.

The results of the QUEST 2.0 satisfaction survey revealed high overall satisfaction among participants, with a mean score of 3.54 out of 5. Participants rated the device favorably across various dimensions, including ease of use, comfort, and effectiveness. This high level of satisfaction highlights the device's success in addressing the key needs identified during the design phase, particularly in terms of user-friendly features and comfort.

The weak positive correlations between performance measures – such as volume inhaled and marker displacement – and satisfaction scores ($r=0.274$, $p=0.014$) suggest that while higher performance may contribute to enhanced satisfaction, other factors like device design and user experience also play significant roles in shaping overall satisfaction. This aligns with prior findings, where factors such as ease of use, comfort, and perceived effectiveness have been shown to contribute significantly to user satisfaction with assistive technology, beyond just performance outcomes.

As noted in the cross-cultural validation of the QUEST 2.0 instrument by Guerreiro et al., satisfaction with assistive devices is influenced not only by functional performance but also by subjective aspects such as ease of handling and comfort, both of which were positively rated in our study. This emphasizes the importance of a holistic approach to device design, ensuring that both functional and ergonomic needs are met to optimize user satisfaction and engagement.[17]

Clinical implications

The redesigned breathing retraining device demonstrates substantial potential for application in both clinical and home-based rehabilitation settings. Its ability to provide accurate volumetric measurements and real-time visual feedback equips healthcare providers with a reliable tool for tracking patient progress during pulmonary rehabilitation. This feature enables clinicians to make data-driven adjustments to therapy protocols, ensuring tailored interventions that optimize outcomes. Accurate measurement of inhaled volume, coupled with a clear visual display, facilitates precise monitoring of lung function improvement, a critical factor in acute care and long-term rehabilitation settings.

Biofeedback systems that provide real-time feedback during respiratory training have shown significant efficacy in improving patient engagement and therapeutic outcomes, as highlighted by Shi et al.[18] The integration of such biofeedback mechanisms into our device fosters patient awareness of progress, motivating adherence to prescribed therapy regimens. This active involvement enhances the effectiveness of pulmonary rehabilitation, reducing the likelihood of therapy discontinuation and improving overall health outcomes.

The device's simplicity and ease of use further enhance its versatility, making it highly suitable for home-based therapy programs. Portable and user-friendly devices, as noted by Shi et al., significantly improve adherence to respiratory exercises outside clinical environments.[18] In patients with chronic respiratory conditions such as COPD, home-based pulmonary rehabilitation has been effective in maintaining lung function and preventing exacerbations. The innovative design of this device allows seamless integration into such programs, offering an accessible and effective solution for long-term rehabilitation needs.

The Phase 1: Need Analysis highlighted critical gaps in existing breathing retraining devices through a review of current literature and focus group discussions with a multidisciplinary panel of surgeons, physicians, and physiotherapists across India. These consultations revealed that while breathing retraining devices are widely recommended for patients post-surgery or with chronic respiratory conditions, many existing models fail to meet the diverse needs of these populations. For instance, patients recovering from surgery often face difficulty performing deep sustained inhalations, while those in advanced stages of rehabilitation require tools to strengthen respiratory muscles.

To address these challenges, a novel device was conceptualized with features such as volumetric measurement, one-handed usability, and portability. The integration of these features provides a solution for independent respiratory exercises, addressing both the physical and psychological barriers to effective pulmonary rehabilitation. By incorporating expert insights and addressing limitations identified in the literature, this innovative device has the potential to significantly improve patient outcomes and recovery trajectories.

The development process in Phase 2 focused on creating a lightweight, portable, and user-friendly breathing retraining device. PVC material was chosen for its durability and lightweight properties, ensuring that the device remains easy to handle. Compared to heavier materials like wood, metal, or glass, PVC reduced the overall weight, improving usability across diverse patient groups. To further enhance user comfort, the device was designed as a single unit to minimize biome-

chanical challenges, such as excessive shoulder flexion, during use.

Key design specifications were informed by human anatomical and physiological considerations. The horizontal mouthpiece was set at 20 cm with an internal diameter of 2 cm, optimized for visual far-field capacity (25 cm) and ergonomic handling. The vertical cylinder, measuring 17 cm in height, featured a diameter of 0.5 cm to minimize flow resistance, reflecting the average diameter of human bronchioles. This ensured streamlined airflow and accurate inhalation measurements.

The inclusion of visual feedback mechanisms, such as a foam-based floating disc calibrated with a diameter 1 mm smaller than the cylinder, provided precise volumetric measurements. The disc, marked for volume gradations, ensured accurate tracking of inhaled volume while preventing tilting. These features enhanced user engagement and motivation by offering clear, goal-oriented feedback, a critical element in pulmonary rehabilitation.

The results from the effectiveness and feasibility testing phase revealed a statistically significant positive correlation between marker displacement (in centimeters) and inhaled volume ($p<0.001$). The mean inhaled volume measured was 2.13 L, while the mean marker displacement was 5.38 cm. These findings validate the device's mechanical design, where the floating disc reliably reflects inhalation volume, ensuring precise biofeedback.

These results align with the foundational work by Barach et al., which demonstrated that cylinder height provides a simple yet effective measure of lung capacity.[19] Their study supports the reliability of cylinder-based designs for assessing respiratory function in healthy individuals. Similarly, the current findings underscore the practicality of using cylinder height and marker displacement as robust indicators of lung function, further solidifying the device's utility in pulmonary rehabilitation.

The integration of these features into a single device bridges the gap between clinical needs and patient usability. By facilitating precise measurements and reducing the effort required for operation, the device supports therapeutic goals while enhancing patient satisfaction. This innovative design, validated through rigorous testing, offers a scalable solution for respiratory rehabilitation, with applications in diverse settings, including acute care, chronic respiratory management, and home-based therapy programs.

Study limitations

While this study provides strong evidence for the effectiveness and feasibility of the redesigned breathing retraining device, certain limitations must be acknowledged. The study was conducted exclusively among healthy adults aged 18 to 60 years, excluding clinical populations such as individuals with chronic respiratory conditions or post-surgical patients, limiting its generalizability. Additionally, the testing phase focused on short-term assessments without evaluating long-term outcomes, necessitating further longitudinal studies.

The absence of a control group limits comparative analysis against existing technologies, and convenience sampling may introduce selection bias despite an adequate sample size. Finally, while user satisfaction was measured using QUEST 2.0, qualitative feedback from participants was not fully analyzed, leaving opportunities to gain richer insights into user experiences and preferences.

Conclusion

This multiphasic study successfully developed and validated an innovative breathing retraining device that combines training and measurement functionalities. By enhancing inspiratory volume with visual biofeedback and providing precise volumetric data, the device bridges the gap between conventional spirometers and respiratory trainers. Its dual-purpose design offers a next-generation solution for respiratory therapy, pulmonary rehabilitation, and sports medicine, benefiting both clinical and home-based users.

Future research will focus on evaluating its long-term efficacy in clinical populations and exploring digital enhancements to further broaden its applications.

Declarations

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Author contributions

Conceptualization, P.D. and P.R.; Methodology, P.D.; Software, C.P.; Validation, P.D., P.R. and K.P.; Formal Analysis, P.D. and P.R.; Investigation, P.D.; Resources, P.D.; Data Curation, P.D.; Writing – Original Draft Preparation, P.D.; Writing – Review & Editing, P.D.; Visualization, P.D.; Supervision, P.D.; Project Administration, P.D. and K.P.; Funding Acquisition, P.D.

Conflicts of interest

The authors declare no conflicts of interest relevant to this study.

Data availability

The datasets generated and/or analyzed during the current study are not publicly available because the patent for the prototype device is published but not yet granted. Publishing the data at this stage would compromise the patent process. However, the data are available from the corresponding author on reasonable request.

Ethical approval

The ethical approval was acquired from the Institutional Ethics Committee, School of Physiotherapy, RK University (ECR/259/Indt/GJ/2016/RR-21).

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REVIEW PAPER

Comparative efficacy of topical microbicides in the prevention of HIV transmission – results from a systematic review and network meta-analysis

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ABSTRACT

Introduction and aim. Preventing new HIV infections is crucial, particularly for women and girls at high risk. Vaginal microbicides offer a female-controlled HIV prevention method. This systematic review evaluated the comparative efficacy of topical microbicides in preventing HIV transmission.

Material and methods. Electronic databases were searched up to May 2024 for randomized controlled trials (RCTs) comparing topical microbicides versus placebo/no treatment in sexually active women. The primary outcome was the incidence of HIV. A random effects network meta-analysis (NMA) was employed. Relative ranking was assessed using surface under the cumulative ranking curve (SUCRA) probabilities.

Analysis of literature. Thirteen RCTs were included in the review comparing the dapivirine ring, the tenofovir gel, BufferGel, PRO 2000, Carraguard, cellulose sulfate, or SAVVY against placebos. Compared to placebo, only dapivirine significantly reduced HIV incidence (risk ratio (RR) 0.71 [95% CI 0.56 to 0.91]). Dapivirine was superior to BufferGel (RR 0.61 [95% CI 0.39 to 0.94]) and SAVVY (RR 0.52 [95% CI 0.28 to 0.97]). Dapivirine ranked highest in efficacy (SUCRA=0.93), followed by tenofovir (SUCRA=0.76). In general, consistent network results with some small study effects.

Conclusion. This study supports the use of the vaginal dapivirine ring for HIV prevention over SAVVY or BufferGel. More high-quality trials are needed to validate the efficacy of tenofovir gel.

Keywords. HIV, topical microbicides, vaginal microbicides

Introduction

Human immunodeficiency virus (HIV) infections remain a global health challenge, particularly in regions with high prevalence rates and limited access to prevention and treatment interventions. In 2022, approximately 39 million people lived with HIV, with 1.3 million new infections and 630,000 reported deaths reported. Of particular concern, in 2022, globally, 46% of all new HIV infections occurred among women and girls, with approximately 4,000 adolescent girls and young women aged 15–24 years who became infected with HIV every week.[1]

To control the HIV epidemic, the prevention of new infections is of crucial importance. Although preexposure

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prophylaxis (PrEP) is an effective option, it requires strict daily adherence to oral medications.[2] Another approach, male-controlled prevention methods, such as condoms and circumcision, offer limited power to women in managing their own exposure to HIV infection. Other prevention methods, such as postexposure prophylaxis and behavioral changes, also exist, but preventing HIV transmission remains a major public health challenge, particularly with respect to sexually active women.

Microbicides are defined by the World Health Organization (WHO) as "compounds that can be applied inside the vagina or rectum to protect against sexually transmitted infections (STIs), including HIV".[3] Vaginal microbicides, in particular, represent a female-controlled approach to prevent HIV, especially in situations where women are unwilling or unable to negotiate condom use. Microbicides would empower women to protect themselves, because they are a potential preventive option that they can easily control themselves and, most importantly, do not require the cooperation, consent, or even knowledge of the partner. Various topical microbicide formulations are available and the field is advancing with recent developments, particularly the role of nanotechnology, specifically dendrimers, in new formulations, which have shown promise in preclinical studies.[4,5]

In terms of clinical studies, numerous randomized controlled trials (RCTs)[6] have been conducted to evaluate the efficacy and safety of a variety of topical microbicide formulations including surfactants (SAVVY, nonoxynol-9), vaginal defense enhancers (BufferGel), entry inhibitors (Carragurard and PRO 2000) and antiretroviral drugs (tenofovir gel, dapavirine ring). In 2021, a Cochrane systematic review was published on the efficacy of topical microbicides in preventing sexually transmitted infections. The review included 12 trials with 32,464 participants.[6] The trials evaluated a wide range of compounds and delivery mechanisms, including gels, creams and intravaginal rings, each with its unique pharmacokinetic and pharmacodynamic properties. In the review, dapivirine demonstrated potential to prevent HIV, while other microbicides had limited success. Dapivirine may be particularly attractive due to its use in slow-release devices, which are effective for longer durations compared to vaginal gels and creams that must be applied before and/or after each sexual encounter.[7] Overall, while some of the trials produced encouraging results, others were inconclusive or even contradictory, resulting in a fragmented evidence base that hinders the identification of optimal microbicide candidates to be recommended for widespread distribution and use.

Aim

A major challenge in synthesizing evidence from existing RCTs is the lack of head-to-head comparisons of different microbicide formulations. Typically, trials evaluate a single candidate microbicide against a placebo, making it difficult to compare the relative effectiveness of different interventions. Consequently, there is a pressing need in this situation to use a comprehensive comparative analysis method that integrates data from multiple trials and allows indirect comparisons, thereby ranking available treatment options based on their efficacy. In this systematic review and network meta-analysis, our aim was to comprehensively evaluate the efficacy of available topical microbicides in preventing HIV transmission.

Material and methods

The protocol for this study was registered with Open Science Framework (https://osf.io/5fknw). This review followed the PRISMA extension statement for systematic reviews that incorporate network meta-analyses.[8]

Search strategy and study selection

We identified relevant studies by systematic search of PubMed, EMBASE, and the Cochrane CENTRAL Register of Controlled Trials from January 2020 to the 7th of May 7, 2024. The search was restricted to studies published from 2020 onward because studies published up to 2019 could be identified from previously published systematic reviews.[6] In addition, we manually checked the reference lists of published systematic reviews. In this study, we did not consider conference abstracts. The general search strategy is provided in Appendix S1. Two reviewers (E.L. and K.K.) independently performed screening of titles and abstracts for relevance and then selected the studies for inclusion after examining the full text of the potentially eligible articles. Any discrepancies were resolved by discussion with a third reviewer (S.V.). We included randomized controlled trials (RCTs), which followed participants for at least 12 months and compared the use of topical microbicides including detergent-like products (surfactants), vaginal defense enhancers, entry inhibitors, and antiretroviral drugs with placebo or without treatment. Eligible participants were sexually active nonpregnant heterosexual women (i.e. women who have sex with men), 16 years and above in any setting, who had no laboratory-confirmed HIV at baseline. Studies investigating nonoxynol-9 were excluded from the review because current evidence from a WHO report indicates that nonoxynol-9-containing spermicides do not protect against HIV infection and may even increase the risk of HIV infection in women who use these products frequently.[9] The outcome of interest in this review was laboratory-confirmed incidence of HIV. We excluded quasirandomized trials because such studies produce effect sizes that indicate more extreme benefits when compared with randomized trials.[10]

Data extraction and quality assessment

Data extraction was performed independently by two reviewers (EL and N.N.) using standard data extraction

forms. For the result, we used the initial number of participants randomized to each trial arm and performed the analyzes regardless of how the authors of the original trials had analyzed the data (intention-to-treat principle).[10] Two reviewers (EL and S.S.) independently assessed the risk of bias within each study using the Cochrane Risk of Bias tool.[11] The tool covers the following domains: selection bias (random sequence generation, allocation concealment), performance bias (blinding of participants and personnel), detection bias (blinding of outcome assessment), attrition bias (incomplete outcome data), reporting bias (selective reporting) and other bias. Each domain was assessed and categorized into low, high, or unclear risk of bias. Discrepancies were resolved by consensus. Each study will be classified with an summary risk of bias assessment according to Cochrane's criteria:[11] "low risk of bias" if the study is assessed as low risk across all key domains; "unclear risk of bias" if the study is assessed with low or unclear risk across all key domains; and "high risk of bias" if the study is assessed with a high risk of bias in one or more key domains.

Data synthesis and statistical analysis

The outcome measure was estimated as the risk ratio (RR), which is the ratio between the incidences of HIV in the intervention arm and those in the control arm along with a 95% confidence interval (CI). For direct comparisons, we performed standard pairwise meta-analyses using the DerSimonian and Laird random effects model to estimate the pooled effect size.[12] If a direct comparison was based on two or more trials, we assessed heterogeneity between trials using I^2 statistics. We used a random-effects NMA using a consistency model within a frequentist approach to incorporate indirect evidence with direct evidence.[13] 'Control or placebo' was used as a common comparator in the network model. The inconsistency assumption was evaluated using the global inconsistency test by fitting design-by-treatment into the inconsistency model.[13] The network inconsistency assumption, which refers to a disagreement between direct and indirect estimates, was evaluated using the loop-specific approach.[14] We used surface under the cumulative ranking curve (SUCRA), which estimates the probabilities for all treatments to obtain a treatment ranking based on efficacy.[15] Higher SUCRA scores (ranging from 0 to 1) indicated that the intervention has a high likelihood of being best. We examined the effects of the small study using a comparison adjusted for comparison.[13] To assess the robustness of our findings, we performed sensitivity analyzes excluding small-sized (<25th percentiles) trials[16] and high risk bias trials. For statistical analysis, we used Stata version 16.0 (StataCorp, College Station, TX, USA). We used the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) approach, adapted to network meta-analysis, to rate the quality of evidence.

Analysis of the literature

The database search resulted in 1440 records. After eliminating 15 duplicates, 1425 titles and abstracts were selected based on the predefined eligibility criteria. Subsequently, 16 records underwent further screening, but all were excluded because they did not assess HIV incidence as an outcome or investigated oral pre-exposure prophylaxis medications only. Consequently, the 13 studies incorporated into this network meta-analysis were those previously included in other systematic reviews (Fig. 1).

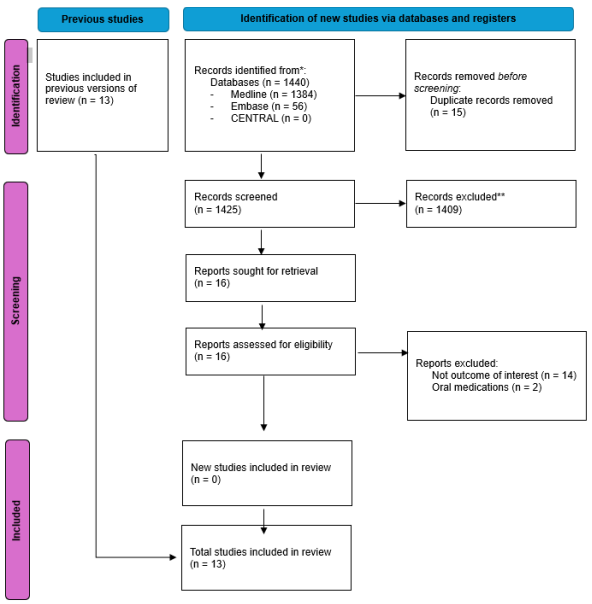


Fig. 1. PRISMA flow diagram

Characteristics of studies

The characteristics of the 13 included studies are summarized in Table 1. All 13 trials were parallel RCTs conducted in sub-Saharan Africa, one study also having a site in the USA and another in India.[7,17-28] The vaginal microbicides tested included BufferGel and PRO 2000 (1 trial, 3101 women), Carraguard (2 trials, 6602 women), cellulose sulfate (2 trials, 3069 women), C31G (SAVVY) (2 trials, 4295 women), dapivirine ring (2 trials, 4588 women), PRO 2000 (1 trial, 9385 women), and tenofovir gel (3 trials, 4958 women).[17,18,25-28] All microbicides were compared to placebo in these RCTs.

Two trials used vaginal rings,[7,20] while the others used vaginal gels. In the ring trials, women in the intervention group used vaginal rings containing 25 mg of dapivirine, worn continuously for a month, and replaced at each monthly follow-up visit. In two trials,[25,26] women used 1% tenofovir gel, inserting one dose within 12 hours before and another after vaginal sex, with a maximum of two doses over a 24-hour period. In the third study,[22] the tenofovir gel was inserted up to one hour before intercourse. The gels were supplied in prefilled single-use applicators. For the five other types of vaginal microbicide gels (cel-

lulose sulfate, SAVVY, PRO 2000, BufferGel, and Carraguard), women were instructed to insert the gel within an hour before vaginal intercourse. All of those gels were provided in pre-filled single-use applicators.

Figures 2 and 3 show the risk of bias assessment for each included study. Most of the studies were rated as overall low risk of bias. The two studies rated as overall high risk of bias were due to incomplete outcome data.

Fig. 2. Risk of bias assessment for each included study

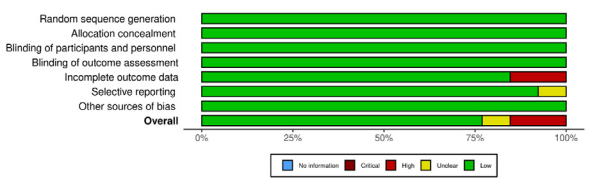


Fig. 3. Summary of the risk of bias assessment for included studies

Network meta-analysis findings: HIV incidence

All 13 RCTs provided dichotomous data for HIV incidence. The resulting network plot is provided in Figure 4. The network meta-analysis suggested that, compared to placebo, dapivirine (RR, 0.71 [95%CI, 0.56 to 0.91]) statistically significantly reduces the risk of acquiring HIV infection (Fig. 5). The other microbicides, when compared with placebo, appear to result in little to no difference in the risk of acquiring HIV. In this category were tenofovir (RR 0.83, [95% CI 0.66, 1.03]), Carraguard (RR, 0.89 [95% CI 0.68,1.16]), PRO 2000 (RR, 0.92 [95%CI, 0.71,1.19]), BufferGel (RR, 1.17 [95%CI, 0.81,1.70]), cellulose sulphate (RR, 1.20 [95%CI, 0.72,1.99]), and SAVVY (RR, 1.37 [95%CI, 0.77, 2.44]) (Fig. 5). These findings are similar to those obtained using standard pairwise meta-analyzes (Fig. 5 and 6).

Table 1. Summary of characteristics of included studies

ID	Author, year	Country	Age	Intervention	Comparator	Trial discontinued
1	Abdool Karim, 2010	South Africa	18 to 40 years	Tenofovir 1% gel and condom	Placebo gel and condom	No
2	Abdool Karim, 2011	Malawi, South Africa, Zambia, Zimbabwe, and USA	18 years and older	1. BufferGel 2. 0.5% PRO 2000	1. Placebo gel 2. No gel	No
3	Baeten, 2016	Malawi, South Africa, Uganda, and Zimbabwe	18 to 45 years	Dapivirine 25mg vaginal ring	Placebo vaginal ring	No
4	Delany-Moretlwe, 2018	South Africa	18 to 30 years	Tenofovir 1% gel	Placebo gel	No
5	Feldblum, 2008	Nigeria	18 to 35 years	C31G (SAVVY) 1.0% gel and condom	Placebo gel	Yes
6	Halpern, 2008	Nigeria	18 to 35 years	Cellulose sulphate and condom	Placebo gel and condom	Yes
7	Marrazzo, 2015	South Africa, Uganda, Zimbabwe	18 to 45 years	Tenofovir 1% gel	Placebo gel	Yes
8	McCormack, 2010	South Africa, Uganda, Zambia, and Tanzania	18 years or older, (> 16 years in Tanzania and Uganda)	1. PRO 2000 2% gel and condom2. PRO 2000 0.5% gel and condom	Placebo gel and condom	Yes – 2% gel
9	Nel, 2016	South Africa, Uganda	18 to 45 years	Dapivirine 25mg vaginal ring	Placebo	No
10	Peterson, 2007	Ghana	18 to 35 years	C31G (SAVVY) 1% and condom	Placebo and condom	Yes
11	Skoler-Karpoff, 2008	South Africa	16 years and older	Carraguard gel and condom	Placebo gel and condom	No
12	Van Damme, 2008	South Africa, Uganda, Benin, and India	18 years and older	Cellulose sulphate 6% gel and condom	Placebo gel and condom	Yes
13	Carraguard, Phase II South Africa Team 2010	South Africa	18 years and older	Carraguard	Placebo	No

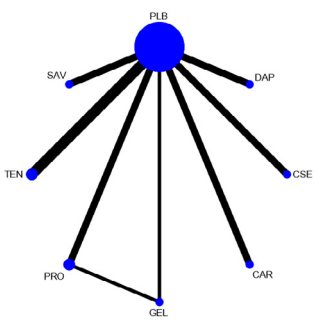


Fig. 4. Network plot, notes: The size of the nodes corresponds to the number of trials that study the treatments. Directly comparable treatments are linked with a line; The thickness of the line corresponds to the number of trials that assess the comparison, DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir

When we evaluated comparative efficacy among different microbicides, dapivirine was superior in reducing the risk of HIV infection compared to BufferGel (RR, 0.61 [95%CI, 0.39, 0.94]) and SAVVY (RR, 0.52 [95%CI, 0.28, 0.97]) (Fig. 4). No statistically significant differences were observed between other interventions. The SUCRA plot revealed that dapivirine (SUCRA=0.93) was ranked first for efficacy followed by tenofovir (SUCRA=0.76), Carraguard (SUCRA=0.64), PRO 2000 (SUCRA=0.60), BufferGel (SUCRA=0.25), cellulose sulfate (SUCRA = 0.25) and SAVVY (SUCRA=0.15) (Fig. 7).

CAR	NA	NA	NA	NA	NA	NA	0.89 (0.79,1.11)
0.75 (0.42,1.32)	**CSE**	NA	NA	NA	NA	NA	1.15 (0.59,2.25)
1.25 (0.87,1.80)	1.68 (0.96,2.95)	**DAP**	NA	NA	NA	NA	0.71 (0.57,0.89)
0.76 (0.48,1.20)	1.02 (0.54,1.90)	0.61 (0.39,0.94)	**GEL**	1.49 (0.99,2.22)	NA	NA	1.05 (0.73,1.52)
0.97 (0.67,1.41)	1.30 (0.74,2.29)	0.78 (0.54,1.11)	1.28 (0.87,1.89)	**PRO**	NA	NA	0.88 (0.63,1.25)
0.65 (0.34,1.23)	0.87 (0.40,1.88)	0.52 (0.28,0.97)	0.86 (0.43,1.70)	0.67 (0.36,1.26)	**SAV**	NA	1.35 (0.70,2.58)
1.08 (0.76,1.53)	1.45 (0.83,2.51)	0.86 (0.62,1.20)	1.42 (0.92,2.19)	1.11 (0.79,1.55)	1.66 (0.89,3.08)	**TEN**	0.82 (0.65,1.05)
0.89 (0.68,1.16)	1.20 (0.72,1.99)	0.71 (0.56,0.91)	1.17 (0.81,1.70)	0.92 (0.71,1.19)	1.37 (0.77,2.44)	0.83 (0.66,1.03)	**PLB**

Fig. 5. Pairwise (upper right portion) and network (lower left portion) meta-analytic results for HIV incidence, notes: results are expressed as risk ratios (95% CI). For pairwise meta-analyses, RR <1 indicates that the treatment specified in the row is more effective. For the NMA, RR <1 indicates that the treatment specified in the column is more effective. Green shaded results indicate statistical significance, DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir

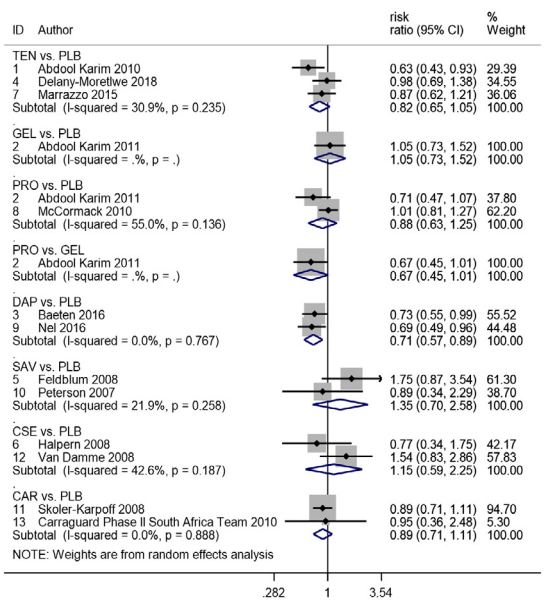


Fig. 6. Results of the meta-analysis of the pairwise analysis, DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir

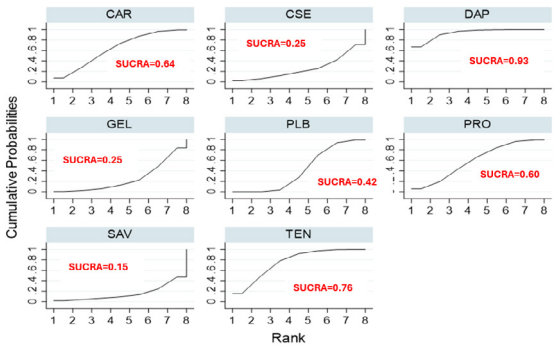


Fig. 7. SUCRA ranking of efficacy, DAP – dapavirine, CAR carraguard, CSE – cellulose sulfate, GEL – BufferGel, PLB – placebo, PRO PRO 2000, SAV – SAVVY, TEN – tenofovir

Network consistency and small study effects

The global inconsistency using the 'design-by-treatment' interaction model and the loop-specific approach did not demonstrate evidence of inconsistency (Fig. 8). The comparison-adjusted funnel plot demonstrated some evidence of small study effects (Fig. 9).

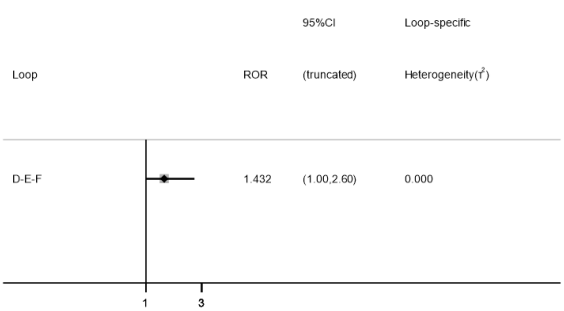
Test of global inconsistency

```
Testing for inconsistency:
 ( 1)  [_y_F]des_EF = 0

           chi2(  1) =    2.22
         Prob > chi2 =    0.1360
```

Note: Global inconsistency in a network can be evaluated and detected using inconsistency models. These models differ from the consistency models by relaxing the consistency equations and allowing intervention effects to vary when estimated directly and indirectly. A p-value less than 0.05 indicates the presence of inconsistency.

Loop-specific approach



Note: The loop-specific approach considers only triangular and quadratic loops. In this case, only one triangular loop is formed (Gel-PRO-PLB) as in Figure 1 (network plot). The plot shows that only one triangular loop is formed and there is no statistically significant inconsistency as the confidence intervals for RoRs are compatible with zero inconsistency (RoR is close to 1)

Fig. 8. Inconsistency testing

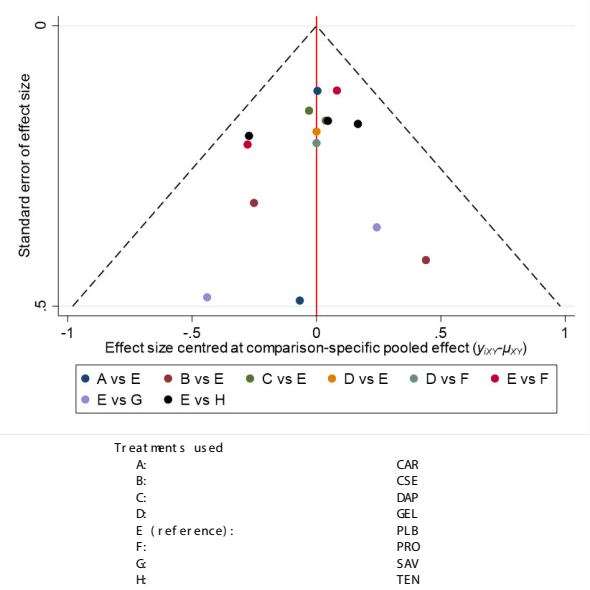


Fig. 9. Comparison-adjusted funnel plot, DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir

Sensitivity analysis and GRADE summary of evidence

The results were not affected by the sensitivity analyzes based on excluding small-sized studies and trials of high risk of bias (Table 2). Overall, the quality of evidence based on the application of GRADE criteria to the findings of the NMA was generally rated as very low to moderate quality (Table 3). We had moderate confidence in estimates supporting the use of vaginal dapivirine compared to placebo in terms of reducing the risk of HIV acquisition.

Table 2. Sensitivity Analyses for primary outcome*

Comparison	Primary	Excluding high ROB trials	Excluding small sized studies
		Risk ratio (95% CI)	
CAR vs PLB	0.89 (0.68, 1.16)	0.89 (0.69, 1.15)	0.88 (0.67, 1.19)
CSE vs PLB	1.20 (0.72, 1.99)	1.54 (0.82, 2.90)	1.30 (0.62, 2.11)
DAP vs PLB	0.71 (0.56, 0.91)	0.71 (0.56, 0.90)	0.71 (0.53, 0.94)
GEL vs PLB	1.17 (0.81, 1.70)	1.18 (0.82, 1.69)	1.13 (0.80, 1.75)
PRO vs PLB	0.92 (0.71, 1.19)	0.92 (0.71, 1.19)	0.90 (0.68, 1.30)
SAV vs PLB	1.37 (0.77, 2.44)	0.89 (0.34, 2.30)	1.32 (0.67, 2.98)
TEN vs PLB	0.83 (0.66, 1.03)	0.83 (0.67, 1.03)	0.80 (0.61, 1.34)

* DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir

The GRADE approach adapted to network meta-analysis was used to rate the quality of evidence into four levels: high, moderate, low, and very low quality. In this approach, direct estimates from RCTs rated at high quality and can be graded down to moderate, low, and very low quality based on risk of bias, indirectness, imprecision, inconsistency, and publication bias. The rating of the quality of the indirect estimates starts at the lowest rating of the two direct estimates that contribute to the indirect estimate of the comparison of interest as first-order loops. In the presence of intransitivity, indirect estimate can be further rate down from the lower of the confidence ratings of the contributing direct comparisons. Finally, if both direct and indirect evidence is available, then the higher of the two quality ratings can be assigned to the quality rating for NMA estimates.

Table 3. Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) Summary of evidence*

Comparisons	Direct evidence (from pairwise meta-analysis)		Indirect evidence (from node-splitting)		Network meta-analysis	
Partial/ complete resolution	RR [95% CI]	Quality of evidence	RR [95% CI]	Quality of evidence	RR [95% CI]	Quality of evidence
CAR vs PLB	0.89 (0.79, 1.11)	Low	NA	NA	0.89 (0.68, 1.16)	Low
CSE vs PLB	1.15 (0.59, 2.25)	Very low	NA	NA	1.20 (0.72, 1.99)	Very low
DAP vs PLB	0.71 (0.57, 0.89)	Moderate	NA	NA	0.71 (0.56, 0.91)	Moderate
GEL vs PLB	1.05 (0.73, 1.52)	Low	-0.7705791 (SE 0.4411362)#	Very low	1.17 (0.81, 1.70)	Very low
PRO vs PLB	0.88 (0.63, 1.25)	Low	NA	NA	0.92 (0.71, 1.19)	Low
SAV vs PLB	1.35 (0.70, 2.58)	Very low	NA	NA	1.37 (0.77, 2.44)	Very low
TEN vs PLB	0.82 (0.65, 1.05)	Low	NA	NA	0.83 (0.66, 1.03)	Low
DAP vs GEL	NA	NA	0.61 (0.39, 0.94)	Low	0.61 (0.39, 0.94)	Low
DAP vs SAV	NA	NA	0.52 (0.28, 0.97)	Low	0.52 (0.28, 0.97)	Low
DAP vs TEN	NA	NA	0.86 (0.62, 1.20)	Low	0.86 (0.62, 1.20)	Low

Randomized controlled trials (RCTs) without important limitations are rated high on the GRADE scale. However, the above results are from long-term observational follow-up of RCTs with proper random sequence generation during the intervention phase. Hence, the initial quality rating starts with moderate level.
a. risk of bias
b. imprecision
c. Imprecision (close to null effect)
d. Based on rating of the two pairwise estimates that contribute to the indirect estimate (first-order loop)
e. Inconsistency
f. Intransitivity
g. Indirectness

* DAP dapavirine, CAR – carraguard, CSE cellulose sulphate, GEL – BufferGel, PLB placebo, PRO – PRO 2000, SAV SAVVY, TEN – tenofovir, # – log form

Discussion

This systematic review and network meta-analysis evaluated the efficacy of available topical microbicides in preventing HIV transmission. Despite our search for new RCTs from 2020 to 2024, no new studies were identified. The absence of recent publications may be attributed to several factors. There have been no significant breakthroughs in the development of widely available topical microbicides for HIV prevention, and challenges related to efficacy, safety, and user acceptability continue to impede their broad adoption.[29,30] Furthermore, recent research trends, as highlighted in the AIDS 2024 Research Roundup,[31] highlighted a focus toward long-acting injectable PrEP rather than topical microbicides. However, it is crucial to recognize that the field is evolving. For example, a safety study for a vaginal ring containing dapivirine and levonorgestrel is currently underway,[32] and several preclinical studies are investigating various combinations of antiretroviral drugs (eg, dapivirine, islatravir) with hormonal contraceptives (e.g., ethinylestradiol, etonogestrel) that demonstrated potential.[30] There may also be ongoing clinical trials or preclinical studies not yet widely published. Further exploration and continued monitoring of emerging studies are essential to understanding the future direction of research in this area.

Conventional meta-analysis has previously indicated that the vaginal dapivirine ring may be an effective intervention for preventing HIV.[6,33] Other interventions such as tenofovir, carraguard, cellulose sulfate, BufferGel, PRO 2000 and SAVVY have also been tested for this purpose. But in the absence of adequate head-to-head trials, their relative efficacy remains unclear. Conventional pairwise meta-analyzes offer only limited information because they compare interventions in pairs and do not use all available data to inform decision-making optimally. In contrast, NMA combines direct and indirect evidence from a network of RCTs to compare the efficacy of all available interventions. Thus, NMA improves the precision of the efficacy estimates, even if there are no direct comparisons. This is the first systematic review with NMA to assess the comparative effectiveness of topical microbicides for HIV prevention.

Overall, the findings of this NMA revealed that dapivirine ranks first for efficacy and was the only statistically significant intervention compared to placebo in preventing HIV. This aligns with the findings of a Cochrane systematic review, which indicated that dapivirine probably reduces the risk of developing HIV infection, while other topical microbicides may result in little or no difference in the risk of acquiring HIV.[6] Additionally, this NMA contributes strong evidence that dapivirine is statistically significantly more effective than SAVVY and BufferGel.

The only current licensed pharmaceutical HIV prevention methods are injectables or daily oral pills for PrEP.[2,34] While these PrEP methods are safe and effective when used as prescribed, maintaining a daily pill regimen or receiving regular injections can be challenging for some people. Consequently, other forms of HIV prevention, such as microbicides, are being developed and studied. Microbicides may offer specific advantages for some women, as they can be used without requiring negotiation with a sexual partner, making them preferable to condoms for HIV prevention. Given the high risk of HIV for women and girls in many regions,[1] it is crucial to have an effective, appealing, women-initiated HIV prevention method. Almost half of the global population living with HIV is women, who primarily contract the virus through heterosexual contact.[35,36] Evidence, including findings from this NMA and previous systematic reviews,[6,33] suggests that the vaginal dapivirine ring likely reduces the risk of HIV acquisition in heterosexual women.

One main issue with topical microbicides is adherence. The dapivirine ring, which is longer-acting and only needs to be left in place for a month, has potential advantages over coitally dependent or daily-use products. However, data from the two dapivirine trialsindicated poor adherence of the rings, as measured by residual amounts of dapivirine in those rings.[37,38] Reports of non-adherence included removing the rings for sex, bathing, in menses or extended periods, and reinserting them shortly before clinic visits. Reasons reported for non-adherence included hygiene concerns, external influences, and interest solely in study benefits, with some women removing the ring to get pregnant or use other vaginal products. Despite these challenges, two expanded open-label trials involving HIV-negative women who had participated in previous phase 3 trials demonstrated that the dapivirine ring is acceptable. These trials reported a reduction in the risk of HIV seroconversion under conditions closer to real-world settings, with less frequent clinic visits and HIV tests than the more rigorously controlled RCTs.[37] These findings support the probability that improved adherence will occur once women are aware of the ring's efficacy and safety of the ring and, in turn, suggest that the dapivirine ring is a feasible and acceptable HIV prevention method for women.

In 2020, the European Medicines Agency issued a positive scientific opinion on the monthly dapivirine vaginal ring.[39] Subsequently, the WHO recommended the ring as part of combination prevention strategies for women at high risk of acquiring HIV.[40] The dapivirine ring has been approved in Zimbabwe and several other countries in eastern and southern Africa, with additional approvals pending.[41] If further approved by national regulatory agencies, the monthly dapivirine ring would offer women a discreet and long-acting HIV prevention option that they can control, enhancing its potential as

a vital tool in reducing HIV transmission among women worldwide. Another key consideration is the economic implications, as cost-effectiveness studies for the dapivirine ring are currently limited.[42,43] More research is needed to evaluate its cost-effectiveness in different settings, considering factors such as user demand and adherence.

In this NMA, tenofovir ranked second but was not statistically significant. The comparative efficacy of dapivirine and tenofovir is also not statistically significant. The direction of effect indicates a beneficial effect of tenofovir in preventing HIV, although this finding did not achieve statistical significance. As such, additional high-quality clinical trials are needed to further evaluate the effectiveness of tenofovir.

Study limitations

This review is the first to integrate data from multiple trials into a network meta-analysis to evaluate the efficacy of topical microbicides in preventing HIV transmission. However, several limitations must be acknowledged. Our search strategy was limited to English-language publications only, thereby excluding relevant studies published in other languages. Findings should be interpreted with caution due to heterogeneity between studies, which arises from variations in microbicide formulations, study locations, sample sizes, and follow-up durations. Most of the trials were conducted in low and middle-income countries, primarily in Africa, with varying durations of follow-up. Given the lower incidence of HIV among women in high-income countries, studies assessing the effectiveness of these interventions may be predominantly limited to sites in low-income countries. Thus, the generalizability of the findings in various countries or regions with diverse socioculturral contexts is limited. The most promising interventions to prevent HIV infection were dapivirine and tenofovir, respectively, both exclusively tested in African trial sites. However, the limited sample size and number of studies may restrict the broad applicability of these findings. Furthermore, there is a potential for publication bias, with some evidence suggesting small study effects.

Conclusion

In conclusion, the findings of this review support the use of the dapivirine vaginal ring for the prevention of HIV, demonstrating its superiority over SAVVY and BufferGel. Integration of dapivirine into comprehensive HIV prevention programs holds promise for significantly reducing HIV transmission rates, particularly in regions where women face disproportionate risks. Although tenofovir gel shows promise, more research and high-quality clinical trials are necessary to confirm its efficacy.

Supplementary materials

S1: Search Strategy

Declarations

Funding

No funding was received for conducting this study.

Author contributions

Conceptualization, E.L. and S.V.; Methodology, E.L. and S.V.; Formal Analysis, E.L. and S.V.; Investigation, E.L., S.S., S.K., N.N., and S.V.; Data Curation, S.V.; Writing – Original Draft Preparation, E.L., F.S., and S.V.; Writing – Review & Editing, E.L., S.S., S.K., F.S., N.N. and S.V.; Visualization, S.V.; Project Administration, E.L.

Conflicts of interest

The authors have no competing interests to declare thatare relevant to the content of this article.

Data availability

All data relevant to the review are included in this published article and its supplementary information files. In this review, no new data was generated in this review, as it is based on previously published randomized controlled trials.

Ethics approval

Not applicable.

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REVIEW PAPER

Probiotics for the prevention of antibiotic-associated diarrhea – an umbrella review of meta-analyses of randomized controlled trials

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ABSTRACT

Introduction and aim. Antibiotic therapies induce diarrhea by disrupting the intestinal microbiota, prompting research into probiotics to prevent antibiotic-associated diarrhea (AAD). The aim of this study was to systematically identify and summarize meta-analyses of randomized controlled trials (RCT) on probiotics for AAD prevention.

Material and methods. Databases including PubMed, EMBASE, Epistemonikos, and the Cochrane Database were searched up to December 11, 2023. Systematic reviews and meta-analyses of RCTs on probiotics for AAD prevention in any age group were included. Meta-analyses were re-performed to calculate pooled risk ratios (RR) with 95% confidence intervals (CI). Evidence quality was assessed using GRADE criteria.

Analysis of the literature. The review included 16 articles with 39 unique meta-analyses. Probiotics reduced AAD risk across various groups: adults (RR 0.47, 95% CI 0.40–0.56), all ages (RR 0.58, 95% CI 0.50–0.68), and outpatients (RR 0.49, 95% CI 0.36–0.66) with a moderate level of evidence. For the use of any probiotics in pediatrics, the initial high-quality evidence (RR 0.48, 95% CI 0.44–0.63) was downgraded to moderate after a sensitivity analysis excluding small studies.

Conclusion. Probiotics are beneficial in preventing AAD, but evidence quality varies from low to moderate. High-quality trials are needed to identify the most effective probiotic species and strains, dosages, and target patient populations.

Keywords. antibiotic-associated diarrhea, *Lactobacillus*, probiotics

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Introduction

Diarrhea is a common adverse effect of antibiotic therapies, primarily attributed to significant disruptions in the intestinal microbiota induced by most antibiotics.[1] The direct adverse effects of antibiotics on the intestines involve disruptions in digestive function stemming from diminished concentrations of gut bacteria or the proliferation of pathogenic microorganisms.[1,2] Antibiotic-associated diarrhea (AAD) is characterized by the occurrence of three or more unformed stools per day, manifesting within hours to up to 8 weeks after initiating antibiotic treatment.[1] Studies indicate that the prevalence of AAD ranges from 5% to 35% among individuals exposed to antimicrobials, with variations based on the antibiotic class, host health, and susceptibility to pathogens.[3] In most cases, AAD is benign and can be resolved with symptomatic treatment. However, when AAD is attributable to a *Clostridium difficile* infection, symptoms tend to be more severe, potentially leading to a fulminant, relapsing, and occasionally fatal pseudomembranous colitis.[3,4] The consequences of AAD extend beyond the immediate health impact, contributing to prolonged hospital stays and increased medical costs, particularly in cases involving *C. difficile* infection.[3,5-7]

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'.[8] Given that AAD primarily arises from an imbalance in the natural intestinal flora, research has concentrated on exploring the advantages of introducing living organisms, such as probiotics, to reinstate the normal flora. Various strains from bacterial species have been tested in clinical studies for the prevention and/or treatment of AAD including those from the *Bacillus, Bifidobacterium, Clostridium, Lactobacillus, Lactococcus, Leuconostoc,* and *Streptococcus* genera. Additionally, the fungi *Saccharomyces boulardii* has been investigated for its potential impact on AAD.[9] Among the probiotics, *Lactobacillus rhamnosus* strain GG and *S. boulardii* strain CNCM I-745 were the most extensively studied.[10,11]

Several systematic reviews and meta-analyses have demonstrated the benefits of probiotics in the prevention of AAD in different populations.[12–16] Umbrella reviews make it feasible to summarize the evidence from multiple meta-analyses on the same topic and enable the grading of evidence.[17–19] To date, there has been little synthesis of the strength and quality of this evidence in aggregate. This umbrella review aims to systematically identify relevant meta-analyses of randomized controlled trials (RCTs) of probiotics for the prevention of AAD, summarize their findings, and assess the strength of evidence.

Material and methods

The protocol of this review was registered with PROSPERO (CRD42023465792). We report following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA).[20]

Search strategy and eligibility criteria

We conducted a comprehensive search on PubMed, EMBASE, Epistemonikos, and the Cochrane Database of Systematic Reviews (CDSR) from database inception to December 11, 2023 (S1). We also manually searched the cited references of the retrieved articles and reviews. The process of study selection was independently carried out in EndNote by two reviewers. After removing duplicates, the titles and abstracts of the identified articles were screened for relevance. Full-text articles of potentially eligible studies were retrieved and assessed against the eligibility criteria. Any discrepancies in the selection process were resolved through discussion with a third reviewer.

We included studies that fulfilled the following eligibility criteria: systematic reviews and meta-analyses of RCTs investigating the effects of probiotics for the prevention of AAD in any population of any age. No restriction was applied for comparators. In instances where multiple meta-analyses addressed the same research question, we selected the meta-analysis with the largest dataset, as previously described.[17,21,22] Articles without full-text and meta-analyses that did not provide sufficient or adequate data for quantitative synthesis were excluded.

Data extraction and quality assessment

Data extraction and quality assessment were conducted independently by two reviewers. Discrepancies were resolved through consensus by engaging in discussions with the third reviewer. The quality of the meta-analyses was evaluated using AMSTAR-2 (A Measurement Tool to Assess systematic Reviews), where quality of the meta-analysis is rated into four categories – high, moderate, low, or critically low.[23]

Data synthesis

Effect sizes were categorized based on the population, intervention, comparator, and outcomes to create a list of unique meta-analyses (association). For each association, we extracted effect sizes of individual studies included in each meta-analysis and re-performed the meta-analyses to calculate the pooled effect sizes as risk ratio (RR) with corresponding 95% CIs using the DerSimonian and Laird random-effects model, or the Hartung-Knapp-Sidik-Jonkman approach for meta-analyses with less than five studies.[24,25] $p<0.05$ was considered statistically significant in 2-sided tests. Heterogeneity was evaluated using the I^2 statistic. The evidence for small-study effects was assessed by the Egger regression asymmetry test.[26] $p<0.10$ was taken as statistical evidence of the presence of small-study effects. Statistical analyses were conducted using Stata version 16.0 (StataCorp, Texas, USA).

We assessed the quality of evidence per association by applying the GRADE criteria (Grading of Recommendations, Assessment, Development, and Evaluations) in five domains, including (1) risk of bias in the individual studies, (2) inconsistency, (3) indirectness, (4) imprecision, and (5) publication bias.[27] We graded the strength of evidence (high, moderate, low, and very low) using GRADEpro version 3.6.1 (McMaster University).

Sensitivity analyses

Sensitivity analyses were performed for those meta-analyses graded as high quality in the primary analysis by excluding small-size studies (<25th percentile) and excluding primary studies having a high risk of bias rated by the Cochrane's risk of bias 2 tool (RoB 2) for RCTs from the identified associations.[28,29]

Analysis of the literature

In total, we identified 1617 articles, scrutinized 78 full-text articles, and included 19 eligible articles (1.18%) for preliminary data extraction (Fig. 1). After the selection criteria for the overlapping meta-analyses were applied, 16 articles were ultimately selected for evidence synthesis.[13,15,30-43] Agreement between reviewers for eligibility of articles was excellent (κ statistic=0.8). The list of excluded articles after applying the selection criteria for the overlapping meta-analyses is provided in Table S2.

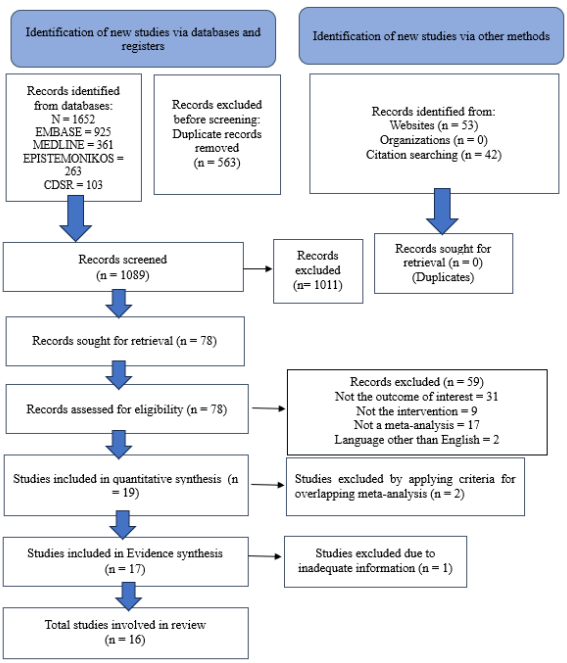

Fig. 1. PRISMA flow diagram

Description and summary of meta-analyses

A total of 39 unique meta-analyses were identified from 16 articles.[13,15,30–43] The included meta-analyses were published between 2002 and 2022. The median number of studies per meta-analysis was 10 (interquartile range [IQR], 6–22) and the median meta-analysis sample size based on 38 meta-analyses was 1847 (IQR: 795–4014). Based on the AMSTAR-2 methodological quality rating, the meta-analyses were classified as follows: six were graded as high quality (15.4%), sixteen as moderate quality (41%), thirteen as low quality (33.3%), and four as critically low quality (10.3%) (Table 1). The descriptive characteristics of meta-analyses were provided in Table 1 and Tables S3 and S4.

Grading of meta-analyses

A summary of evidence of all 39 meta-analyses is presented in Table S4. Ten were supported by a very low level of evidence (25.6%), followed by low (15 meta-analyses (38.5%)), moderate (8 meta-analyses (20.5%)), and high (1 meta-analysis (2.6%)) levels of evidence in the primary analysis. Thirty-two of the 39 meta-analyses (82.05%) were statistically significant at p≤0.05 based on random-effects models and demonstrated the use of probiotics reduce the risk of AAD (Table S4).

Three meta-analyses demonstrated that the use of probiotics as a whole reduced the risk of AAD in adults (RR 0.47, 95% CI 0.40 to 0.56), any age group (RR 0.58, 95% CI 0.50 to 0.68) and outpatients (RR 0.49, 95% CI 0.36 to 0.66) with moderate level of evidence (Figure 2). The associations between risk reduction of AAD and the use of *S. boulardii* (RR 0.50, 95% CI 0.38 to 0.64) in adults, the use of bifidobacteria (RR 0.33, 95% CI 0.29 to 0.39) in pediatrics and the use of *Lactobacillus GG* and *S. boulardii* together (RR 0.40, 95% CI 0.28 to 0.59) in any age group patients[41] were also graded as moderate level of evidence.[13,31,32,43] One meta-analysis finding was supported by high quality evidence (use of probiotics as a whole in the pediatric population (RR 0.48, 95% CI 0.44 to 0.63)) in the primary analysis.[36] However, a sensitivity analysis that excluded studies with small sizes downgraded the evidence to moderate quality (Table S5).

Discussion

This umbrella review systematically identified 39 unique meta-analyses of RCTs investigating the efficacy of probiotics for the prevention of AAD and ascertained the overall strength of evidence using GRADE approach.

Overall, findings from this umbrella review indicated that using probiotics, in general, reduces the risk of AAD. However, there is a caveat – the finding is based on a limited number of high-quality RCTs. The overall strength of evidence from this umbrella review is moderate to low. Despite the promising findings, it is essential to acknowledge that systematic reviews and meta-analyses have consistently reported favorable findings with moderate quality of evidence with regard to the effects of probiotics on the prevention AAD, primarily attributed to concerns related to trial quality, specifically the high risk of bias for some studies, and the notable differences in the spectrum of probiotics and

Table 1. Characteristics of meta-analyses*

Author, year	Population	Intervention	Comparison	Outcome	No of studies	Total participants	AMSTAR
Agamennone et al., 2018	Any age group	Probiotic dairy products	Placebo	Incidence of AAD	7	488	Critically low
Agamennone et al., 2018	Any age group	Probiotic food supplements (Non-dairy products)	Placebo	Incidence of AAD	25	3232	Critically low
Blaabjerg et al., 2017	Outpatients of any age group	Any probiotics	Placebo	Incidence of AAD	17	3631	Low
Blaabjerg et al., 2017	Outpatients of any age group	*Saccharomyces boulardii*	Placebo	Incidence of AAD	5	1139	Low
Blaabjerg et al., 2017	Outpatients of any age group	*Lactobacillus acidophilus* and *Bifidobacterium lactis*	Placebo	Incidence of AAD	2	455	Low
Blaabjerg et al., 2017	Outpatients of any age group	Any probiotics	Placebo	Incidence of WHO defined diarrhoea	7	1724	Low
Cremonini et al., 2002	Any age group	*Lactobacillus* GG, *Saccharomyces boulardii*	Placebo	Incidence of AAD	7	881	Critically low
Hempel et al., 2012	Any age group	Any probiotics	Placebo	Incidence of AAD	63	11811	Moderate
Hempel et al., 2012	Any age group	Genera blends	Placebo	Incidence of AAD	25	3446	Moderate
Hempel et al., 2012	Any age group	*Genus, Bacillus*	Placebo	Incidence of AAD	2	367	Moderate
Hempel et al., 2012	Any age group	*Genus, Enterococcus*	Placebo	Incidence of AAD	3	1448	Moderate
Hempel et al., 2012	Any age group	*Genus, Lactobacillus*	Placebo	Incidence of AAD	17	2534	Moderate
Hempel et al., 2012	Any age group	*Genus, Saccharomyces*	Placebo	Incidence of AAD	15	3940	Moderate
Jafarnejad et al., 2016	Inpatients	Any probiotics	Placebo	Incidence of AAD	22	6435	Low
Jafarnejad et al., 2016	Elderly	Any probiotics	Placebo	Incidence of AAD	5	3434	Low
Jafarnejad et al., 2016	Adults	*Lactobacillus* sp.	Placebo	Incidence of AAD	22	5828	Low
Jafarnejad et al., 2016	Adults	*Saccharomyces boulardii*	Placebo	Incidence of AAD	11	1832	Low
Jafarnejad et al., 2016	Adults	*Bifidobacteria* sp.	Placebo	Incidence of AAD	13	4511	Low
Kale-Pradhan et al., 2010	Paediatric and adult patients	*Lactobacillus*	Placebo	Risk of AAD	10	1862	Moderate
Ritchie et al., 2012	Any age group	Any probiotics	Placebo	Incidence of CDD	6	NA	High
Szajewska et al., 2005	Adults and children	*Saccharomyces boulardii*	Placebo	Incidence of AAD	5	1076	High
Szajewska et al., 20154	Adults and children	*Lactobacillus rhamnosus* GG	Placebo	Incidence of AAD	12	1499	High
Videlock et al., 2012	Any age group patients treated with antibiotics	Any probiotics	Placebo	Incidence of AAD	34	4138	Moderate
Guo et al., 2019	Paediatric patients treated with antibiotics (any dose)	Any probiotics	Placebo/control	Incidence of AAD	33	6352	High
Guo et al., 2019	Paediatric patients treated with antibiotics (5 billion CFU)	Any probiotics	Placebo/control	Incidence of AAD	20	4038	High
Guo et al., 2019	Pediatric patients treated with antibiotics	*Lactobacillus rhamnosus (strains: GG, ATCC53103 and E/N, Oxy, Pen)*	Placebo/control	Incidence of AAD	6	686	High
Kale-Pradhan et al., 2010	Pediatrics	*Lactobacillus*	Placebo	Risk of AAD	4	585	Moderate
Szajewska et al., 2015	Children	*Lactobacillus rhamnosus* GG	Placebo	Incidence of AAD	4	381	High
Szajewska et al., 2006	Pediatric inpatients or outpatients	Any probiotics	Placebo	Risk of AAD	6	766	High
Videlock et al., 2012	Pediatric patients treated with antibiotics	Any probiotics	Placebo	Incidence of AAD	10	1246	Moderate
Xu et al., 2017	Pediatrics	*Bifidobacterium*	Placebo	Risk and treatment of AAD	30	7225	Critically low
Zhang et al., 2022	Elderly on antibiotics	Any probiotic	Placebo	Incidence of AAD	8	4691	Moderate
Zhang et al., 2022	Elderly inpatients	Any probiotic	Placebo	Incidence of AAD	6	624	Moderate
Zhang et al., 2022	Elderly inpatients	Probiotic given during antibiotic treatment	Placebo	Incidence of AAD	5	420	Moderate
Avadhani et al., 2010	Adult hospitalized population	Any probiotic	Placebo	Incidence of AAD	8	1220	Moderate
Avadhani et al., 2010	Adult hospitalized population	Any probiotic	Placebo	Incidence of CDAD	4	471	Moderate
Liao et al., 2020	Adult inpatients and outpatients	Any probiotic	Placebo	Incidence of AAD	36	9312	Low
Jafarnejad et al., 2016	Adults	Any probiotic	Placebo	Incidence of AAD	25	3826	Low
Videlock et al., 2012	Adult patients treated with antibiotics	Any probiotic	Placebo	Incidence of AAD	24	2921	Moderate

* AAD – antibiotic associated diarrhea, CDAD – *Clostridium difficile* associated diarrhea

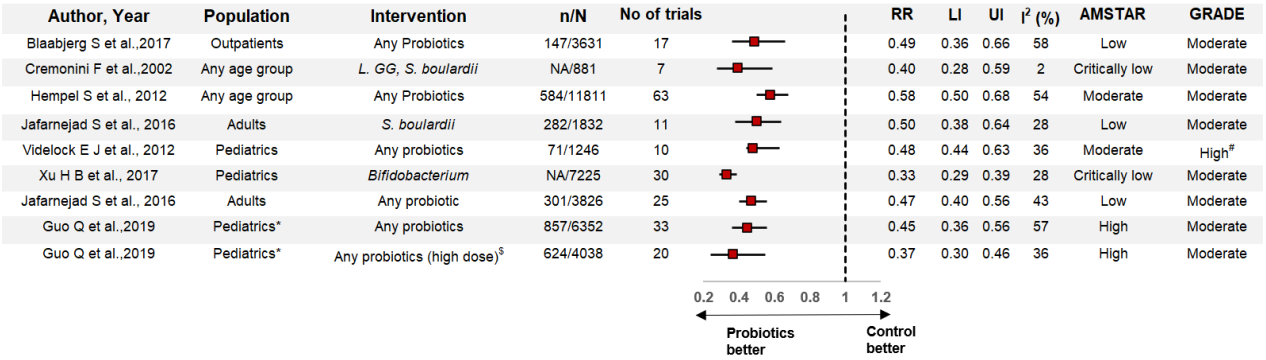

Fig. 2. Meta-analyses graded as moderate to high-quality in primary analysis (*Pediatric patients treated with antibiotics with a follow-up of 5 days to 12 weeks; $high dose (≥ 5 billion CFU/day); #the certainty of evidence of this meta-analysis downgraded to moderate quality after sensitivity analysis (L. GG – *Lactobacillus rhamnosus* GG*; S. boulardii – Saccharomyces boulardii;* NA – not available; RR – risk ratio; LI – lower confidence interval; UL – upper confidence interval; n – number of cases; N – total number of patients))

the conditions of use examined in RCTs.[12–14] As noted in this review, the diversity in probiotics extends to variations in strains and species, including genera blends, as well as differences in potency, dosage, and duration of use. Additionally, variations in study populations, including differences in age groups, types of infections, and patient settings (inpatient vs. outpatient), further complicate the interpretation of results.

Probiotics are increasingly popular globally, with a market expected to reach $52 billion by 2030.[44] They are easily accessible and come in various appealing flavors and dosage forms, including capsules, powders, liquids, and others. These products often contain a mix of microbial strains, primarily from genera like *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces,* rather than single strains.[45] Probiotics exert their health benefits through a range of mechanisms that can be nonspecific, species-specific, or strain-specific.[8,46] Nonspecific effects mechanisms, such as inhibiting the growth of pathogenic microorganisms in the gastrointestinal tract, producing bioactive metabolites, and reducing luminal pH in the colon, vary widely among strains, species, or even genera. Species-specific mechanisms include vitamin synthesis, gut barrier reinforcement, bile salt metabolism, enzymatic activity, and toxin neutralization. Strain-specific mechanisms, which are rare, may involve cytokine production, immunomodulation, and effects on the endocrine and nervous systems. Notably, within the same species, distinct strains can have vastly different activities and biological effects.[46] Additionally, combinations of various strains can result in different activities, as certain microbial activities rely on interactions between different strains.[47] Given these variations, recommendations for probiotic use should be specific to the individual and/or combination of species and strains. In this review, the majority of included studies focused on any ('non-specific') probiotics. Among the meta-analyses graded as moderate to high quality, only three studies examined the use of specific genera or species.[32,41,43] In addition to considering species and strains, probiotics may also exert dose-dependent effects. While commercially available probiotic formulations typically contain at least 10^6 CFUs,[48] the optimal dose, frequency, and duration of probiotic use to achieve clinical effect of preventing AAD is unclear. In this review, the probiotic dosage regimen varied across studies, making cross-comparisons difficult. Therefore, future RCTs exploring effects of specific probiotic species and strains, as well as the optimal dose and duration of treatment are warranted.

Another challenge arises due to a lack of thorough evaluations specifically targeting probiotic-related adverse events. While RCTs generally report low rates of adverse events, it is important to highlight that several case studies have documented serious adverse events, particularly in vulnerable patients. A survey of specific populations examined in clinical trials identified an instance of invasive disease in an immunocompromised patient, and concerns were also raised about the safety of probiotics in children receiving intensive care and adults with severe acute pancreatitis.[49-51] These reports emphasized the necessity for improved documentation regarding the safety of probiotics. Consequently, the current state of evidence underscores the limitation in drawing firm conclusions regarding the safety of probiotics in managing AAD.

Overall, it remains to be answered, specifically which probiotics, what dosage regimen, and in which population the use of probiotics will be safe and beneficial in managing AAD. Currently, there is no global consensus on the clinical use of probiotics for AAD. The American Gastrointestinal Association suggests certain strains and combinations of probiotics in adults and children on antibiotic treatment, but this recommen-

dation is conditional (not strong) due to limited supporting evidence. The recommendation also note that patients who are concerned about financial costs or potential harms (e.g., immunocompromised), and who have a low risk of developing *C. difficile* infection (e.g., outpatients in the community), may opt not to use any probiotics.[47] The World Gastroenterology Organization[52] and Infectious Diseases Society of America[53] offer similar recommendations citing insufficient high quality evidence on the efficacy and safety of probiotics in AAD. In Asia, clinical practice echo a comparable standpoint. For instance, a position statement from the Malaysian Society of Gastroenterology and Hepatology generally affirms the efficacy and safety of specific probiotic strains in AAD, including *C. difficile*-associated diarrhea. However, it also advises careful scrutiny of claimed health benefits and supportive evidence before advocating their clinical use.[54] In the recent European Society for Pediatric Gastroenterology, Hepatology and Nutrition position paper, it is suggested that high doses (≥5 billion CFU per day) of *S. boulardii* or *L. rhamnosus* GG may be considered for preventing AAD in outpatients and hospitalized children with specific risk factors, though the evidence is of moderate certainty.[55] The paper also notes that the strain designation of *S. boulardii* was unclear in many of the trials. Therefore, until high quality evidence becomes available, healthcare professionals and consumers should exercise caution and carefully weigh the potential benefits and risks associated with probiotic use. Addressing the identified gaps through higher quality trials is essential before definitive recommendations can be made regarding the role of probiotics in AAD.

Study limitations

The present umbrella review has several limitations that should be acknowledged. Firstly, the search strategy was confined to English language publications only, which may have led to the exclusion of relevant studies published in other languages. The findings of this review are based on moderate to low-quality evidence. Therefore, the results should be interpreted with caution, and further research is needed to confirm the findings of this review.

Conclusion

The findings of this study support the use of probiotics for the prevention of AAD. However, the quality of the evidence was mostly low to moderate. More high-quality trials are warranted in this area. Future research should focus on identifying the most effective probiotic species and/or strains, optimal dosage regimens, and specific patient populations (e.g., adults/children, inpatient or outpatient) that will benefit from probiotic use.

Supplementary materials

S1: Search Strategy, Table S2: Excluded studies and reason for exclusion, Table S3: Description of probiotics, Table S4: GRADE Meta-analysis, Table S5: Sensitivity analysis

Declarations

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Author contributions

Conceptualization, E.L. and S.V.; Methodology, E.L., S.T.S. and S.V.; Formal Analysis, S.T.S, S.K. and S.V.; Investigation, E.L., M.K.M., P.V.I., S.S., S.K. and S.V.; Resources, E.L.; Data Curation, S.V.; Writing – Original Draft Preparation, E.L., S.T.S. and S.V.; Writing – Review & Editing, E.L., S.T.S., M.K.M., S.S., P.V.I., S.K., S.M.C., Y.Y.L. and S.V..; Visualization, S.V.; Project Administration, E.L.

Conflicts of interest

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Ethics approval

Not applicable.

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REVIEW PAPER

The X-ray repair cross-completing gene 1 (XRCC1) polymorphisms and lung cancer incidence – a confirmatory umbrella review of observational evidence

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ABSTRACT

Introduction and aim. Lung cancer (LC) is a leading cause of cancer-related deaths worldwide, with X-ray repair cross-complementing gene 1 (XRCC1) playing a crucial role in DNA repair and influencing LC risk through genetic mutations. Despite numerous meta-analyses, results have been inconsistent. This study systematically evaluated existing meta-analyses to clarify the association between XRCC1 gene variations and LC.

Material and methods. A comprehensive literature search was conducted using Scopus, Web of Science, Embase, and Cochrane databases. The present Umbrella review followed PRISMA and MOOSE guidelines. The AMSTAR tool assessed the methodological quality of the included studies.

Analysis of the literature. A total of 28 data sets were analyzed: 9 for the rs25487 (codon 399), 11 for the rs1799782 (codon 194), and 8 for the rs25489 (codon 280) polymorphisms. Significant associations were found with odds ratios ranging from 0.93 to 1.92 ($p<0.05$) in 16 data sets. XRCC1 rs25487/codon 399 and rs1799782/codon 194 were strongly linked to LC risk, while rs25489 (codon 280) was not. Twelve datasets showed significant heterogeneity, and publication bias was not detected in 24 datasets. Most meta-analyses demonstrated high methodological quality.

Conclusion. These findings suggest that XRCC1 (rs25487/codon 399 and rs1799782/codon 194) gene polymorphisms have the potential to serve as biomarkers for the early identification and management of LC risk.

Keywords. case-control, DNA repair, genetic susceptibility, lung cancer, meta-analysis, XRCC1 gene

Introduction

Lung cancer (LC) is the most prevalent form of cancer and the leading cause of cancer-related deaths around the world. In 2020, it was responsible for more than 1.7 million fatalities and 2.2 million new cases.[1] LC primarily manifests in two types: non-small cell LC (NSCLC) and small cell LC (SCLC). NSCLC is the most common type, comprising approximately 80-85% of cases including squamous cell carcinoma, adenocarcinoma, and large cell carcinoma.[2,3] SCLC represents 10-15% of LC and typically exhibits faster growth and spread compared to NSCLC.[4] The fatality rate for LC is remarkably high, with

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more than 80% of individuals dying of the illness. The main factors contributing to this high fatality rate are delayed detection and cancer's high degree of malignancy.[5] The prevalence of active smoking or past smoking among patients on the day of diagnosis varies from 60% to 90% across different global regions. Extensive epidemiological evidence has conclusively identified tobacco smoking and second-hand smoke exposure as predominant risk factors for the development of LC.[6] Among patients who have never smoked tobacco, it is identified that 19% of female LC cases are observed, in contrast to 9% in male LC cases.[7] Other factors associated with a higher risk of developing LC include exposure to environmental air pollutants like ozone, particular matter (PM) nitrogen oxides, dietary habits and supplements, alcohol consumption, low physical activity, air pollution, workplace exposure, and sulfur dioxide.[7,8] One possible rationale is that prolonged exposure to air pollution could raise the risk of LC by causing oxidative damage, which occurs as a result of inflammatory injury and the generation of reactive oxygen species (ROS), although the findings from population studies continue to be debated due to variations in study designs and participant numbers.[9] However, certain studies have shown that the development of LC is influenced by the interplay of an individual's genetic predisposition and environmental risk factors. In addition, scientific evidence suggests a strong connection between genetic makeup and the onset of LC.[10] Lack of early diagnosis often leads to fatalities from LC, as the disease is frequently identified only in advanced stages. Effective treatment of LC requires a comprehensive knowledge of the disease's underlying causes, reliable early diagnostic techniques, and the appropriate use of medications.[11] Thus, early detection of LC is vital, particularly for high-risk groups such as smokers and individuals employed in oil fields or other industries with toxic exposures. There is an urgent necessity to uncover new biomarkers for this purpose.[12] Precise diagnosis is crucial for tailored LC treatments. Therefore, identifying sensitive biomarkers for early detection is essential.

Recent research has focused on genetic markers to anticipate cancer onset. Single nucleotide polymorphisms (SNPs) are emerging as potential indicators of cancer risk. Previous studies have associated variations in DNA repair genes with increased LC susceptibility.[13,14] The human genome is protected by the DNA repair mechanism against ongoing harm caused by oxidizing and alkylating chemicals, ionizing radiation, nicotine and cigarette smoke, environmental exposures, and occupational hazards.[15] The complex DNA repair machinery in the human body includes several intricate systems, with base excision repair (BER) playing a vital role. BER is tasked with repairing minor DNA lesions, such as those caused by ionizing radiation, potent alkylating agents, and cellular metabolic byproducts like ROS that can harm DNA bases.[16] DNA glycosylases are a group of enzymes that remove the damaged base from the DNA. Subsequently, AP endonucleases, including APE1 enzymes, are tasked with breaking the phosphodiester link at the site of damage. This cleavage produces a 5'dRP and 3'OH, which are used for DNA repair and joining. The base excision repair (BER) process has long and short repair paths. X-ray repair cross-completing gene 1 (XRCC1) is crucial for BER, binding to DNA ligase III and polβ. It also helps detect DNA breaks with PARP.[16,17]

XRCC1 is situated on the human chromosome 19q13.2-13.3 and contains three fairly common polymorphic codons at positions 399(Arg/Glu), 194(Arg/Trp), 280(Arg/His), which have the potential to impact the amino acid sequence.[18] Cells deficient in the XRCC1 gene may exhibit increased sensitivity to ultraviolet light, mitomycin, ionizing radiation, and hydrogen peroxide. Nonetheless, XRCC1 plays a crucial role in the BER system. Consequently, it is plausible to suggest that gene expression could influence cancer development.[19] The relationship between specific gene polymorphisms XRCC1 (rs25487/codon 399, rs1799782/codon 194, and rs25489/codon 280) and the development of LC remains unclear. Extensive research, including meta-analyses and case-control studies, has been undertaken to explore this connection. Research findings on the link between these genetic variations and LC risk are inconsistent. Some studies have found no connection, while others have identified a significantly elevated risk. Given the complexities and inconsistencies in the existing literature regarding the association between XRCC1 polymorphisms and LC risk, an umbrella review is proposed.

Aim

This review aims to systematically collate and assess the findings from multiple meta-analyses, providing a holistic understanding of the genetic factors involved in LC susceptibility. These studies are synthesized to clarify the conflicting evidence surrounding XRCC1 (rs25487/codon 399, rs1799782/codon 194, and rs25489/codon 280) polymorphisms and their implications for LC risk. This comprehensive approach not only addresses existing inconsistencies but also provides insights that could inform clinical practice regarding genetic screening and risk stratification in LC patients. The study findings may contribute as a biomarker to identifying high-risk populations and improving early detection strategies, which are crucial for enhancing patient outcomes.

Material and methods

An umbrella review was performed, by systematically gathering and assessing systematic reviews and meta-analyses focused on a particular research topic. The umbrella review followed PRISMA (Preferred Reporting Item for Systemic Review and Meta-Analysis)

and MOOSE (Meta-Analysis of Observational Studies in Epidemiology) guidelines. Before its initiation, the review protocol was registered in PROSPERO (ID: CRD42024571433).

Literature search

A comprehensive search of the literature was performed across a various online databases, including Scopus, Web of Sciences, Embase, and Cochrane databases of systemic review. The focus was on meta-analyses of case-control studies investigating LC and the XRCC1 gene, without any time limitations. The literature search was independently conducted by two authors, Velmurugan and Subbaraj using the MeSH terms and keywords "Lung Cancer," "XRCC1," "gene polymorphisms," "Meta-Analysis," "adenocarcinoma," "non-small cell LC," "neoplasm," and "case-control. Duplicate data were removed before the screening phase. Each retrieved article reviewed at the title, abstract, and full-text level to assess eligibility, and any discrepancies between the authors were resolved through discussion to reach a consensus.

Inclusion and exclusion criteria

Meta-analyses were selected based on the following inclusion criteria: (i) The study included only meta-analyses using case-control studies (ii) The study explored the link between XRCC1 polymorphisms and LC risk. (iii) The scope of this study was limited to the English language (iv) The studies reported pooled odds ratios (ORs) with 95% confidence intervals (CIs) to quantify the strength of the relationship. The exclusion criteria comprised protocols, reviews, editorials, conference proceedings, and abstracts as these sources do not provide the primary data or quantitative outcomes. In the cases of multiple meta-analyses reported similar findings, the analysis with the highest number of included studies was prioritized. However, no studies were excluded based on this criterion, as all relevant analyses contributed valuable insights to our review.

Data extraction

The data from the selected papers was independently collected by two authors, Velmurugan and Subbaraj who used standardized form to collect details on primary author, publication year, number of studies, study design, total cases and controls, participant ethnicity, and genotyping methods. Furthermore, ORs and their CIs for each qualifying meta-analysis were obtained from all available genetic models. Furthermore, the extracted outcomes encompassed p-values for the overall pooled effect, Egger's test for publication bias, and the I^2 statistic for heterogeneity. Additionally, we documented the quality assessment criteria used in the selected meta-analyses. Data were organized and managed using Microsoft Excel.

Assessment of methodological quality

The methodological quality of the included meta-analyses of case-control studies was assessed using the "A MeaSurement to Assess Systemic Reviews (AMSTAR) tool". The AMSTAR tool comprises 16 items, each question can be answered with 'Yes", "No" and "Partially yes". A score of 1 for a positive response and 0 points for other responses. The total score is the accumulation of these 16 items. A score of ≥8 is deemed as high quality, 4-7 points indicate moderate quality, and a score of ≤3 or lower reflects low quality.[20] Disagreements regarding AMSTAR ratings were addressed through discussion.

Data analysis

The outcome data, ORs, and their corresponding 95% CI from each of the published studies were extracted for conducting available meta-analyses. A p-value<0.05 indicated statistically significant findings in the pooled meta-analysis. Heterogeneity was assessed using the I^2 and Q statistics at a significance level of p<0.1. The potential publication bias was also examined using Egger's test, also at a significance level of p<0.1. Instead of recalculating summary estimates with 95% confidence intervals, the existing effect sizes and 95% CI for each variable were directly extracted.

Analysis of the literature

Search results

The flow diagram in Figure 1 illustrates the process of selecting articles, starting with the identification of 250 articles and the removal of duplicates.

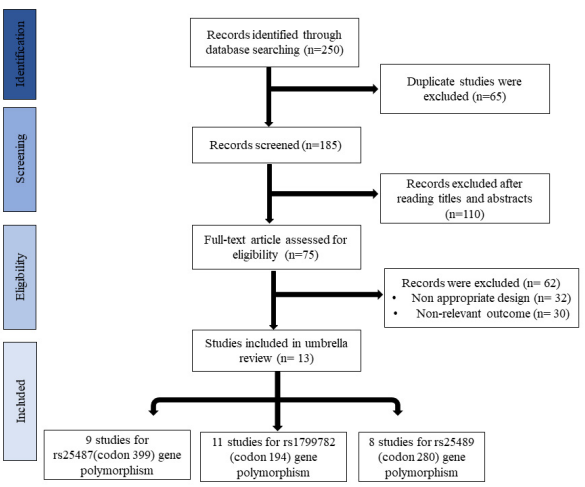

Fig. 1. Flowchart of literature screening for X-ray repair cross-completing gene 1 gene polymorphisms and lung cancer risk

Following a comprehensive assessment, 13 primary meta-analyses were discovered.[21-33] These analyses delved into the connections between XRCC1 rs25487/codon399, rs1799782/codon 194, and rs25489/codon

Table 1. Summary of Odds Ratios (OR) with 95% Confidence Intervals (95% CI) for each meta-analysis on lung cancer risk associated with XRCCI rs25487 (codon 399) polymorphism across various genetic models

SNPs	*Study*	*Ethnicity*	*Genotyping method*	*No. of studies included in the meta-analysis*	*Case/Control*	*Contrast*	*OR (95% CI)*	*Heterogeneity (p-value)*	*Publication bias (Egger's P)*	*Quality assessment scale and outcome*
rs25487 (codon 399)	Chen et al.,2015	Caucasian	PCR-RFLP	10	2187/3453	Arg/Gln + Gln/Gln	0.93 (0.82-1.04)	0.7	Absence of publication bias	NO
	Li et al., 2014	Asian, Caucasian	PCR-RFLP	31	5701/6924	Arg/Gln + Gln/Gln	0.974 (0.905- 1.049)	0.022	0.217	NO
	Wang et al., 2014	Asian, Caucasian, African, Mixed	PCR-RFLP	46	23,033/26,225	M vs. C	1.06 (1.01-1.12)	<0.001	Absence of publication bias	NO
						MM vs. CC	1.19 (1.05-1.34)	<0.001	Absence of publication bias	
						MM vs. CM+CC	1.19 (1.06-1.33)	<0.001	Absence of publication bias	
						MM+CM vs. CC	1.04 (0.98-1.10)	<0.001	Absence of publication bias	
	Huang et al., 2013	African, Asian, Caucasian	PCR-RFLP, TaqMan	41	14,156/16,667	Recessive	1.57 (1.02-2.42)	0.026	0.767	NO
						Dominant	1.00 (0.94-1.07)	0.009	0.546	
						Additive model	1.05 (0.93-1.19)	0.003	0.984	
	Dai et al., 2012	Asian, Caucasian	PCR-RFLP	39	12,303/14,965	Arg/Gln	0.98 (0.92-1.06)	0.03	0.76	NO
						Gln/Gln	1.05 (0.91-1.21)	0.0005	0.49	
						Arg/Gln + Gln/Gln	1.00 (0.93-1.07)	0.009	0.78	
	Kiyohara et al., 2010	African, Asian, Caucasian, Mixed	Sequencing, Replication	24	8,684/10,913	Arg/Gln	0.97 (0.89-1.05)	0.153	Absence of publication bias	NO
						Gln/Gln	1.00 (0.86-1.17)	0.004	Absence of publication bias	
	Zheng et al., 2009	Asian	PCR-RFLP	8	1242/1343	Arg/Gln + Gln/Gln vs. Arg/Arg	1.16 (1.00-1.36)	0.07	0.148	NO
	Wang et al., 2009	Asian, Caucasian	PCR-RFLP	30	10214/12599	Arg/Gln	1.00 (0.95-1.06)	0.05	0.407	NO
						Gln/Gln	1.06 (0.89-1.25)	<0.0001	0.992	
						Arg/Gln + Gln/Gln	1.00 (0.92-1.09)	0.005	0.343	
	Kiyohara et al., 2006	Asian, Caucasian	PCR-RFLP	18	7385/9380	Arg/Gln	0.99 (0.93-1.06)	0.026	0.052	Assessed homogeneity of the study population
						Gln/Gln	0.94 (0.80-1.11)	0.179	Absence of publication bias	

Table 2. Summary of Odds Ratios (OR) with 95% Confidence Intervals (95% CI) for each meta-analysis on lung cancer risk associated with XRCCI rs1799782 (codon 194) polymorphism across various genetic models

SNPs	*Study*	*Ethnicity*	*genotyping method*	*No. of studies included in the meta-analysis*	*Case/Control*	*Contrast*	*OR (95% CI)*	*Heterogeneity (P-value)*	*Publication Bias (Egger's P)*	*Quality assessment scale and outcome*
rs1799782 (codon 194)	Chen et al., 2015	Caucasian	PCR-RFLP	6	857/2108	Arg/Trp + Trp/Trp	0.94 (0.73-1.21)	0.44	Absence of publication bias	NO
	Zhang et al., 2014	Asian, Caucasian	Illumina, PCR-RFLP, TaqMan	25	8,876/11,210	Trp vs. Arg	0.97 (0.92-1.03)	0.3	0.33	NO
						ArgTrp vs. ArgArg	0.92 (0.85-0.98)	0.017	0.12	
						TrpTrp vs. ArgArg	1.07 (0.92-1.23)	0.38	0.65	
						(TrpTrp + ArgTrp) vs. ArgArg	0.93 (0.87-1.00)	0.047	0.25	
						TrpTrp vs. (ArgTrp + ArgArg)	1.08 (0.94-1.25)	0.255	0.5	
	Li et al., 2014	Asian, Caucasian	PCR-RFLP	16	1793/2190	Arg/Trp + Trp/Trp	0.948 (0.872- 1.030)	0.118	0.336	NO
	Huang et al., 2013	African, Asian, Caucasian	PCR-RFLP, TaqMan	23	7,426/9,603	Recessive	1.23 (1.05-1.44)	0.216	0.416	NO
						Dominant	0.96 (0.86-1.07)	0.042	0.588	
						Additive model	1.22 (1.04-1.44)	0.107	0.555	
	Dai et al., 2012	Asian, Caucasian	PCR-RFLP	22	7,534/9,753	Arg/Trp	0.93 (0.86-1.00)	0.46	0.093	NO
						Trp/Trp	1.19 (1.01-1.39)	0.23	0.83	
						Arg/Trp + Trp/Trp	0.96 (0.89-1.03)	0.24	0.22	
	Huang et al., 2011	Chinese	Taqman, PCR-RFLP	10	3303/3594	Trp/Trp Vs. Arg/ Trp + Arg/Arg	1.31 (1.13-1.53)	0.007	0.869	NO
	Kiyohara et al., 2010	African, Asian, Caucasian, Mixed	Sequencing, Replication	13	4,431/6,320	Arg/Trp	0.89 (0.79-1.00)	0.467	Absence of publication bias	Assessed homogeneity of the study population
						Trp/Trp	1.15 (0.80-1.67)	0.51	Absence of publication bias	Assessed homogeneity of the study population
	Jiang et al., 2010	Chinese, Italian, European	PCR-RFLP	22	7515/9560	Arg/Trp VS Arg/Arg	0.91 (0.85- 0.99)	0.49	0.56	NO
						Trp/Trp VS Arg/Arg	1.22 (1.04-1.44)	0.11	0.07	
						Trp/Trp + Arg/Trp VS Arg/Arg	0.95 (0.88-1.02)	0.12	0.26	
	Wang et al., 2009	Asian, Caucasian	PCR-RFLP	16	4848/6592	Arg/Trp	0.88 (0.79-0.97)	0.37	0.183	NO
						Trp/Trp	1.07 (0.85-1.33)	0.21	0.21	
						Arg/Trp + Trp/Trp	0.91 (0.83-1.00)	0.21	0.34	
	Zheng et al., 2009	Asian	PCR-RFLP	6	1020/1038	Arg/Trp + Trp/Trp vs. Arg/Arg	1.06 (0.89-1.27)	0.74	0.794	NO
	Kiyohara et al., 2006	Asian, Caucasian	PCR-RFLP	9	3714/5385	Arg/Trp	0.89 (0.78-1.03)	0.392	Absence of publication bias	Assessed homogeneity of the study population
						Trp/Trp	1.19 (0.76-1.86)	0.407	Absence of publication bias	NO

Table 3. Summary of Odds Ratios (OR) with 95% Confidence Intervals (95% CI) for each meta-analysis on lung cancer risk associated with XRCCI rs25489 (codon 280) polymorphism across various genetic models

SNPs	*Study*	*Ethnicity*	*genotyping method*	*No. of studies included in meta-analysis*	*Case/Control*	*Contrast*	*OR (95% CI)*	*Heterogeneity (P-value)*	*Publication Bias (Egger's P)*	*Quality assessment scale and outcome*
rs25489 (codon 280)	Chen et al., 2015	Caucasian	PCR-RFLP	5	894/1133	Arg/His + His/His	1.13 (0.73-1.76)	0.06	Absence of publication bias	NO
	Li et al., 2014	Asian, Caucasian	PCR-RFLP	10	611/708	Arg/His + His/His	1.03 (0.85- 1.25)	0.036	0.569	NO
	Guo et al., 2013	Asian, European	PCR-RFLP	16	8,736/9,924	His vs. Arg	1.00 (0.84-1.20)	0.967	0.05	NO
						HisHis vs. ArgArg	1.53 (1.08-2.16)	0.016	0.06	
						HisHis vs. ArgArg/ArgHis	1.55 (1.10-2.19)	0.012	0.07	
						HisHis/ArgHis vs. ArgArg	0.96 (0.79-1.17)	0.702	0.08	
	Huang et al., 2013	African, Asian, Caucasian	PCR-RFLP, TaqMan	16	6,211/6,763	Dominant	1.04 (0.83-1.29)	0.001	0.292	NO
						Recessive	1.30 (0.71-2.37)	0.065	0.52	
						Additive model	1.46 (0.99-2.15)	0.146	0.292	
	Dai et al., 2012	Asian, Caucasian	PCR-RFLP	12	5,292/5,934	Arg/His	0.98 (0.88-1.10)	0.17	0.69	NO
						His/His	1.42 (0.89-2.26)	0.33	0.26	
						Arg/His + His/His	0.99 (0.89-1.11)	0.08	0.53	
	Kiyohara et al., 2010	African, Asian, Caucasian, Mixed	Sequencing, Replication	13	4,431/6,320	Arg/Trp	0.89 (0.79-1.00)	0.467	Absence of publication bias	Assessed homogeneity of the study population
						Trp/Trp	1.15 (0.80-1.67)	0.51	Absence of publication bias	
	Zheng et al., 2009	Asian	PCR-RFLP	3	599/402	Arg/His + His/His vs Arg/Arg	0.63 (0.28-1.41)	0.0002	0.08	NO
	Kiyohara et al., 2006	Asian, Caucasian	PCR-RFLP	7	3640/3981	Arg/His	1.03 (0.88-1.20)	0.741	Absence of publication bias	Assessed homogeneity of the study population
						Arg/His or His/His	1.06 (0.91-1.23)	0.564	Absence of publication bias	

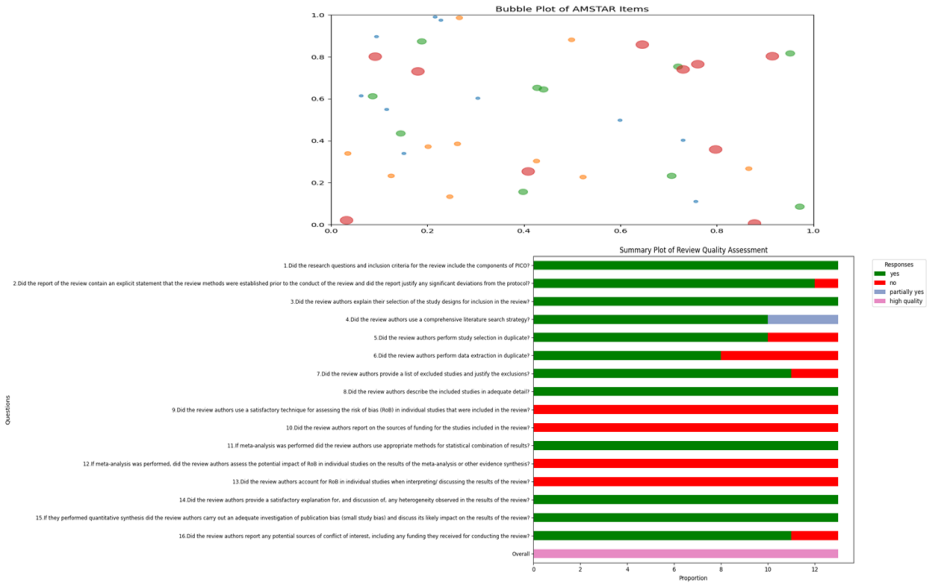

Fig. 2. Quality assessment of the included studies using the AMSTAR tool. The bubble plot illustrates associations with color-coding based on their classification output, with bubble size indicating the annual number of citations received by the study. Additionally, the bar plot summarizes the assessment

280 polymorphisms and the risk of LC. The study design included only case-control studies in all meta-analyses, a total of 504 case-control studies. These 13 qualified papers covered 28 meta-analyses across three main areas: XRCC1 rs25487/codon399 polymorphism (n=9), XRCC1 rs1799782/codon 194 polymorphism (n=11), and XRCC1 rs25489/codon 280 polymorphism (n=8). The eligible articles were published between 2006 and 2015. There was a median of 6,798 case subjects and a larger median of 8,072 control subjects per meta-analysis. Ten studies contained data from the Asian population, while data from 9 studies were included for Caucasians. Additionally, 3 studies included data from the African population, 2 from mixed populations, 2 from the European population, 2 from the Chinese population, and 1 from the Italian population. The majority of the meta-analyses did not conduct methodological quality assessments, although some studies assessed the homogeneity of the study population. The included primary studies were predominantly high-quality trials and their control groups generally adhered to Hardy-Weinberg equilibrium (HWE). Detailed characteristics of the eligible studies are provided in Tables 1, 2, and 3.

Heterogeneity and publication bias

Out of the 28 meta-analyses conducted, the Q test revealed that 16 datasets exhibited no significant heterogeneity among the studies ($p \geq 0.1$), while 12 displayed substantial heterogeneity ($p < 0.1$). In terms of publication bias, 24 outcomes did not demonstrate statistical evidence of it ($p \geq 0.1$), whereas 4 outcomes indicated the presence of publication bias ($p < 0.1$) based on Egger's test.

Quality assessment of included meta-analyses

A methodological quality assessment of the 13 included articles was conducted using the AMSTAR criteria. The review achieved a total score of 8-12 out of 16, corresponding to a percentage score of 75%, classifying it as high quality. This indicates that the review was conducted with a strong methodological approach, reflecting robustness in its design and execution (Fig. 2).

The review's strengths included the prior registration of a protocol, which ensured transparency and minimize bias, and a comprehensive literature search that effectively captured a wide range of relevant studies. Additionally, the review rigorously assessed the risk of bias in the included studies and provided clear disclosure of any potential conflicts of interest, enhancing the credibility of its findings. Despite these strengths, the review had a few minor limitations. It did not perform a sensitivity analysis, which could have explored the robustness of the results under different assumptions. Additionally, the review provided incomplete information on the sources of funding for the included studies, which could affect the interpretation of potential biases. Overall, however, the high AMSTAR score confirms that this review is of high quality (Table 4). These minor limitations do not significantly detract from its reliability, making it a valuable and credible source of evidence in its field.

Summary and description of association for XRCC1

The study evaluated the relationships between the XRCC1 rs25487/codon399, rs1799782/codon 194, and rs25489/codon 280 polymorphisms and the onset of LC by analyzing various genetic models including dominant, recessive, and codominant models, as well as specific allelic forms (homozygote and heterozygote). In

Table 4. Quality assessment of systematic reviews and meta-analyses: Response to AMSTAR checklist questions and overall quality rating*

Study	*Q1*	*Q2*	*Q3*	*Q4*	*Q5*	*Q6*	*Q7*	*Q8*	*Q9*	*Q10*	*Q11*	*Q12*	*Q13*	*Q14*	*Q15*	*Q16*	*AMSTAR Score (%)*	*Over all*
Chen et al., 2015	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	75	High quality
Zhang et al., 2014	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	75	High quality
Li et al., 2014	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	68.75	High quality
Wang et al., 2014	Yes	Yes	Yes	Partially yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	68.75	High quality
Huang et al., 2013	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	75	High quality
Guo et al., 2013	Yes	Yes	Yes	Yes	Yes	NO	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	68.75	High quality
Dai et al., 2012	Yes	Yes	Yes	Yes	NO	NO	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	62.5	High quality
Huang et al., 2011	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	75	High quality
Kiyohara et al., 2010	Yes	Yes	Yes	Partially Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	68.75	High quality
Jiang et al., 2010	Yes	Yes	Yes	Partially Yes	NO	NO	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	NO	50	High quality
Zheng et al., 2009	Yes	Yes	Yes	Yes	NO	NO	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	62.5	High quality
Wang et al., 2009	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	Yes	Yes	75	High quality
Kiyohara et al., 2006	Yes	NO	Yes	Yes	Yes	NO	NO	Yes	NO	NO	Yes	NO	NO	Yes	Yes	NO	50	High quality

* AMSTAR – assessment of Methodological Quality of Systematic Reviews, Q – question

this study, all meta-analyses calculated ORs with 95% CIs for all meta-analyses to determine the connection between variations in the XRCC1 gene and the risk of LC. Among the 28 data sets examined, 16 (57.14%) revealed statistically significant summary findings, with ORs between 0.93 to 1.92 ($p<0.05$). These strong correlations were observed across different genetic models, covering 3 comparisons: 5 (31.25%) for the XRCC1 rs25487/codon399 polymorphism, 8 (50%) for the XRCC1 rs1799782/codon 194 polymorphism, and 3 (18.75%) for the XRCC1 rs25489/codon 280 polymorphisms. Additionally, 12 (42.86%) of the datasets examined produced statistically non-significant summary results, with odds ratios ranging from 0.93 to 1.92 ($p > 0.05$). Noteworthy associations were identified across different genetic models, involving 3 comparisons: 4 (33.33%) for the XRCC1 rs25487/codon399 polymorphism, 3 (25%) for the XRCC1 rs1799782/codon 194 polymorphisms, and 5 (41.67%) for the XRCC1 rs25489/codon 280 polymorphisms. A summary of the findings is depicted in Fig. 3. The findings indicate that XRCC1 rs1799782/codon 194 gene polymorphisms demonstrate a strong association with LC risk. Additionally, a more significant association was discovered between XRCC1 rs25487/codon399 polymorphisms and LC risk. However, the current study suggests that XRCC1 rs25489/codon 280 polymorphisms may not be strongly linked to susceptibility to LC risk.

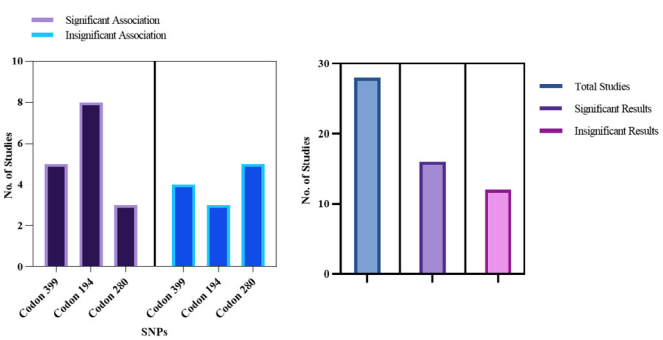

Fig. 3. A summary of the results from the current study investigating the relationship between X-ray repair cross-complementing gene 1 (XRCC1) gene polymorphisms and the risk of lung cancer

Discussion

LC develops through complex pathways, encompassing multiple stages and a multifaceted process. The precise cause of LC remains incompletely comprehended.[34] Various genes are linked to the onset of LC. Furthermore, the levels of gene expression related to DNA damage repair significantly influence the likelihood of developing malignant tumor.[35] The XRCC1 gene is one of about 20 genes involved in fixing DNA damage caused by radiation or certain chemicals. It helps repair bro-

ken DNA strands and damaged DNA bases.[36] XRCC1 is crucial for repairing broken DNA. It facilitates the rejoining of DNA strands by interacting with polynucleotide kinase (PNK) and coordinating the repair process. Hence, this gene is regarded as a pivotal candidate gene that influences susceptibility to LC.[37] Although many comprehensive population studies have explored the correlation between XRCC1 gene variants and the likelihood of developing LC, the results have been inconsistent and conflicting. Karaagac et al. identified XRCC1 gene polymorphisms as prognostic markers for survival and recommended considering them as predictive markers for guiding treatment decisions in patients with NSCLC.[38] Likewise, Minina et al. found a higher LC risk in a coal-mining population associated with the XRCC1 rs25487/codon399 genetic variant.[39] According to Yang et al., specific variants of the XRCC1 gene (rs25487/codon399) are associated with varying responses to radiotherapy and its side effects in NSCLC patients. These findings highlight the potential of DNA repair genes as predictors of treatment effectiveness.[40] Al-Rawi et al. also indicated that the XRCC1 rs1799782/codon 194 may be associated with LC within this specific population group.[41] The study revealed that naphthalene and phenanthrene exposure were positively associated with LC risk and that the XRCC1 rs25487 (codon 399) polymorphism modified this risk through PAH-gene interaction analysis.[42] Similarly, Ezzeldin et al. reported that gene-gene and gene-environment interactions involving the XRCC1 gene polymorphism indicated an elevated LC risk. However, no association was found between XRCC1 rs25487 and LC in combination with ERCC1 or CHRNA3 variants, and the association varied across different ethnic groups. Further studies are needed to clarify these findings.[43] The findings by Karaagac et al. suggested NSCLC patients with certain SNPs exhibit a higher stage and more advanced disease at initial diagnosis, with XRCC1 and TP53 gene polymorphisms predicting metastasis risk, supporting their use in biomarker assessment.[44] The current study's findings are in line with these previous discoveries.

This study involved a systematic identification and analysis of 13 meta-analyses of observational studies to evaluate the relationship between XRCC1 polymorphisms and LC development. The findings reveal a consistent and strong relationship between the XRCC1 rs1799782/codon 194 gene polymorphisms and an increased susceptibility to LC. Moreover, a clear association was identified between the XRCC1 rs25487/codon399 polymorphisms and LC risk in the majority of the studies analyzed.[21,22,24-33] In contrast, the majority of studies did not find a significant association between XRCC1 rs25489/codon 280 polymorphisms and LC susceptibility.[23,30] Additionally, several factors could have influenced the results, such as the limited number of studies, small sample sizes, absence of study quality assessment, and HWE deviations. The considerable heterogeneity found across the original studies is a primary contributor to these discrepancies. Among the 28 datasets analyzed, 12 exhibited notable heterogeneity, while 16 demonstrated a lack of heterogeneity. The diverse range of adjusted factors such as age, gender, environmental exposure, alcohol consumption, family history, treatment usage, race, and smoking habits across studies might introduce bias and heterogeneity, affecting the reliability of our analysis. Additionally, of the 28 datasets analyzed, 24 showed no evidence of publication bias, while four indicated potential publication bias, suggesting that some negative study results might be published. Selection bias may influence the findings, when the researchers give priority to publishing positive results, resulting in an overabundance of such outcomes in academic literature. This bias can distort the overall understanding of the relationship between XRCC1 polymorphisms and lung cancer risk. Information bias is another concern, as inconsistencies in how genetic variants and lung cancer outcomes were measured across studies could introduce inaccuracies. For example, differences in the definitions of lung cancer stages or diagnostic criteria may lead to varying conclusions regarding the role of XRCC1 polymorphisms. Additionally, the studies included in our review were rated as high quality according to AMSTAR criteria. They demonstrated strong adherence to methodological standards, including rigorous protocol registration, comprehensive literature searches, and thorough risk of bias assessments. These factors contribute to the reliability and robustness of this current findings, enhancing the overall validity of the meta-analysis results.

Unlike traditional systematic reviews or meta-analyses, umbrella reviews offer a broader perspective by summarizing the findings of multiple studies on a particular phenomenon or research question.[45] The present study is the first to apply this strategy for a comprehensive critical evaluation of the published associations between XRCC1 rs25487/codon399, rs1799782/codon 194, and rs25489/codon 280 gene polymorphisms and LC incidence. Additionally, the present study included primary studies with notably large sample sizes, which minimized the potential for bias compared to smaller studies. Moreover, the genotype distributions for the majority of control SNPs aligned with HWE, thereby strengthening the robustness of our findings.

In advanced NSCLC, patients are often treated with platinum-based chemotherapy, which induces DNA damage. The XRCC1 gene polymorphism is also a predictor of clinical outcomes in NSCLC patients receiving platinum-based chemotherapy.[46] The study reported by Bushra et al. identified the XRCC1 G>A (rs25487) polymorphism as a predictive biomarker in advanced NSCLC

treated with platinum-based chemotherapy. This variant is significantly associated with severe toxicities and therapeutic responses.[47] The results suggest that the XRCC1 rs25487-GG genotype is associated with better overall response rates (ORR) in NSCLC patients undergoing platinum-based chemotherapy. However, no association was found between XRCC1 rs1799782 and the clinical outcomes of platinum-based chemotherapy.[48] This study elucidates the role of XRCC1 gene polymorphisms in the development of LC, potentially enhancing our understanding of genetic susceptibility to this disease. By identifying specific genetic markers, this research could improve risk assessment, facilitate early detection, and inform personalized treatment strategies for individuals at higher risk. The findings underscore the importance of conducting studies across diverse populations to capture variability in XRCC1 polymorphisms and their interactions with environmental factors in LC etiology.

The umbrella review has limitations including the exclusion of relevant data published in other languages due to analysis being restricted to meta-analyses published in English. Presence of heterogeneity in the meta-analyses, possibly due to selection bias and other factors. Discrepancies in participant demographics and controlled variables across studies contribute to heterogeneity. Furthermore, the individual XRCC1 SNPs were focused, omitting haplotype analyses due to a lack of available data. Future research should explore XRCC1 haplotypes to provide a more comprehensive understanding of their association with lung cancer risk. Most studies included in the review were conducted in Asian and Caucasian populations. Due to insufficient studies across diverse groups, we did not perform a subgroup analysis by ethnicity. Future research should focus on larger, multi-ethnic samples to improve generalizability.

Conclusion

This umbrella review seeks to comprehensively examine the collective evidence from multiple systematic reviews and meta-analyses on the association between XRCC1 gene variations and the development of LC. This comprehensive approach aimed to provide a consolidated analysis of the available literature to better understand the relationship between XRCC1 gene variations and LC susceptibility. The present review shows that there is a substantial link between XRCC1 (rs25487/codon399 and rs1799782/codon194) gene polymorphisms and the risk of LC. However, there is no noticeable link between the XRCC1 rs25489/codon gene polymorphisms and the onset of LC. Furthermore, the observed associations may vary across different demographic factors, such as ethnicity, age, gender, and tobacco habits. For instance, certain populations may exhibit an increased susceptibility to the effects of these polymorphisms due to environmental interactions and lifestyle factors, including smoking, which is a well-established risk factor for LC. Gender-specific responses to these genetic variations could also influence risk, with emerging evidence suggesting differential susceptibility between males and females.

Therefore, further investigation is necessary to clarify and authenticate these results. A thorough investigation of the potential connection between genetic and environmental factors requires methods such as longitudinal cohort studies to track changes over time, genome-wide association studies to identify specific genetic variants, environmental exposure assessments using geographic information systems, and multidisciplinary collaboration among researchers in genetics, epidemiology, and environmental science. Discovering genetic markers has the potential to aid in risk assessment, early detection, and personalized treatment strategies. Therefore, these findings emphasize the importance of conducting comprehensive research across varied populations.

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Declarations

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Author contributions

Conceptualization, G.K. and K.G.; Methodology, S.V.; Software, S.V.; Validation, G.K., K.G. and R.R.; Formal Analysis, R.R.; Investigation, S.V.; Resources, K.G.; Data Curation, K.G.; Writing – Original Draft Preparation, S.V.; Writing – Review & Editing, G.K.; Visualization, G.K.; Supervision, G.K.; Project Administration, G.K.; Funding Acquisition, G.K.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Data availability

Data and materials used/analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

This study does not involve experiments with animals or human subjects.

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REVIEW PAPER

A systemic review and meta-analysis of the effect of virtual reality training on balance in the elderly to prevent falls

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ABSTRACT

Introduction and aim. Virtual reality (VR) is used in various healthcare treatments. This review evaluates virtual reality therapy (VRT) for balance rehabilitation to prevent falls in older adults.

Material and methods. Randomised control trials from January 2013 to May 2024 were searched in databases like PubMed and Web of Science. Data were extracted and analysed using RevMan 5.4 software.

Analysis of the literature. The review included 12 studies with an average of 56 participants aged 50-80 years. Treatments lasted 4–10 weeks with 2-5 sessions per week, each 30-60 minutes. Meta-analyses of five studies using the berg balance scale (BBS) showed a weak impact on balance (Z=2.07, p=0.04; SMD=1.05, 95% CI [0.06, 2.05], p<0.0001). Conversely, the Timed Up and Go (TUG) test showed a more positive impact (Z=2.25, p=0.02; SMD=-0.74, 95% CI [-1.39, -0.09], p<0.001), with a difference of 4.4 higher in the experimental group than the control group.

Conclusion. VRT shows promising effects in balance and gait training for older adults, but further clinical trials are needed to compare its impact with other therapies.

Keywords. virtual reality, balance, ageing, BBS, TUG

Introduction

Ageing is a natural, progressive, and irreversible process that affects the visual, somatosensory, and vestibular systems. Globally, more than 35% of individuals over 65 experience gait imbalance.[1–3] A combination of intrinsic factors such as reduced balance, mobility, and functional skills and extrinsic or environmental factors are predictors of falls in older adults.[4] Falls, prevalent in 30% of the population, are considered one of the main reasons for hospitalisations, morbidity, and mortality.[5–8] Furthermore, the psychological fear of falls limits activities and lowers the overall quality of life.[9] Therefore, correcting or reducing the intrinsic and extrinsic risk factors associated with ageing can help prevent falls in these individuals.[4]

Though balance can be assessed with sophisticated technologies using force platforms to study the kinetics and kinematics of movement, researchers recommend more functional methods that challenge a person's postural control for this purpose.[10] Hence, scales, such as the Berg Balance Scale (BBS), the Timed-Up and Go test (TUG), the Performance Oriented Mobility Assessment

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scale (POMA), the Functional Reach test, the Clinical Test of Sensory Integration for Balance (CTSIB), Dynamic Gait Index (GTI),[10] and the Balance Evaluation Systems Test (BESTest)[11] are used to assess an individual's balance and gait. BBS and TUG are common methods for balance and gait assessment due to their reliability, validity, and ease of implementation. However, the intrinsic complexity of fall risk calls for a comprehensive evaluation approach.. Researchers have recommended concurrently using multiple assessment tools to assess risk and tailor strategies to improve gait imbalances.[12]

Multiple approaches and interventions, such as strength training,[13,14] mobility training,[15] *Tai Chi,*[16] task-specific activities training,[17] Otago exercises,[18] aerobic training,[19] Pilates,[20] and audio-visual feedback training[21] have significantly improved balance in older adults. Recently, newer strategies, such as computer games and virtual workouts, have shown a favourable impact on the balance training of this population.[22,23]

Virtual reality (VR) is a sophisticated end-user immersive interactive simulation system that motivates users through real-time simulations to perform complex tasks in real-life scenarios.[24,25] VR systems are widely used in gaming, and modified versions have been extensively utilised in medical education and healthcare. VR systems are advantageous as the VR therapeutic tools sense the patient's movements and provide audio-visual feedback that engages their sensorimotor systems to move and control items as if they were real.[26–31] The augmented and interactive experiences within the virtual environments allow learners to apply therapeutic learning in their daily lives and help enhance the quality of movements and self-confidence.[26,27] This strategy encourages patients to complete the rehabilitation tasks with interest and enjoyment.[30,31] Virtual reality therapy (VRT) has proven to be a cost-effective therapeutic approach, providing exceptional benefits for individuals, especially those in rural areas.[32–34] Its compatibility with telehealth services further amplifies its reach to the masses, making it an effective and powerful tool in healthcare.[34]

Although numerous studies have demonstrated the effectiveness of VRT in enhancing balance and preventing falls among the elderly, there is considerable heterogeneity in the intervention protocols and types of VR tools employed.[35–38] Most previous reviews have not thoroughly analysed the assessment tools used across various studies or conducted a meta-analysis.[39] Additionally, the inclusion criteria of these studies have varied significantly. For instance, Bleakley et al. included both healthy elderly individuals and those with cognitive deficits, whereas de Amorim et al. focused on individuals with balance impairments.[37,38] Rodríguez-Almagro et al. included healthy individuals in VR combined with occupational therapy (OT) and rehabilitation but did not conduct a meta-analysis.[36] Consequently, the inconsistent outcomes raise questions about the effectiveness of this unique balance therapy method in older adults.[40,41]

Given the need for in-depth research with consistent methodologies, types of interventions, and scales used to compare the effects of VRT to traditional exercises on the balance of older adults, there is a significant gap in the knowledge of this emerging technology and treatment. Studies examining the positive and negative effects of VRT in rehabilitation will broaden the scope of geriatric rehabilitative care.

Aim

Therefore, our study aims to systematically review the existing literature on physical therapy interventions involving VR and conduct a meta-analysis of the BBS and TUG scales frequently used in these studies. This article is the first to conduct a meta-analysis on VR in older adults. This study will help determine the effectiveness of VRT in improving balance in older adults and thereby preventing falls.

Material and methods

Search strategy

We used the (PICOTS) framework, i.e., population, intervention, comparison, outcomes, timeframe, and study type, to develop the inclusion criteria. We searched the Scopus, PubMed, Web of Science, EMBASE, CINAHL, ICTRP databases, CT.gov, and grey literature electronically using controlled vocabulary and the following keywords: virtual reality or "VR," augmented reality or "AR," "Wii Fit," video games, "Nintendo," older adults or elderly, balance measures or outcome measures, postural reactions, and postural stability. The search strategy was discussed with the institutional librarian. We also conducted an additional grey literature and manual search of dissertations and unpublished literature to include all the relevant articles for further review.

Selection criteria

We included peer-reviewed randomised control trials (RCT) and experimental studies with full-text articles published in English between January 2013 and May 2024 for final inclusion. We excluded editorials, commentaries, conference abstracts, observational studies reviews, grey literature, and non-English studies. The data screening and selection of articles is mentioned in the PRISMA flow diagram below (Fig. 1).

Two reviewers independently screened the titles and abstracts of articles based on the keywords and search strategy. The reviewers resolved the discrepancy in scoring through discussions to reach a consensus. All trials classified as relevant by either of the two reviewers were retrieved. A third reviewer resolved disagreements

through discussions, and the third and fourth reviewers helped compile the data into a final set. Figure 1 above provides the summary of the search articles and study analysed.

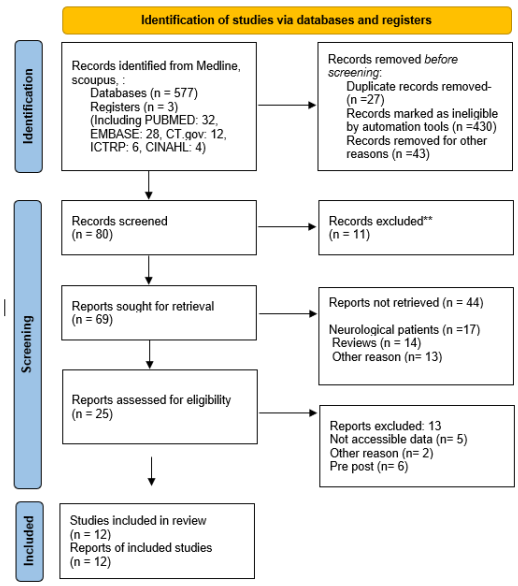

Fig. 1. PRISMA flow diagram

After full data extraction, the reviewers independently assessed the risk of bias using the RoB 2 tool.[40] We assessed the studies' bias based on random sequence generation, allocation concealment, blinding of outcome assessment, incomplete outcome data, intention-to-treat analysis, and follow-up. Items were classified as 'low risk' when clearly described, 'adequate or high risk' when not adopted, and 'inadequate and unclear' when not clearly defined or missing in the text.[41] The "incomplete outcome data" was also classified as 'high risk' if the drop-out rate was higher than 20%.[41]

Data extraction and analysis

Data from relevant articles were extracted on Excel sheet and further synthesised using the RevMan 5.4® software to calculate the mean differences at 95% confidence intervals (95% CIs) for continuous data and (95% CI) for the dichotomous discrete data. Table 1 summarises different studies, durations, sample sizes, ages, and protocols.

Results

Study characteristics

Out of 580 records identified from databases, 69 articles were retrieved for full-text review, and 12 RCTs met the selection criteria and were included in the final analysis, as summarised in Table 1. The combined analysis of the reviewed studies spanned healthy individuals between ages 45 and 75 years with a demographic focus on older adults with a mean age ranging from 60.6 to 69.1 years. The sample sizes ranged between 10 and 90, with 10 studies having less than 30 participants, two having sample sizes near 80, and only one having a sample size of 281 participants. The reviewed studies compared the virtual training protocols to tailored conventional training protocols for balance, coordination, strength, and mobility commonly employed in clinical practice. The studies exhibited variations in weeks and session lengths. Notably, 14.29% of studies[42–44] considered session lengths of 12 weeks (about three months) appropriate for better outcomes with VRT, 3.57% of studies showed that 3-week VRT sessions[15] and 4.76% of studies showed that 4-week VRT sessions[45] proved impactful.

The included studies' experimental and control group intervention sessions ranged between 4 and 10 weeks, with a weekly frequency of two to three times and an average duration of 30 minutes to an hour per session. Two studies demonstrated results intending to treat, eight had a post-intervention follow-up, and two did not mention either intention to treat or post-intervention follow-up.

Types of interventions

Participants in the studies were administered strengthening and balance exercises, treadmill walking, static and dynamic balance training, lower leg strengthening, and balance and coordination training with or without VR equipment. Some studies conducted VR training using the Xbox 360 platform and the Wii Fit Nintendo system for their motion detection and activity tracking features. In contrast, others used the Kinect Xbox 360 for a three-dimensional augmented reality experience.[5,19,46–51]

As shown in Table 1, the studies showed variability in the type of intervention and the analysed outcomes. Of the selected 12 RCTs, two were parallel trials where the control group received no intervention. The experimental groups in these trials received either brain exercises (BE), physical exercises (PE), lumbar stabilisation exercises (LSE), or virtual reality exercises (VRE).[46,52] Overall, 46% of studies (n=6) did not provide any intervention for the control group participants, and 62% (n=8) provided different exercise protocols such as ball exercises, treadmill walking, cognitive training, Otago exercises, leg strengthening with balance training, balance training alone, and balance with coordination exercises. Of the different VR gadgets used in these studies, six (46%) used the Wiifit Nintendo, and five (38%) used Xbox and Kinect.

One study demonstrated enhanced postural control with VRT versus traditional exercises.[50] Likewise, another study showed VRT's role in improving balance and coordination in elderly patients.[51] In yet another study, researchers used a smartphone-running application that mirrored the video from the smartphone on a curved 65-inch screen TV for VR training.[53] The results

Table 1. Data extraction sheet

No	Author	Study design	Sample Size	Age group	Control Group	Parallel group	VR tools used for the experimental group	Method protocol	Duration	Variables assessed	Results	Conclusion
1	Kyeongjin Lee (2021)[53]	RCT	56	VRGT 81.01±6.89 Control group 79.47±6.15	Gait training without virtual reality	NA	Gait training with virtual reality. A smartphone running application - The video from the smartphone was mirrored on a curved 65-inch screen TV placed in front of the treadmill.	5 times a week for 4 weeks	50 min	Balance	OLS (p= 0.000), BBS (p= 0.634), FRT (p= 0.448), and TUG (p= 0.002)	In the VRGT group, the balance ability variable showed a significant decrease in the one-leg-standing test and a significant improvement in the Timed Up and Go test. With respect to spatiotemporal gait parameters, velocity and step width decreased significantly in the VRGT group (p<0.05), and stride length and step length were significantly improved in the VRGT group (p<0.05).
2	Prasertsakul et al. (2018)[50]	RCT	10	40–60 years, age = 51.5±6.61 years, age = 55.0±5.72 years	Balance training	NA	Kinect sensor Dual Task Virtual Reality Balance Training	thrice weekly for four weeks	45 min	Balance	Five standing tasks were assessed: 1. Standing unsupported with eyes open (EO); p<0.05. 2. Standing unsupported with eyes closed (EC); p<0.05. 3. Standing with both feet together; p<0.05. 4. Tandem stance; p<0.05. 5. One-leg stance; p<0.05	VRT facilitates better postural control and contributes to fall prevention in the elderly
3	Žukienė et al. (2018)[51]	RCT	20	mean age 82.8 and 80.4 years	Balance - coordination training	NA	Microsoft Xbox 360 gaming device with Kinect	ten sessions (three months)	30-45 min per session	Balance and coordination	Tinetti Performance Oriented Mobility Assessment; p<0.05, Berg Balance Scale; p<0.05, The Activities-Specific Balance Confidence (ABC) Scale; has no statistical significance, unbalanced coordination samples (Schmitz); p<0.05	The use of virtual reality has a positive effect on the development of balance and coordination
4	Htut et al. (2018)[46]	Randomised Parallel Trial	84 (47 M &37 F)	65-85 years	No intervention	Two parallel groups BE- Brain exercises, and PE-strength and balance exercises	Ten games from X-box 360	thrice weekly for eight weeks	30 min	Balance, muscle strength, cognition, and fall prevention	BBS; p<0.001, TUG; p<0.001, TUGC, MoCA; p<0.001, FES-I; p<0.001, 5TSTS; p<0.001, HGS; p<0.005, TUG-cog; p<0.001	VRE produced measurable improvements in physical and cognition scores
5	Dockx et al. (2017)[56]	RCT	281	above 65 years	Treadmill walking	NA	X Box one Kinect-treadmill augmented training	thrice weekly for six weeks	45 min	Attitudes towards fall prevention	USQ questionnaire p<0.001	Older people's attitudes towards fall prevention exercise with VR were positively influenced by their experience
6	Tsang and Fu (2016)[55]	RCT	79 (31 M & 48 F)	mean age 82.3±3.8 and 82.0±4.3 years	Leg strengthening and balance training	NA	Wii Fit balance training games included Soccer Heading, Table Tilt, and Balance Bubble.	thrice weekly for six weeks	1 hour	Balance	BBS; p<0.001, timed-up-and-go test; p= 0.434, and limits of stability test; p<0.01.	WiiFiT balance training group achieved better balance performance
7	Park et al. (2015)[23]	RCT	30 (19 M & 5 F)	Above 65 years	Ball exercise	NA	Wii Fit	thrice weekly for eight weeks	30 min	Balance	TUG; p<0.05, step length; p<0.05, sway speed; p<0.05	Step length increased significantly, and the average sway speed and Timed-Up and Go time significantly decreased.
8	Jung et al. (2015)[52]	Randomised Parallel Trial	24 (24 F)	mean age 73.6±2.4; 74.3±3.5 and 74.3±2.1 years	No intervention	LSE - lumbar stabilisation	The Nintendo Wii Sports	twice weekly for eight weeks	30 min	Balance and risk of falls	BBS; p<0.001, FRT; p<0.001, TUG; p<0.001, CV; p<0.001, MVHC; p<0.001	Significant clinical improvements in lower extremity balance and mobility

9	Schwenk et al. (2014)[45]	RCT	33	mean age 84.3±7.3 and 84.9±6.6 years.	No intervention	NA	24-inch computer screen, V2.54, and five wearable inertial sensors	twice weekly for four weeks	45 min	Balance	30-second standing with feet close together (but not touching) with eyes open (EO); p = .007 and eyes closed (EC); p =0.010. The CoM sway area (cm2) during EO stance. Physical performance was quantified by the Alternate-Step-Test (AST); p = .037, Timed-up-and-go (TUG); p = .024, and Gait assessment; p = .902.	Study findings guide future exercise interventions integrating wearable sensors for guided game-based training in home and community environments.
10	Cho et al. (2014)[22]	RCT	32	mean age 73.1±1.1 and 71.7±1.2 years	No intervention	NA	The Nintendo Wii Fit balance board and a CD	thrice weekly for eight weeks	30 min	Balance	Subjects' balance with their eye open; p<0.001 and closed; p<0.001 was measured using the Romberg test on a Bio-rescue (RM INGENERIE, France).	Virtual reality training is effective at improving the balance
11	Bieryla and Dold (2013b)[15]	RCT	9	mean age 82.5±1.6 and 80.5±7.8 years	No intervention	NA	Wii Balance Board with Wii Fit	thrice weekly for three weeks	30 min	Balance	BBS; p = 0.037, FAB scale; p = 0.529, FR; p = 0.779 and TUG; p = 0.174	Balance training with Nintendo's Wii Fit may improve balance.
12	Yoo et al. (2013)[42]	RCT	21	mean age 72.90 (3.41)	Otago exercises	NA	Augmented reality environment (graphic and vision-based web-camera)	thrice weekly for twelve weeks	40 min	Fall prevention	BBS; p<0.001, GAITRite system was used to measure spatiotemporal parameters including gait velocity; p<0.001, cadence; p<0.001, step length; p<0.05, and stride length; p<0.05, FES-I; p<0.05	Augmented reality-based Otago exercise is effective for improving balance, gait, and fall efficacy

significantly improved the TUG test and gait parameters.[53]

Similarly, balance training with Nintendo's Wii Fit [15] and augmented reality-based Otago exercises improved balance and gait and reduced falls in older women.[42]

Assessment parameters

Table 1 demonstrates that the studies used scales such as the BBS, TUG, POMA, and ABC tests to assess balance, gait, and fear of falls in older adults. Seven studies used BBS, and five used TUG as measuring tools to evaluate the post-intervention balance. Almost all studies showed significant improvement in the scale assessments after VR training compared to regular exercises.

Risk of bias

The risk of bias analysis of the studies using the RoB 2 tool demonstrated that nine studies had a low risk of bias. Three studies showed bias in blinding and performance, and three showed unclear bias in the randomisation and allocation. Please refer to Figure 2 and 3.

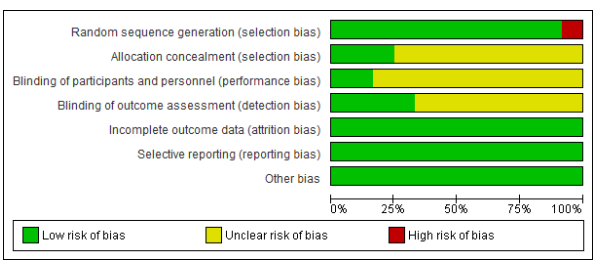

Fig. 2. Risk of bias graph: review authors' judgments about each risk of bias item presented as percentages across all included studies.

Key findings

The selected studies demonstrated that of the individuals who underwent VRT, 92% showed improved static and dynamic balance (n=12) as measured using the BBS (39%, n=5) and the TUG (39%, n=5) as the main tools to measure the outcomes. Two studies had insufficient data for the BBS assessment. Hence, of the seven studies that evaluated balance using the BBS, only five had complete data.[42,46,52,54,55] The post-intervention results of the studies showed improved BBS scores of 4.29±1.27 and TUG test scores of 2.65±1.20 seconds in the VRT group compared to the control group. Our meta-analysis of five studies[42,46,52,54,55] using *RevMan*® 5.4 revealed the BBS's overall effect z= 2.07 (P=0.04) with a Standardized Mean Difference (SMD) of 1.05 at 95% confidence interval (CI) [0.06, 2.05], p<0.001 showing a weak effect of VR training in the experimental group as compared to the control group. The Chi-square test (Chi^2=39.73), df=4 (p<0.00001) and I^2 value (90%) indicated significant heterogeneity, suggesting considerable variability in the true effect of VR training on BBS across the included studies. Please refer to Figure 4 below and supplementary Figure S1.

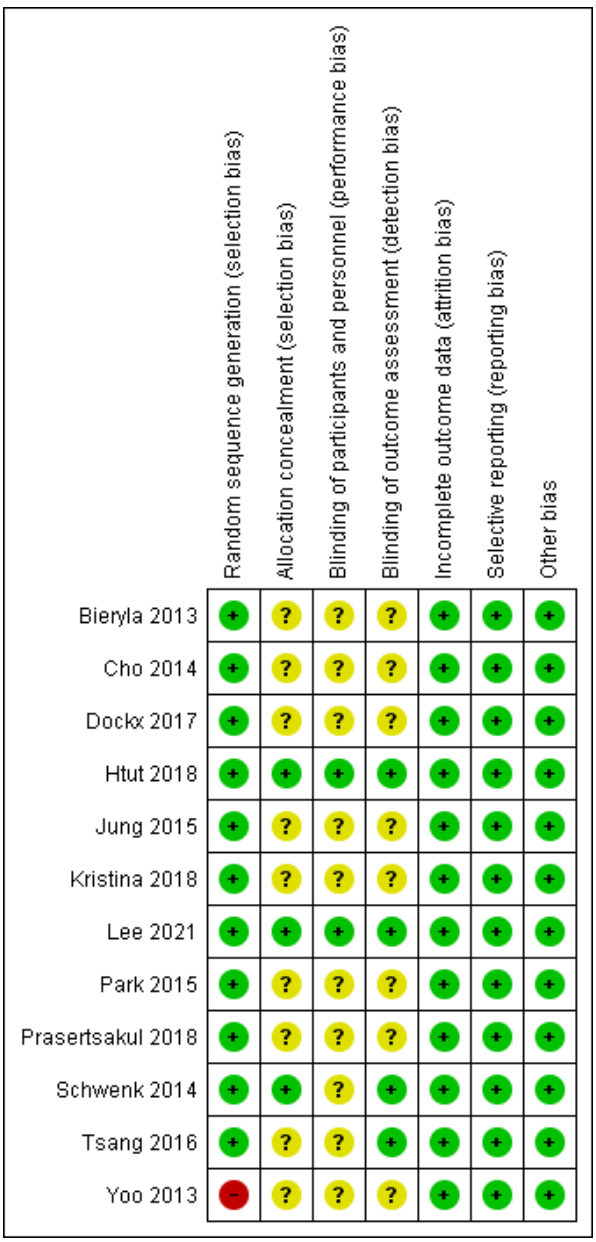

Fig. 3. Risk of bias summary: review authors' judgments about each risk of bias item for each included study

The TUG test meta-analysis for VRT (please refer to Figure 5), based on five different studies, revealed the effect size as z= 2.25 (p=0.02) with the SMD as -0.74, at 95% CI [-1.39, -0.09], P<0.001 indicating that the experimental group showed improved results on the TUG test.[23,45,46,52,55] However, statistically significant heterogeneity was found between studies (Tau^2 = 0.41, Chi^2 = 18.63, df=4 (p=0.0009); I^2=79%, suggesting that the actual effect sizes may vary considerably across studies. The mean TUG Scale score in the experimental group was 18.2 compared to the control group score of 13.8. (Figure 5 and supplementary Figure S2).

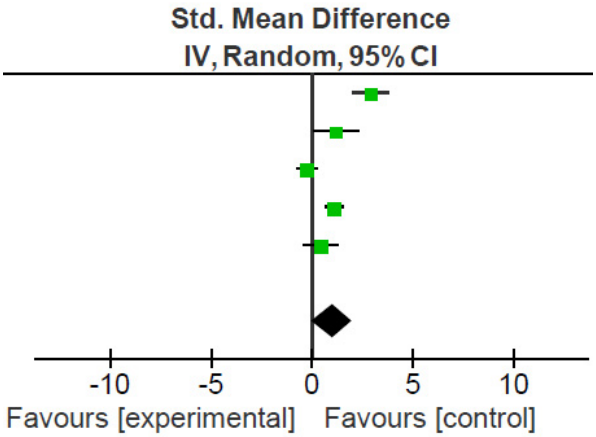

Fig. 4. Forest plot for meta-analysis of studies reporting BBS

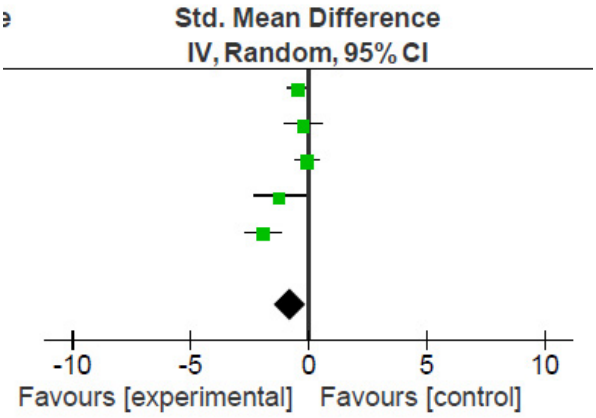

Fig. 5. Forest plot for TUG

Discussion

The analysis of the selected studies demonstrated that exposure to VR can improve physical and cognitive scores,[46,56] positively impacting the individual's physical and psychological well-being.[44] Additionally, high-functioning virtual reality games helped manage the individual's fear of falls.[43] The findings of our study are consistent with previous systematic reviews on VR training.[36–39] However, similar to earlier studies, we found it challenging to single out the most effective treatment method, assessment tool, and types of VR equipment to recommend. Individual study objectives and available technology influenced the selection of VR equipment in all studies. Researchers chose devices based on their motion detection capabilities, interaction, target population, user-friendliness, and the intended level of immersion and engagement – all studies aimed to construct appealing virtual worlds to enhance intervention effectiveness and collect relevant data.

The diverse assessment scales used across the studies reflected the multidimensional nature of balance, mobility, and related factors. The results from various studies demonstrate that selecting a singularly superior measurement scale proves challenging as some scales have distinct advantages over others.[15,22,23,42,45,46,50–53,55,56] Our meta-analysis found that balance training did not improve on the BBS scale assessment but showed significant improvement on the TUG test. Our meta-analysis findings differ from those of de Amorim et al., who reported improved balance training measurements with the BBS scale but similar TUG test results.[37] This difference suggests that the effectiveness of a measurement scale is context-dependent and conditional to the research objectives, the population under study, and the specific variables being measured.[42,55,56] Therefore, measurement scales should be selected based on the intervention or research objective. The BBS scale can assess an individual's ability to sustain balance during specific tasks.[15,23,42,52,53,55] The TUG test can capture functional mobility, which is particularly relevant in research settings.[23,46,47,52,55] The Falls Efficacy Scale (FES) and the ABC scale, both psychological scales, can give valuable insights into the individual's confidence in performing daily activities without the fear of falling.[44]

Enhanced balance and mobility, confirmed by improved step length, sway speed, and TUG scores,[23] underlined the positive impact of physical therapy using VR versus traditional ball exercises on physical performance and balance in older adults.[48,52] Better results with VR training can be attributed to its immersive, interactive, safe, and controlled environments that simulate real-life scenarios, enhancing participants' engagement and motivation.[22,23,53] VR allows precise control over the training environment and tasks, enabling tailored and progressive challenges that can adapt to the individual's performance.[22,23,47] This adaptability can lead to more effective and personalised training outcomes.[44,55] Furthermore, the technological advantage of VR allows for creating consistent and repeatable training scenarios, which can be challenging to achieve with traditional methods.[46,51] Such engaging experiences created by VR games lead to increased patient adherence and effort during training sessions.[51,53] Apart from the treatment acceptability, VR systems provide immediate feedback and collect detailed data on performance metrics, which can be used to adjust training protocols in real-time training.[22,23,45,52]

Studies have reported improved dynamic postural control and functional ability in institutionalised elderly individuals with VRT using video games.[47] These video games encourage the patients to adapt to the game challenges, integrating diverse movement patterns and moving in different directions. Hence, incorporating wearable sensors for guided game-based training in home and community environments may help improve the patient's cognitive and lower limb motor control.[22,42] The reviewed studies conclusively showed the growing acceptance of VR technology as a helpful tool for testing

and improving the balance, mobility, and other characteristics of the older population.

Our study has several strengths, primarily due to our rigorous methodology and meta-analysis inclusion. We used strict selection criteria, and two reviewers independently assessed the articles and thoroughly assessed their quality and risk of bias, which is also a crucial strength of our study. Although we have reported the positive effects of VRT in improving balance in older adults for fall prevention, our study has some limitations. The meta-analysis included only the BBS and TUG scores that produced low effect sizes due to lower sample sizes and heterogenicity of the studies. Thus, variations in sample sizes, methods, interventions, and durations of the reviewed studies may have influenced the results.

Future studies focussing on the meta-analysis of other assessment scales used for the post-intervention balance and gait evaluation of older adults would help conclude the effectiveness of VRT over traditional rehabilitation. Comparing the various assessment scales in a single study is also warranted. There is limited research on comprehensive economic evaluations or cost-effectiveness analyses of VRTs compared to traditional balance training. Cost-effectiveness analysis is essential for informing future funding decisions in rehabilitative care. We recommend that future clinical trials thoroughly compare various VR technologies' initial equipment costs, setup, and operational expenses. This will provide policymakers with the detailed data needed to incorporate VR into standard rehabilitation protocols strategically.

Conclusion

Based on the findings of our systematic review, we can conclude that VRT is a feasible and effective treatment option for improving balance, as assessed by the TUG test in older adults, to prevent falls. While many studies suggest promising effects, based on our meta-analysis, we recommend additional research with rigorous study designs, larger sample sizes and meta-analysis of a more significant number of balance assessment scales to strengthen the evidence. One must also consider the feasibility of implementing VRT for balance rehabilitation programs for older adults.

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Declarations

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Author contributions

Conceptualization, M.R. and S.B.; Methodology, M.R., S.B. and R.D.; Software, R.D.; Validation, R.D., S.B. and M.R.; Formal Analysis, S.B. and R.D.; Investigation, S.B. and R.D.; Resources, S.B. and M.R.; Data Curation, S.B. and R.D.; Writing – Original Draft Preparation, S.B., M.S., M.R., R.D. and T.B.; Writing – Review & Editing, S.B., M.S. and T.B.; Visualization, R.D.; Supervision, M.R., S.B. and T.B.; Project Administration, S.B. and M.R.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The systematic review and meta-analysis data are available in the main manuscript and the supplementary material.

Ethics approval

Dr. D. Y. Patil Deemed University's ethics committee has approved the study under the reference DYPV/EC/448/2020.

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CASE REPORT

Combined COVID-19-related chronic hypoxemia and lack of screening as a double challenge for the management of asymptomatic invasive lung adenocarcinoma

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ABSTRACT

Introduction and aim. Lung adenocarcinoma (LADC) is the most diagnosed histological subtype of lung cancer and the leading cause of cancer death in men in Algeria. Defining the circumstances that preceded the diagnosis improves the management options and reduces its incidence. However, data for this critical period are lacking. We report the case of a patient whose onset of severe COVID-19 and the incidental finding of an undefined LADC overlapped and delayed care of the malignancy.

Case description. We present the case of a 65-year-old man, with invasive LADC discovered during a chest CT scan performed for suspected severe COVID-19. We describe the diagnostic methods and the patient. Histological examination by biopsy required to confirm diagnosis could not be performed due to chronic hypoxemia in the patient, which prevented the complete pathological diagnosis and staging of the disease.

Conclusion. Given the prevalence and aggressiveness of LADC in men in Algeria, our study underscores the critical need to develop screening programs, aimed at identifying the disease in asymptomatic patients, in asymptomatic patients that could significantly improve the chances of successful treatment. This is particularly important because LADC patients often develop serious pathologies that can limit their treatment options. COVID-19 serves as a stark example of such limiting interference, further highlighting the importance of early detection in the management of LADC.

Keywords. chest CT, COVID-19, hypoxemia, LADC, screening

Introduction

Lung adenocarcinoma (LADC), the most common histological subtype of nonsmall cell lung cancer (NSCLC), accounts for more than 40% of cases worldwide.[1] The potential for early diagnosis of LADC (stages I and II) has been confirmed, as it is associated with a significantly good prognosis, with a survival rate of nearly 100% after surgical resection.[2] However, advanced LADC (stages III and IV), with its aggressive behavior and limited therapeutic options, remains a significant cause of

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cancer-related deaths worldwide, resulting in a 5-year overall survival rate of less than 75%.[3]

The circumstances of LADC diagnosis are variable. Although early stage LADC remains asymptomatic for an extended period, advanced LADC is often diagnosed with symptoms such as cough, sputum, and dyspnea.[4] In the general population, incidental finding as a scenario preceding the diagnosis of LADC has been described in many studies when this malignancy can be found at 56% to 66.9% in asymptomatic patients during the investigation of unrelated symptoms or by screening.[5] Interestingly, an estimable percentage of LADC could be discovered incidentally (9.1%).[6] This incidental finding represents an opportunity for the patient to have a better chance of recovery and allows potentially curative treatment, thus improving the overall survival rate.

Between 2020 and 2022, the coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has significantly impacted healthcare systems worldwide.[7-9] The increased use of chest imaging to diagnose COVID-19 during the pandemic has led to the incidental finding of non-COVID-19 related lung lesions, including malignancies.[10,11] In particular, high incidental findings of LADC were also found in patients with COVID-19 at 55.6%.[12] However, the combination of COVID-19 and newly diagnosed malignancies has been described as a complicated onset since concurrent severity of COVID-19 could reduce the chances of patients starting cancer treatment before healing.[13-15] In Algeria, the final statistics reported more than 272 thousand confirmed cases of COVID-19 and over 6 thousand deaths among the Algerian population.

Lung cancer is a real scourge among the Algerian population, since it is the most prevalent cancer among men (19.5%) with high mortality (10.4%).[16] Several descriptive studies of lung cancer have been carried out in Algeria before and after the pandemic. They were mainly conducted to describe demographic, epidemiological or clinicopathological features of different types of lung cancer, including LADC, from the time of pathological diagnosis or the time of treatment in the Oncology Department.[17-25] According to the study of the Lung Cancer Registry in Algeria (LuCaReAl) study, a comprehensive research initiative on lung cancer in Algeria, LADC represents 55% of newly diagnosed lung cancer patients. The LuCaReAl study showed that the proportion of advanced stages at diagnosis was alarming, mainly due to late diagnosis.[22] Unfortunately, we were unable to find information about incidental findings of lung cancer in general or invasive LADC specifically in those reports, and studies that elaborate the clinicopathological and radiological characteristic of incidental lung cancer during the interval of imaging to pathological examination have not been addressed. Thus, the actual effect of the pandemic on the management of newly diagnosed cancer patients among Algerian patients has not been well documented so far and this lack of evidence is shared throughout the world. Therefore, more research to understand the impact of COVID-19 on this category of patients.

Here, we present an example of a case with simultaneous diagnosis of severe COVID-19 pneumonia and newly diagnosed advanced LADC. Chest computed tomography (CT) imaging of COVID-19 diagnosis revealed CT abnormalities that led to the suspicion of advanced lung cancer, which could not be identified by pathology due to COVID-19-related complications. The diagnosis, thus remaining undefined, delayed the oncologic care of the patient. This case may be representative of other cases that deserve to be studied. Additionally, we hope that this report will provide valuable information on the importance of screening for LADC at an early stage before additional medical illness overshadows the clinical picture and minimizes the chances of a cure.

Table 1. Laboratory data of the patient*

Test	Day 2	Day 6	Day 12	Units	Refence range
WBC	4.49	11.24↑	11.41↑	10^3/µL	4–10.2
Neutrophils	83.9↑	89.0↑	52.9	%	43.5–73.5
Lymphocytes	10.9↓	7.61↓	33.8↓	%	15.2–43.3
Red cells	4.90	4.73	5.55	10^6/µL	3.8–6.2
Monocytes	0.23↓	2.68↓	13.3↑	%	3–13
Hemoglobin	10.9↓	11.45↓	11.9↓	g/dL	13–17.5
CCMH	31.5	31.7	31.1	g/dL	31–37
MCV	70.6↓	76.4↓	69.0↓	fL	78–100
MCHC	22.2↓	24.2↓	21.4↓	pg	25–34
Hematocrit	34.6↓	36.1↓	38.3↑	g/dL	40–52
Platelets	189	246.9	425	10^3/µL	120–450
Alanine aminotransferase	42	76↑	40	U/L	10–49
Aspartate aminotransferase	30	32	30	U/L	10–39
D-dimers	766↑	589.6	381.0	ng/mL	>50 years, <age ×10
CRP	91.65↑	--	9.67	mg/L	<10.00

*↓ – the value was below normal,↑ – the value was above normal

Description of the case

On November 28, 2021 (Day 1), a 65-year-old Caucasian male Algerian patient was referred to the emergency unit of our public hospital establishment located in Ain Berda, Annaba, due to shortness of breath, dry cough, fever, chest pain, and a low percutaneous arterial oxygen saturation (SpO_2) at 83% breathing on room air. Due to the endemic location and the history of contact with confirmed positive COVID-19 person a few days before presentation, he was highly suspected of getting a COVID-19 infection. However, a nasopharyngeal test for the COVID-19 antigen was negative. In fact, a serological test of COVID-19 antibodies suggested only an anterior contact with the virus. Therefore, no sufficient evidence could rule out a developing COVID-19 infection and he was admitted to an isolation box. A chest computed tomography (CT), a real-time

polymerase chain reaction (RT-PCR), and a control serological test for the SARS-CoV-2 were prescribed. A routine examination was realized (Table 1), and vital signs were regularly monitored twice (Table 2).

Table 2. Clinical and laboratory data of the patient during hospitalization at COVID-19 unit*

Measure	Fasting blood glucose (g/L) at day	Postprandial plasma glucose (g/L) at night	Oxygen saturation at day (%)	Oxygen saturation at night (%)	Systolic/Diastolic blood pressure at day	Systolic/Diastolic blood pressure at night	Body temperature day/night
Reference range	0.60–1.10	1.10–1.30	> 92%	> 92%	< 90/<140	<90/<140	37/37
Day 4	--	2.36↑	60↓	67↓	--	120/60	-/37.9
Day 5	--	1.48↑	35↓	48↓	100↑/60	120/70	-/37.1
Day 6	1.63↑	2.53↑	30↓	48↓	150↑/80	150↑/100	-/36
Day 7	2.95↑	2.56↑	59↓	43↓	120/70	140/80	39↑/39↑
Day 8	2↑	2.92↑	45↓	54↓	150↑/80	140/80	39↑/39↑
Day 9	2.07↑	1.2	81↓	83↓	170↑/100↑	130/80	37.1/37
Day 10	2.12↑	2.26↑	69↓	76↓	130/80	150↑/90	37.2/37
Day 11	3.14↑	2.44↑	73↓	74↓	140/80	160↑/60	37/37.2
Day 12	3.34↑	2.97↑	67↓	76↓	130/80	130/70	37.1/37.1
Day 13	2.95↑	2.56↑	67↓	82↓	120/70	140/70	37/36.9
Day 14	1.80↑	1.20	81↓	82↓	140/70	140/70	37/36.9
Day 15	1.87↑	2.7↑	81↓	71↓	130/70	130/70	37/37
Day 16	1.46↑	--	--	--	130/80	--	37/36.9

*↓ – the value was below normal,↑ – the value was above normal, hematology and biochemistry laboratory testing were performed two times

On days 2 to 4, initial blood tests did not show a change in white cell blood count (CBC). However, there was an early inversion in the leukocyte formula (neutrophils reached 83.9% and lymphocytes decreased to 10.9%), while platelets were normal. The blood type was O-positive. We noticed low hemoglobin and hematocrit. Blood ionogram (kaliemia and chloremia) and creatinemia were normal. High levels of D-dimers (766 ng/mL) and plasmatic C-reactive protein (CRP, ~91 mg/L) were also noted. The patient presented new-onset hyperglycemia with a high HbA1c level of 7.2%. According to the Hashmi-Asif COVID-19 chart, the calculations of scoring for signs, symptoms, and blood biomarkers for the early presentation of the patient found that he was highly suspected of getting COVID-19 with a score of 24 (Table 3).[26]

On day 5, a serological conversion of COVID-19 antibodies and a positive RT-PCR for COVID-19 were obtained, indicating a developing COVID-19 infection. In addition, contrast-enhanced chest CT revealed severe involvement of both lungs with peripheral, patchy ground-glass opacities (GGO) that extended to more than 75%, consistent with a COVID-19 infection (Fig. 1A) accompanied by localized emphysema (Fig. 1B). No pneumothorax (Fig. 1A) or pulmonary embolism (Fig. 1C) were observed. However, the chest CT also showed a 46.2 × 56 × 69.3 mm solid mass (Fig. 2A and 3B) abutting the thoracic esophagus (Fig. 2B) in the right upper lobe of the lung, suggesting a malignant tumor. Two lymphadenopathies were found. The first was measured up to 0.82 × 15.3 mm and was ipsilateral subcarinal hilar, and the second node in the aortopulmonary window was measured 1.68x17.8 mm (Fig. 2C). Another finding was a 14.7 × 16.8 mm nodule in the left upper lobe, suggesting contralateral pulmonary metastases (Fig. 2D and 3C). A severe has been made of COVID-19 pneumonia diagnosis with a suspected possible advanced lung malignancy has been made.

Table 3. COVID-19 scoring according to Hashmi-Asif COVID-19 Assessment Chart[26,*]

Physical signs and symptoms	Scores		
	Day 2	**Day 6**	**Day 12**
Temperature	(39)>38 score-3	(36)≤37 score-1	(37.1)≤37.5 score-2
Cough	Dry cough score-3	Dry cough score-3	Productive score-2
Fatigue	>2day score-3	>2day score-3	>2day score-3
Nausea and vomiting	Absent score-1	Absent score-1	Absent score-1
Mucus membrane	Dry appearance score-3	Dry appearance score-3	Normal score-1
Total	**13**	**11**	**9**
Blood biomarkers			
Leukocytes (3,800–1,100/μL)	4.49 score-2	11.24 score-2	11.41 score-2
Lymphocytes (1,000–3,900/μL)	10.9 score-3	7.61 score-4	3.8 score-4
Neutrophils (1,900–7,400/μL)	83.9 score-2	89.0 score-2	52.9 score-2
Platelets (150,000–400,000/μL)	189 score-2	246.9 score-2	425 score-1
*Alanine aminotransferase (10–49U/L)	42 score-1	76 score-4	40 score-1
Aspartate aminotransferase (<33 U/L)	30 score-1	32 score-1	30 score-1
Total	**11**	**17**	**12**
Total score	**24**	**28**	**21**

* cumulative scoring ≥13–22/39 is considered at high risk to be diagnosed with COVID-19,
no disease ≤12, mild 13–22, moderate 23–33, severe 34–39

Table 4. Drugs for treatment of the patient during hospitalization*

Drug	Hospital day	Dose	Usage
Cefotaxime	Day 6–day 10	1 g BID	iv
Ciprofloxacin	Day 11–day 16	200 mg BID	iv
Enoxaparin	Day 6–day 16	0.8 UI BID	sc
Dexamethasone	Day 6–day 16	8 mg BID	im
Budesonide	Day 6–day 16	1 mg BID	inh
Omeprazole	Day 6–day 16	40 mg QD	im
Paracetamol	Day 6–day 16	1 g every 8h if fever	iv
Macrogol 4000	Day 6–day 16	10 g TID	po
Nicardipine	Day 11	1 mg once	iv
Neutral Protamine Hagedorn insulin	Day 6–day 16	18 UI BID	sc

* QD – one a day, BID – twice a day, TID – three times a day, sc – subcutaneous, po – per os; iv – intravenously, inh – inhalation, im – intramuscular

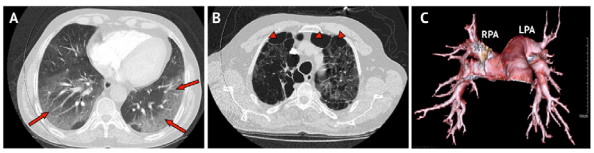

Fig. 1. A: Representative axial-enhanced chest CT scans (lung window) in a 65-year-old man showed a peripheral GGO pattern in the right and left middle lobes, B: Representative axial enhanced CT scans showing multiple localized emphysema and anterior pulmonary bullae (arrows headed) in bilateral upper lobes, C: The rendering of 3D pulmonary volume for enhanced CT images obtained with early injection time showed that the texture of the main pulmonary artery became thicker, presenting an intense homogenous enhancement. On the contrary, the trachea, right pulmonary artery, left pulmonary artery, segmental lobar and sous-segmental arteries were normal

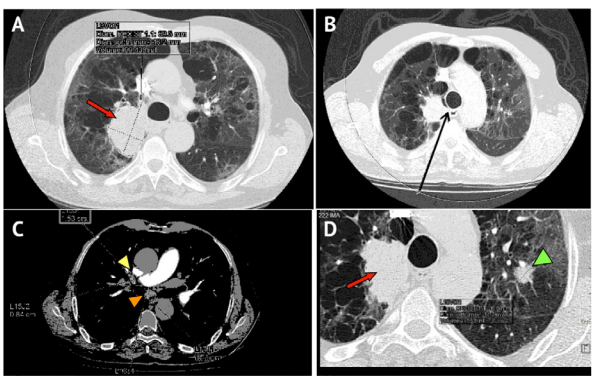

Fig. 2. A: Representative axial images showing a tumor process of the right upper lobe (arrow) that occupies the posterior segment and leans against the mediastinal pleura. The mass measured 46.2×56 mm in the axial plane, B: and was in close contact with the esophagus (arrow), C: CT imaging also showed that the process was associated with an ipsilateral subcarinal hilar node measuring up to 0.82×15.3 mm (yellow arrowhead) and another in the aortopulmonary window measuring 1.68×17.8 mm (orange arrowhead), D: Another suspicious pulmonary nodule was discovered in the left upper lobe measuring 14.7×16.8 mm.

The patient was transferred to the COVID-19 department to be initially treated for his severe pneumonia. With no notable medical history, he was a former smoker with a Brinkman index of 800 (40 years of smoking 20 cigarettes per day, one pack-year) and had quit smoking ten years earlier. Meanwhile, the physical examination found expectorations, marked diminished vesicular murmur throughout the chest, marked finger crabbing, constipation, and hypertension (grade 1). Low-flow oxygen therapy through a nasal cannula at 5 L/min was administered. He underwent 12 days of comprehensive treatment (Table 4) until his recovery. After completion of therapy, no apparent side effects were observed with the above drugs, except for the antibiotic agent, cefotaxime, since the patient reported dizziness and vertigo on day seven a few minutes after the injection of cefotaxime. Given its possible neurological side effects, cefotaxime was stopped and replaced with ciprofloxacin (Table 2).

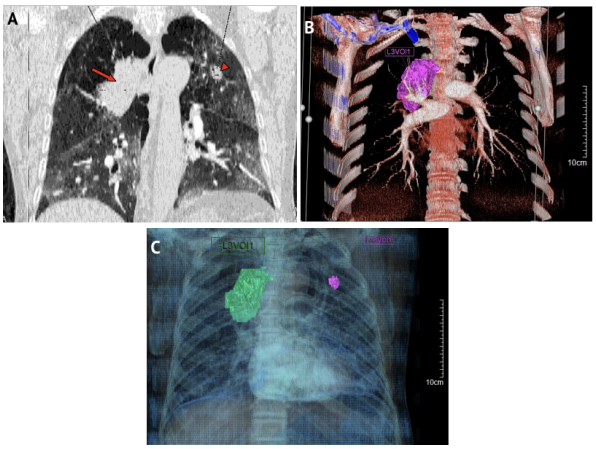

Fig. 3. A: Representative coronal images showing increased lung emphysema with diffuse GGO in the middle and inferior lobes and the tumor process of the right upper lobe (arrow) leaning against the mediastina and the nodule (head arrow) in the left upper lobe suggesting metastasis, B: 3D volume rendered in left rotation image from our patient demonstrating pulmonary tumor (in magenta) extended in height to 69.3 mm, C: 3D volume rendered image demonstrating right tumor (in green) and pulmonary nodule (in magenta)

From day 5 to day 8, the patient's clinical status deteriorated. He presented fever (>39°C, axillary temperature), intense chest pain, and a steady decrease in SpO2 to 30% (Table 4). Supplemental oxygen was given by combining a nasal cannula (at 5 L/min) with a nonrebreather mask (at 15 L/min). After this critical period, his symptoms were partially relieved, and we found improvement in SpO_2 (81–85%) breathing room air. The patient was put back on low oxygen therapy with a nasal cannula at 5 L/min. Additionally, the improvement of biological parameters was generally (except for a transient increase in alanine aminotransferase on day 6, Table 1). Applying the Hashmi-Asif Covid-19 chart again, we found that the transition in the patient's clinical status was correlated with the score values, which raised to 27 (day 6) and decreased with improving the situation of the COVID-19 situation to 21 (day 12) (Table 3).

On day 16, he was discharged from the hospital and was referred to the Oncology Department for a more extensive evaluation of the incidental finding on chest CT.

On February 2, the patient enrolled in the Oncology department of the Annaba Cancer Center. The oncologist prescribed a CT-guided biopsy to perform

a histopathological examination of the incidental mass. Unfortunately, the patient remained hypoxemic and required long-term oxygen therapy (LTOT) for approximately two months using an oxygen nasal cannula, which prevented biopsy.

One month later (March 02), a follow-up chest CT revealed a marked improvement in the GGO pattern, with diffuse bilateral interstitial lesions of sequelae appearance (data not shown). Moreover, where the CT images showed that the lymphatic nodes remained unchanged in form and size, the solid mass was found to be increased (87 mm× 60 mm ×86 mm). We thus estimated the tumor growth rate by calculating the tumor volume doubling time (TVDT) based on the chest CT scan measurements initially found before three months, using the Schwartz formula of exponential growth, where V1 and V2 were the largest diameters on the CT scan measured at two different times, and the time interval between two measurements: TVDT=(t×log2)/[log (V2/V1)].[27] We found that the TVDT for the tumor was 67 days.

On April 10, when its oxygenation status became routine, the patient underwent a CT-guided biopsy. The histopathological examination of the material collected from the solid mass confirmed a diagnosis of non-small cell poorly differentiated (high-grade) partially necrotic carcinoma of the lung. Immunohistochemistry stains showed positivity cytokeratin 7 and thyroid transcription factor-1 with negative cytokeratin 20, cluster of differentiation 56, and p40 protein. The final pathological diagnosis confirmed the mass as a solid adenocarcinoma. The oncologist prescribed substantial exams to classify the cancer. Abdominal and cerebral CT did not reveal additional features. Using technetium 99m-labeled methylene diphosphonate (99mTc-MDP), skeletal scintigraphy was performed. No other suspicious features suggestive of metastatic bone lesions were found on the abdomen, the brain, or the pelvis. According to the classification of tumor node metastases (TNM) of primary lung cancer, 8th Edition.[28] We diagnosed lung adenocarcinoma with clinical stage T3N2M0. Appropriate treatment was initiated in the Oncology department, and he began follow-up for 19 months of chemotherapy. He died in December 2023 from complicated onset and metastasis in the brain and liver.

Discussion

Taking into account the clinical, radiological and epidemiological characteristics, this case study is consistent with previous descriptions of common severe COVID-19 worldwide. The patient presented two symptoms commonly seen among this category of patients: cough (70%) and dyspnea (66%).[29] Indeed, chest CT shows a typical GGO pattern that extends to more than 75% of his lungs, consistent with pulmonary abnormalities found in severe COVID-19 infection, even if he did not present other specific patterns, such as consolidation or pericardial effusion.[30-32] Epidemiologically, this case study shared several risk factors and comorbidities identified in the literature as factors to develop severe COVID-19. To begin with, his age, more than 60 years, is a confirmed risk factor for severe COVID-19.[33] Second, he was a former smoker with a Brinkman index greater than 400 (800), two factors that are known to cause an increased burden of severe COVID-19 outcomes, and sustained mechanical ventilation.[34-36] In addition to these factors, the patient developed a new-onset hyperglycemia described as one of the critical complications observed during patients with severe COVID-19 (41 %).[37,38] Furthermore, of all comorbidities he presented, the LADC was the most burdensome risk factor for COVID-19 severity.[39,40] Regarding this malignancy, we found a gap in the data that described the incidental finding of lung cancer during COVID-19 infection, including tumor characteristics and severity of COVID-19, as well as the course of events from the first imaging to oncology management. Wang and colleagues study conducted in China in 2024 was the only study that reported the incidence of lung cancer as an incidental finding since they identified that among 24390 patients who underwent a chest CT for diagnosis of COVID-19, 0.3% had undefined lung cancer. Interestingly, LADC was the predominant histological type discovered in this cohort, accounting for 55.6%.[12] However, this study relied on data from an epidemiological analysis that lacked several clinicopathological characteristics and did not capture the complete clinical picture of a given case, as mentioned in our study. In addition to this single large-scale study, only one clinical case, presented by Iadevaia and her co-workers, reported incidental lung cancer with severe COVID-19 was shown to be a 42-year-old female, non-smoker.[11] The patient has improved clinically and physically after eight days of onset without long-term COVID-19 complications.[11] Her oncological treatment appears to have been timely, as she promptly received a pathological diagnosis of a micropapillary infiltrating LADC and began chemotherapy. In comparison, although our case recovered from the severe infection and was discharged after ten days of hospitalization, the pathological diagnosis of the suspected LADC was unfortunately delayed by three months. During this time, he experienced prolonged hypoxemia that required LTOT. This finding is consistent with many cohort studies, which have described the persistence of chronic hypoxemia in the early postacute phase of COVID-19, requiring LTOT up to six months after discharge.[41,42] In addition, hypoxemia related to COVID-19 has been proven to result from damage to both the pulmonary parenchyma and vasculature.[43] In this case, hypoxemia should be added to

the list of factors that negatively affect lung cancer care in COVID-19-affected patients, mainly as a barrier to accessing definitive cancer diagnosis for the patient, as mentioned by Malalasekera and colleagues or by delaying the timely diagnosis and staging of the disease, according to Barata and colleagues.[44-46]

Consistent with previous studies conducted before the pandemic, we found that the solid LADC reported here presented typical aggressive clinicopathologic features, including a larger tumor size at initial presentation, lymph node metastasis, lymphovascular and pleural invasion, as well as a fast growth.[47-49] Nevertheless, the tumor presented a TVDT of 67 days that was shorter than the median TDVT found in the literature for solid LADC ranging from 140 days to 229 days.[50,51] LADC size is closely related to the hypoxic state of the tumor microenvironment.[52] Interestingly, hypoxia was shown to be a common characteristic shared in both COVID-19 and LADC; both pathologies are known to harbor hypoxia-associated genetic signatures that prompt altered energy metabolism.[53-55] We can hypothesize that hypoxemia as a long-term COVID-19-related complication could accelerate LADC growth rate of LADC. Future studies are needed to confirm this hypothesis.

Before the COVID-19 pandemic, many countries have established recommendations to define the intervals to be respected between the incidental finding of NSCLC and other points in the care trajectory. For example, the suggested mean time from suspicious imaging to pathological diagnosis should be between 14 and 21 days, while the median time from the first abnormal radiographic finding to treatment should be between 52 and 84 days.[56-59] Considering that LADC can get a rapid TVDT, even patients newly diagnosed with an early LADC can be shifted to an invasive one without rapid management due to interfering factors during the imaging-pathological examination interval. A more comprehensive study is necessary to provide more details about the factors impacting the management of LADC.

Incidental LADC detection is common and does not present a novelty. However, our study's strength is that it constitutes the first report of incidental discovery of invasive LADC in Algeria. Therefore, it is interesting because it makes us aware that incidental discovery is not well described and reported. In fact, clinical scenarios after incidental diagnoses of lung cancer, as well as their radiological characteristics, are lacking in Algeria, both before and after the pandemic period. This may impact the evaluation of specific variations in this group of patients that could affect the management of LADC, especially in the presence of comorbidities such as COVID-19. In this case, LADC growth is an evolving process and guidelines may be suggested to plan an appropriate follow-up examination and management. We can advise physicians to use the European collaborative group report for individual incidental findings statements as an evidence-based approach for reporting and managing incidental findings.[60] A CT follow-up examination can explore the natural chronologic evolution of LADC. Low-dose chest CT (LDCT) can be helpful in the first diagnosis and in predicting tumor growth patterns and aggressiveness of suspected malignant suspected LADC.[61] We invite other teams to describe their findings on incidental lung cancer to improve our understanding and improve patient outcomes.

According to the National Anticancer Plan, interventions to reduce the burden of lung cancer in Algeria are limited to controlling tobacco consumption of the risk factor tobacco.[20] However, given the high incidence of invasive LADC among the Algerian population, which reduces the chances of treatment success and survival, the health system must make judicious decisions to reinforce this preventive approach with an early diagnosis procedure to reduce the incidence of aggressive forms. Thus, screening should be an obvious first choice for LADC early detection. Indeed, it is worth noting that the observed decrease in mortality from pulmonary malignancy in developed countries is due mainly to the increase in lung cancer screening in smokers and non-smokers with other risk factors.[62,63] However, it is noticeable that more different screening methods can be applied if chest-X-ray or sputum is a method that has proved ineffective and that is not recommended as a screening tool. LDCT remains the only possibility. Several screening studies, such as the National Lung Screening Trial (NLST) and the NELSON Trial, have shown the effectiveness of LDCT in improving the early diagnosis of lung cancer but also in reducing specific lung cancer mortality (20% at 6.5 years in NLST, 24% at ten years in NELSON).[64,65] Interestingly, LDCT screening is effective in detecting LADC. In 3339 individual screening trials, lung cancer has found a ratio for cancer detection ratio, with LADC accounting for 88% (40% early stage and 53% invasive).[66] Unfortunately, Algeria does not have a national lung cancer screening program. LDCT screening can be generally disseminated in eligible Algerian populations such as high-risk smokers. However, since LADC is also related to non-cigarette risk factors, considerations should be taken to increase screening, with proper guidelines for implemented LDCT screening involving occupational exposure and other risk factors.[67] In the absence of a formal screening program, we can advise physicians to use the European Collaborative Group report for individual reporting of incidental findings that can be followed during incidental lung cancer discovery as an evidence-based approach for reporting and managing incidental findings.[60]

It should be noted that our study has a few limitations since it represents an isolated case of inciden-

tal cancer during COVID-19 hospitalization in our facility. This makes the result less generalizable. Although the current study describes the characteristics of COVID-19 with LADC, we cannot emphasize whether the COVID-19 lockdown negatively affected its onset. We encourage the community to address the same studies that require large-scale and longer-term observation. We, therefore, propose to examine the frequency of incidental diagnosis among an incident cohort of patients with lung cancer, compare the characteristics of incidentally versus nonincidentally diagnosed patients, and examine common pathways and mechanisms that led to the incidental diagnosis of cancer using registries.

Conclusion

Considering the high incidence of aggressive LADC in Algeria, more attention should be paid to identifying new procedures for its management. The described case shows how important it is to spread the screening principles and the need for studies about incidentally findings of the disease, especially among former tobacco people and professionals from high-risk groups in Algeria. We make a call to highlight the main circumstances leading to the late diagnosis of invasive lung cancer, particularly the lack of screening. Our case study illustrates the importance of implementing a large-scale LDCT screening program for lung cancer in Algeria, which can improve options for early lung cancer detection and follow-up for diagnosis and treatment.

In addition, this description underlines the critical importance of conducting more in-depth studies to obtain more information on the clinicopathological features of incidental findings. These studies are essential not only to define the incidental discovery of cases of lung cancer during the COVID-19 pandemic retrospectively but also to evaluate the long-term effects of the COVID-19 pandemic, such as delayed diagnoses and changes in patient care, on this population. The urgency and importance of this research cannot be overstated.

Declarations

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Author contributions

Conceptualization, R.B.; Methodology, R.B, A.A.D. and A.C; Software, R.B. and A.A.D; Validation, R.B., N.D. and N.H.; Formal Analysis, R.B., A.A.D. and H.D; Investigation, R.B.; Resources, R.B., A.A.D. and H.D; Data Curation, R.B; A.A.D. and H.D; Writing – Original Draft Preparation, R.B.; Writing – Review & Editing, R.B., H.D. and A.M.; Visualization, R.B, H.D. and A.B.; Supervision, R.B.; Project Administration, R.B.

Conflicts of interest

The authors declare no conflict of interest in connection with the study.

Data availability

No additional source data are required.

Ethics approval

This study was approved by the institutional ethics committee of the Public Hospital Establishment and the Ethics Committee of the Cancer Center of Annaba.

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CASE REPORT

Clinical exome sequencing (carrier screening) identifies the gene *INPPL1* in a sporadic case of opsismodysplasia

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ABSTRACT

Introduction and aim. This study presents a case of opsismodysplasia in a family, characterized by skeletal dysplasia and neurological complications in two consecutive neonates.

Description of the case. Genetic analysis revealed that the father carries a likely benign/variant of uncertain significance (VUS) in exon 14 of the *INPPL1* gene (c.1706C>T, p.Thr569Met), while the mother carries a pathogenic variant in exon 15 (c.1809del, p.Trp604GlyfsTer17). These variants follow an autosomal recessive inheritance, confirming carrier status. Additionally, the father is a carrier of a likely pathogenic variant in the *CYP17A1* gene (OMIM*609300), specifically in exon 6 (c.1040G>A, p.Arg347His, heterozygous), affecting 17,20-lyase activity and associated with isolated 17,20-lyase deficiency. Targeted sequencing and Sanger validation elucidated the genetic basis of the condition, emphasizing the importance of genetic testing and counselling in families with a history of genetic disorders. The detected variants in the INPPL1 gene disrupt SHIP2 protein function, contributing to the observed abnormalities.

Conclusion. This study underscores the significance of early genetic diagnosis for reproductive counselling and timely intervention. Further research into opsismodysplasia's genetic mechanisms may lead to improved management and therapies for affected individuals. Overall, this case highlights the critical role of genetic analysis in diagnosing and managing rare genetic disorders, offering insights into personalized care and family planning.

Keywords. exome sequencing, *INPPL1* gene, opsismodysplasia, rare skeletal dysplasia

Introduction

Recurrent pregnancies resulting in offspring with congenital anomalies pose significant clinical challenges and necessitate thorough investigation to elucidate the underlying etiology.[1] We present a case of two consecutive pregnancies with offspring exhibiting similar skeletal deformities and neurological anomalies, despite uneventful antenatal periods and negative prenatal screenings.[2]

Opsismodysplasia (OMIM#258480) is a rare genetic disorder characterized by severe skeletal abnormalities and distinct facial features. It is primarily caused by homozygous or compound heterozygous mutations in the inositol polyphosphate phosphatase like 1 (*INPPL1*) gene (OMIM*600829) located on chromosome 11q13.[3] First described by Zonana et al. in 1977 and later designated under its current name by Maroteaux in 1984, opsismodysplasia presents a complex array of clinical manifestations, posing significant challenges to affected individuals and their caregivers.[4] Individuals with opsismodysplasia often exhibit facial dysmorphism, in-

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cluding microcephaly, delayed ossification, a prominent brow, enlarged fontanels, and flattened vertebrae. Additionally, they may display distinct craniofacial features such as a depressed nasal bridge, diminutive nose, forward-facing nasal openings (anteverted nares), elongated philtrum, and increased distance between the eyes (hypertelorism), accompanied by protruding eyes (exophthalmos). These facial characteristics contribute to the clinical diagnosis of opsismodysplasia.[5]

Beyond facial features, individuals with opsismodysplasia typically present with skeletal abnormalities such as rhizomelic micromelia, characterized by extremely short long bones, as well as abbreviated hands and feet. Other skeletal manifestations include slender thorax, profound platyspondyly, and delayed bone maturation, often leading to significant functional impairment. Respiratory infections are a common concern, with fatalities resulting from respiratory failure documented within the initial years of life. However, extended survival has also been reported, underscoring the variable clinical course of the disorder.

Radiographic examinations play a crucial role in the diagnosis of Opsismodysplasia, revealing characteristic skeletal abnormalities such as abbreviated long bones, delayed epiphyseal ossification, pronounced platyspondyly, and metaphyseal cupping. Additionally, distinctive abnormalities in the metacarpals and phalanges are often observed, further aiding in the clinical assessment of affected individuals.

The recurrence of similar congenital anomalies in two consecutive pregnancies despite negative prenatal screenings underscores the complexity of this case. The absence of identifiable genetic or environmental factors highlights the limitations of current diagnostic modalities in elucidating rare and multifactorial conditions. The presence of periventricular leukomalacia in the first child and underdeveloped gyration with corpus callosum aplasia in the second child suggests a potential neurological basis for the observed anomalies.

Aim

This study presents a case of opsismodysplasia in a family, characterized by skeletal dysplasia and neurological complications in two consecutive neonates.

Description of the case

A detailed family history was taken to identify patterns of inheritance and assess the likelihood of the disorder being passed on. However, the family's medical history did not reveal any notable anomalies. The first child, a female born in April 2015 via Lower Segment Cesarean Section (LSCS) in PrayagRaj, Uttar Pradesh, India, presented with global developmental delay, microcephaly, tented upper lip, cortical thumbs, bilateral congenital talipes equinovarus, overlapping fingers, retrognathia, hypotonia, and respiratory difficulties. Imaging studies revealed periventricular leukomalacia on MRI, indicative of cerebral white matter injury. Despite extensive investigations including karyotyping, biochemical assays, and TORCH screenings, the underlying cause remained elusive. Tragically, the child succumbed to complications after 35 days of life.

The second child, a male born in 2023, exhibited similar skeletal deformities including acyanotic heart disease with an atrial septal defect (ASD) of 3.5 mm. Imaging studies revealed a small brain with underdeveloped gyration and corpus callosum aplasia on MRI, consistent with neurological abnormalities observed in the first child. Despite negative prenatal screenings and unremarkable antenatal evaluations, the child experienced respiratory difficulties and passed away after 30 days of life.

Investigations

Extensive investigations were conducted for both children, encompassing karyotyping, biochemical assays including complete blood count, renal and liver function tests, erythrocyte sedimentation rate, ultrasound imaging, and TORCH screenings. Additionally, imaging studies including X-rays and MRI were performed to assess skeletal and neurological abnormalities.

An Indian family presenting with a clinical diagnosis of Opsismodysplasia was enrolled in this Case study, comprising parents and affected siblings. Ethical approval was obtained from the Institutional Ethical Committee at Banaras Hindu University. The research methodology involved targeted sequencing, specifically capturing and sequencing the protein-coding regions of the genome or genes. This approach enhances the identification of mutations within exonic regions, which are typically more clinically actionable compared to variations in non-coding regions.

Sample collection and DNA extraction

Blood samples were collected from the family members (parents), and genomic DNA was extracted using Pure Link Genomic DNA mini-Kit (Cat No. K1820-01) standard protocols. The DNA samples were isolated for targeted gene capture using a custom capture kit.

Sequencing and data analysis

Sequencing of the captured libraries was performed on the NextSeq 1000 and 2000 Systems Illumina platform, achieving a mean coverage of >80-100X. The obtained sequences were aligned to the human reference genome (GRCh37/hg19) using the BWA program and analyzed using Picard and GATK version 3.6 to identify variants relevant to the clinical indication.[6,7]

Variant annotation and filtering

Gene annotation of the identified variants was conducted using the VEP program (Version 102.0) against the En-

sembl release 87 human gene model. Clinically relevant mutations were annotated using published variants in literature and databases such as ClinVar, OMIM, GWAS, HGMD, and SwissVar. Common variants were filtered based on allele frequency in population databases including 1000 Genome Phase 3, ExAC, EVS, dbSNP147, 1000, an internal Indian population database. Genetic variations that are present in a significant proportion of the general population, usually with a minor allele frequency (MAF) above a certain threshold (e.g., >1%).[8,9]

Variant interpretation

Non-synonymous and splice site variants found in the clinical exome panel consisting of 8332 genes were utilized for clinical interpretation. Silent variations that did not result in any change in amino acid in the coding region were not reported. The effect of non-synonymous variants was assessed using multiple algorithms such as PolyPhen-2, SIFT, Mutation Taster2, Mutation Assessor, and LRT.[9-12]

Validation and clinical interpretation

Validation of identified variants by Sanger sequencing 3500 Series Genetic Analyzers with BigDye™ Terminator v3.1, was recommended to rule out false positives. It was advised to sequence the variants in both affected and unaffected family members to validate their significance (Tables 1 and 2). Genetic counseling was also recommended for further guidance and interpretation of the genetic findings.[13,14]

Table 1. Sanger sequencing analysis results for father's *INPPL1* gene

Gene name	*INPPL1* (Exon 15)
Variation detected in NGS	chr11:71943374C>T (HET); c.1706C>T; p.Thr569Met
Sanger validation result	Present (Heterozygous)

In the Table 1, the Sanger sequencing analysis results for the father's INPPL1 gene are provided. The detected variation corresponds to a substitution of cytosine with thymine at nucleotide position 1706 in exon 15 of the INPPL1 gene, resulting in the amino acid change from threonine to methionine at codon 569 (p.Thr569Met). The Sanger validation confirms the presence of this variant in a heterozygous state.

Table 2. Sanger sequencing analysis results for mother's *INPPL1* gene

Gene name	*INPPL1* (Exon 15)
Variation detected in NGS	chr11:71943766delC (HET); c.1809del; p.Trp604GlyfsTer17
Sanger validation result	Present (Heterozygous)

In the Table 2, the Sanger sequencing analysis results for the mother's INPPL1 gene are outlined. The detected variation corresponds to a deletion at nucleotide position 1809 in exon 15 of the INPPL1 gene, resulting in a frameshift mutation leading to the substitution of tryptophan at codon 604 with glycine and a premature termination codon (p.Trp604GlyfsTer17). The Sanger validation confirms the presence of this variant in a heterozygous state.

NGS result and variant confirmation

NGS analysis identified a heterozygous missense variation in exon 14 of the INPPL1 gene (chr11:71943374C>T), resulting in the amino acid substitution of Methionine for Threonine at codon 569 (p.Thr569Met; ENST00000298229, Table 3). The variant lies in the catalytic domain of the SHIP2 protein, and missense mutations in this domain have been reported to inactivate the phosphatase function of the protein. This variant was detected in the father of the patient. Based on the evidence, this INPPL1 variation was classified as a likely benign variant, warranting correlation with the clinical symptoms Figures 1 and 2.[15]

Table 3. Pathogenic variant identified in the mother and likely benign/VUS variant in father of the patient are presented, the patient's father carries a likely benign/VUS variant in exon 14 (c.1706C>T, p.Thr569Met), while the patient's mother carries a pathogenic variant in exon 15 (c.1809del, p.Trp604GlyfsTer17), these variants follow an autosomal recessive mode of inheritance

Disease	Patient's father	Patient's mother
Opsismodysplasia (OMIM#258480)	Carrier	Carrier
	Mode of Inheritance: AR	Mode of Inheritance: AR
	Gene: *INPPL1* (OMIM*600829)	Gene: *INPPL1* (OMIM*600829)
	Exon 14, c.1706C>T, p.Thr569Met	Exon 15, c.1809del, p.Trp604GlyfsTer17
	Classification: likely benign	Classification: pathogenic
Isolated 17,20-lyase deficiency (OMIM#202110), 17-alpha-hydroxylase/17,20-lyase deficiency (OMIM#202110) Mode of inheritance: AR	Carrier Gene: *CYP17A1* (OMIM*609300) Exon 6, c.1040G>A, p.Arg347His, Heterozygous Classification: likely pathogenic	Non-carrier

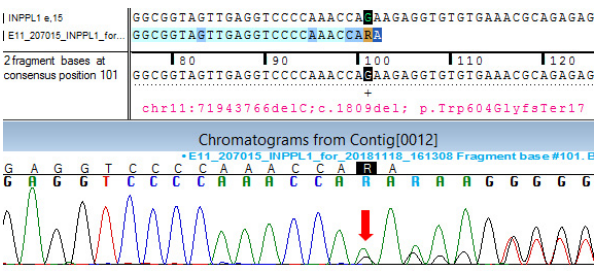

Fig. 1. The figure depicts the sequence chromatogram and alignment illustrating the observed variation in exon 15 of the *INPPL1* gene (chr11:71943766delC; c.1809del; p.Trp604GlyfsTer17), the variation is identified in a heterozygous state in the mother

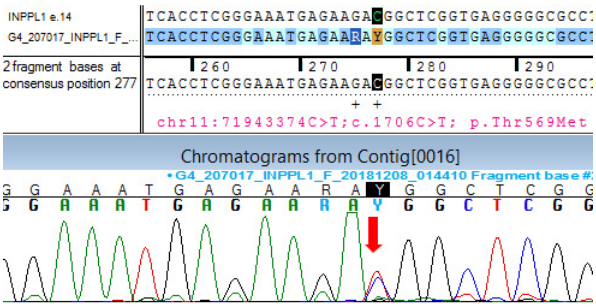

Fig. 2. The figure displays the sequence chromatogram and alignment, highlighting the variation identified in exon 14 of the *INPPL1* gene (chr11:71943374C>T; c.1706C>T; p.Thr569Met), this variation was detected in a heterozygous condition in the father of the patient

ACMG classification of p.Trp604GlyfsTer17 mutation

The p.Trp604GlyfsTer17 mutation identified in exon 15 of the INPPL1 gene is classified as likely pathogenic according to ACMG guidelines. This classification is supported by strong evidence including the presence of a frameshift resulting in a premature termination codon (PVS1), absence from control populations (PM2), and multiple lines of computational evidence supporting a deleterious effect (PP3, PM4, Table 4).

Table 4. Variant interpretation based on prediction tools and ACMG classification

Variant	Prediction tools SIFTa mutation taster (pathogenicity)	ClinVar	ACMG classification
c.1706C>T (p.Thr569Met) in INPPL1 exon 14	Likely benign	Likely benign	Variant of uncertain significance (VUS)
c.1809del (p.Trp604GlyfsTer17) in INPPL1 exon 15	Pathogenic	Pathogenic	PVS1, PM2, PP3, PM4

c.1706C>T (p.Thr569Met) in INPPL1 exon 14: ClinVar support the Likely Benign pathogenicity of the c.1706C>T (p.Thr569Met) variant, and it is classified as likely benign in the Leiden Open Mutation Database. Multiple in-silico analysis tools, such as SIFT (Sorting Intolerant From Tolerant) and Mutation Taster, support a likely benign or VUS classification, indicating that ACMG PP3 is not met. While the PM2 criterion is fulfilled due to the variant's extreme rarity (GMAF=0.00060), the PM1 criterion is not applicable as there is no evidence of a mutational hotspot.

c.1809del (p.Trp604GlyfsTer17) in INPPL1 exon 15: Predicted as pathogenic by various tools and supported by ClinVar. ACMG classification includes PVS1 (null variant in a gene where loss of function is a known mechanism of disease), PM2 (absent from controls), PP3 (multiple lines of computational evidence), and PM4 (protein length changes due to frameshift).

Discussion

The presented case involves a family with two consecutive neonates affected by Opsismodysplasia, a rare genetic disorder characterized by skeletal dysplasia and neurological complications. Genetic analysis revealed that the patient's father carries a likely benign/variant of uncertain significance (VUS) in exon 14 of the *INPPL1* gene (c.1706C>T, p.Thr569Met), while the patient's mother carries a pathogenic variant in exon 15 of the *INPPL1* gene (c.1809del, p.Trp604GlyfsTer17). These variants follow an autosomal recessive mode of inheritance, confirming the parents' carrier status for this condition and this variant requires further confirmation due to its unclear classification.

Additionally, the father was found to be a carrier of a likely pathogenic variant in the *CYP17A1* gene (OMIM*609300), specifically in exon 6 (c.1040G>A, p.Arg347His, heterozygous), which affects 17,20-lyase activity. Isolated 17,20-lyase deficiency is primarily associated with mutations in the *CYP17A1* gene, which encodes for the enzyme cytochrome P450 17α-hydroxylase/17,20-lyase. This enzyme plays a crucial role in steroid hormone biosynthesis, specifically in the production of glucocorticoids and sex steroids. The identification of pathogenic variants to clinically significant variants and subsequent validation by Sanger sequencing provides valuable insights into the genetic basis of opsismodysplasia in this family. The variants detected in the *INPPL1* gene, particularly in exon 14 and exon 15, are known to disrupt the function of the SHIP2 protein, leading to the characteristic skeletal and neurological abnormalities observed in opsismodysplasia.[17-22]

The detection of these variants underscores the importance of genetic testing and counselling in families with a history of genetic disorders.[18] By identifying carrier status in parents, healthcare providers can offer informed reproductive counselling to mitigate the risk of passing on genetic conditions to future offspring. Additionally, early detection of genetic disorders allows for timely intervention and management strategies to improve patient outcomes.[19,20]

Conclusion

In conclusion, the genetic analysis of this family with opsismodysplasia revealed likely pathogenic variants in the *INPPL1* gene, confirming carrier status in both parents. The identification of these variants highlights the role of targeted sequencing and Sanger validation in diagnosing rare genetic disorders and providing valuable genetic counselling to affected families. Moving forward, further research into the genetic mechanisms underlying Opsismodysplasia may lead to the development of targeted therapies and improved management strategies for affected individuals.

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Declarations

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Author contributions

Conceptualization, S.R. and R.S.; Methodology, A.A. And S.M.; Software, A.A. And S.M.; Validation, R.S., S.R. and A.A.; Formal Analysis, R.S.; Investigation, S.R.; Resources, R.S.; Data Curation, S.M.; Writing – Original Draft Preparation, A.A.; Writing – Review & Editing, R.S., S.R. and A.A.; S.M.; Visualization, S.R.; Supervision, R.S.; Project Administration, R.S.; Funding Acquisition, S.R.

Conflicts of interest

There are no conflicts of interest.

Data availability

The detailed datasets analyzed during the current study are available with the corresponding author. In the future, it will be made available on reasonable request. Data are however available from the authors upon reasonable request.

Ethics approval

The studies involving human participants were reviewed and approved by the Ethics Committee of the Institutional Ethical committee before starting the study (No. Dean/2022/EC/3827).

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LETTER TO THE EDITOR

Dual challenges – the growing burden of dengue and its associated co-infections

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Dear Editor,

Dengue is a viral mosquito-borne infection that is transmitted to humans by Aedes mosquitoes; Aedes aegypti and Aedes albopictus, in tropical and sub-tropical areas.[1] It causes a range of nonsevere to severe clinical manifestations.[1] Around 96 million dengue infections are recorded annually with 21,000 deaths worldwide.[2] The four serotypes of dengue virus (DEN1-4) can cause the disease with 25% to 40% heterogeneity.[3] Infection with one serotype provides lifelong immunity against that specific serotype, but subsequent infection by another serotype often creates fatal outcomes if untreated.[3]

The number of Dengue cases are significantly increasing.[4] Between 2000 and 2019, the World Health Organization (WHO) reported a ten-fold increase in dengue infection rate from 500,000 to 5.2 million cases worldwide.[4] The rate elevated to over 7.6 million cases with 3000 deaths by the end of April 2024 which has already exceeded the 4.6 million cases reported in 2023.[5] The disease is now endemic in more than 100 countries in the WHO regions of Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific.[5] This increase can be caused by globalization and increased international travel that facilitate the virus spread from dengue endemic regions to previously unaffected areas as well as the crucial role of climate change in elevating the disease cases.[4,5] Dengue season is found to start 1–2 months earlier due to warmer temperatures, extending the mosquito breeding season and thus making the peak dengue season last longer.[5]

Furthermore, in some endemic regions, dengue co-infection has been reported, making the scenario even worse and complicating patient management.[5] This problem can be illustrated by the significant overlap in geographic regions of dengue, Zika virus, and chikungunya shown in (Fig. 1). Notably, the areas marked brown and blue on the map represent regions that are particularly burdened by cocirculation of two or more of the viruses, highlighting the increased risk of co-infection. Furthermore, the areas indicating transmission of a single virus for now remain susceptible for possible future co-infection due to the mobility of populations and the adaptability of the Aedes mosquito vector. This simultaneous occurrence of dengue co-infections can result in worsening of the symptoms, increasing the severity of the disease and mortality rate.

Additionally, the presence of these simultaneous infections can complicate the diagnosis and treatment course.[5] Due to similar initial symptoms, misdiagnoses and misreporting as monoinfection are possible in the absence of differential laboratory testing.[6] For example, a study in Brazil found that 84.4% of the 828,654 suspected arbovirus cases were initially classified as 'suspected dengue', while only 15.6% were considered 'suspected chikungunya'. However, laboratory confirmed cases showed the actual proportions were 65.9% chikungunya and only 34.1% dengue.[6]

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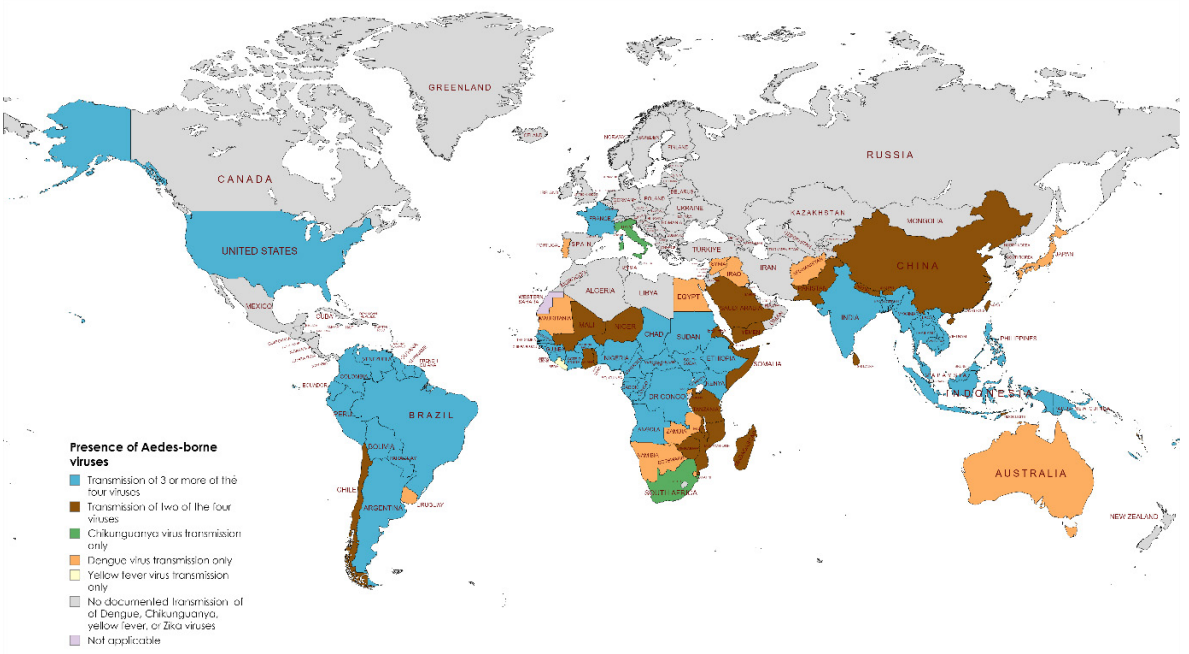

Fig. 1. Countries, territories or areas with previous or current local mosquito-borne transmission of more than one *Aedes*-borne virus (dengue, chikungunya and Zika) as of 30 April 2024

In summary, the increasing trend of dengue infection coupled with increasing cases of its co-infections requires urgent global action. Future studies should focus on addressing preventive strategies and implementing early detection techniques to reduce the risk of dengue and other outbreaks in areas that are currently unaffected. Enhanced surveillance, continued research to find vaccines, early detection of co-infection cases, and integrated clinical management protocols are required to reduce the impact on affected patients and the additional burden on healthcare resources.

Declarations

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Author contributions

Conceptualization, D.K.S.; Data Curation, F.R.K.; Writing – Original Draft Preparation, F.R.K.; Writing – Review & Editing, D.K.S.

Conflicts of interest

All authors declare that they have no conflicts of interest.

Data availability

All data used in this study are publicly available and are obtained from openly accessible websites such as the World Health Organization with the specific sources referenced in the manuscript.

Ethics approval

The study used publicly available data from World Health Organization and no direct human or animal data were used. As the data are openly accessible, no ethical approval was required for the study.

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— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, Ethics Approval, References.

Requirements for case reports submitted to Eur J Clin Exp Med:

— Patient ethnicity must be included in the Abstract under the Case Presentation section.
— Consent for publication is a mandatory journal requirement for all case reports. Written informed consent for publication must be obtained from the patient (or their parent or legal guardian in the case of children under 18, or from the next of kin if the patient has died).

Language Style

Manuscripts must be submitted in English (American or British usage is accepted, but not a mixture of these).

Title page

These sections should appear in all manuscript types:

Title: The title of your manuscript should be concise and informative. It should identify if the study reports (human or animal) trial data, or is a systematic review, meta-analysis or replication study. When gene or protein names are included, the abbreviated name rather than full name should be used.

Author list and affiliations: Authors' full first and last names must be provided. For each affiliation provide the details in the following order: department, institution, city, country. If available, the e-mail address of each author should also be provided. At least one author should be designated as *corresponding author*, and his or her email address and other details should be included at the end of the affiliation section.

Abstract: The abstract should be a total of about 250 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts: *Introduction and aim:* Place the question addressed in a broad context and highlight the purpose of the study; *Material and methods*: Describe briefly the main methods or treatments applied. Include any relevant preregistration numbers, and species and strains of any animals used. *Results:* Summarize the article's main findings; and *Conclusion*: Indicate the main conclusions or interpretations. The abstract should not contain any undefined abbreviations or unspecified references.

Keywords: Three to six pertinent keywords need to be added after the abstract in alphabetical order. We recommend that the keywords are specific to the article, yet reasonably common within the subject discipline.

Back matter

Supplementary materials: Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.

Acknowledgments: Thank all of the people who helped with the research but did not qualify for authorship. Acknowledge anyone who provided intellectual assistance, technical help, or special equipment or materials.

Funding statement: All sources of funding of the study should be disclosed.

Author contributions: Authors must supply an Author Contribution Statement as described in the *Author contributions statements* section.

Conflicts of interest: Authors must supply a competing interests statement. For more details please see *Competing interests policy*.

Data availability: Authors must include a Data Availability Statement in all submitted manuscripts; see *Availability of materials and data* section for more information.

Ethics approval: Example of an ethical statement: "All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of XXX (Project identification code)."

References: References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript. We recommend preparing the references with a bibliography software package, such as EndNote, Reference Manager or Zotero to avoid typing mistakes and duplicated references.

References style

In-text citations and references should be prepared according to the American Medical Association (AMA) style. Each item should be listed in numerical order.

In-text citations

Each reference should be cited in the text using superscript arabic numerals. These superscript numbers should be outside periods. If you are citing sequential references, these should be indicated with a hyphen. Nonsequential references should be separated with commas. There should not be a space between numbers. For example: The degree of respiratory muscles fatigue depends on the applied exercise protocol and the research group's fitness level.[1,2] The greatest load with which a patient continues breathing for at least one minute is a measure of inspiratory muscles strength.[3] Diabetes mellitus is associated with a high risk of foot ulcers.[4-6]

Sample Reference

In listed references, the names of all authors should be given unless there are more than 6, in which case the names of the first 3 authors are used, followed by "et al.". If the source does not have any authors, the citation should begin with the title.

To find the proper abbreviation of journal go to the National Library of Medicine PubMed Journals Database at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Journals.

Page number(s) should be inserted in full (for example: use 111–112, not 111–2).

The following are examples of individual citations made according to the required rules of editing and punctuation:

— **Article from a journal, number of authors from 1 to 6**

Author AA, Author BB, Author CC. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Lee JC, Seo HG, Lee WH, Kim HC, Han TR, Oh BM. Computer-assisted detection of swallowing difficulty. C*omput Methods Programs Biomed*. 2016;134(2):72-78. doi: 10.1016/j.cmpb.2016.07.010

Morris A. New test for diabetes insipidus. *Nat Rev Endocrinol*. 2019;15(10):564-565. doi: 10.1038/s41574-019-0247-x

— ***Article from a journal, number of authors* more *than 6***

Author AA, Author BB, Author CC, et al. Title of article. *Accepted Abbreviated Journal Title*. Year;Volume(Issue):Page-Page. doi (if available)

Gonzalez ME, Martin EE, Anwar T, et al. Mesenchymal stem cell-induced DDR2 mediates stromal-breast cancer interactions and metastasis growth. *Cell Rep*. 2017;18:1215-1228. doi: 10.1016/j.celrep.2016.12.079

Jordan J, Toplak H, Grassi G, et al. Joint statement of the European Association for the Study of Obesity and the European Society of Hypertension: obesity and heart failure. *J Hypertens*. 2016;34:1678-1688. doi: 10.1097/HJH.0000000000001013

— **Websites**

Author AA (if indicated). Webpage title. Name of Website. URL. Published or Updated date. Accessed date.

Cholera in Haiti. Centers for Disease Control and Prevention Web site. http://www.cdc.gov/haiticholera/. Published October 22, 2010. Updated January 9, 2012. Accessed February 1, 2012.

Address double burden of malnutrition: WHO. World Health Organization site. http://www.searo.who.int/mediacentre/releases/2016/1636/en/. Accessed February 2, 2017.

— **Book**

Author AA, Author BB. *Title of Work*. Location: Publisher; Year:Page-Page

Doane GH, Varcoe C. *Family Nursing as Relational Inquiry: Developing Health– Promoting Practice*. Philadelphia, PA: Lippincott Williams & Wilkins; 2005:25-28.
London ML, Ladewig PW, Ball JW, et al. *Maternal & Child Nursing Care*. Upper Saddle River, NJ: Pearson Education; c2011:101-103.
— Chapter in a book
Chapter Author AA. Title of chapter. In: *Name of Book*. Edition Number. Editor AA, ed. Location: Name of Publisher; Year:Page-Page.
Grimsey E. An overview of the breast and breast cancer. In: *Breast Cancer Nursing Care and Management*. 2nd ed. Harmer V, ed. Chichester, UK: Wiley-Blackwell; 2011:35-42.
NOTE: The Editorial Board requires consistent and carefully made references prepared according to the above-mentioned AMA standards. Otherwise, the work will be sent back to the authors.

Preparing figures, schemes and tables

File for Figures and Schemes must be provided during submission and at a sufficiently high resolution (minimum 1000 pixels width/height, or a resolution of 300 dpi or higher). Common formats are accepted, however, TIFF, JPEG, EPS and PDF are preferred.

Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure (not on the figure itself) or above the table. All figures, schemes, and tables should be numbered following their number of appearance (Figure 1, Scheme 1, Figure 2, Scheme 2, Table 1, etc.).

Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text.

All table columns should have an explanatory heading. To facilitate the copy-editing of larger tables, smaller fonts may be used, but no less than 8 pt. in size. Tables must be provided in an editable format in appropriate place in the main text. Tables provided as jpeg/tiff files will not be accepted. Do not submit your tables in separate files.

Abbreviations

The journal requires using only standard abbreviations. Common abbreviations such as DNA and RNA do not require definitions. Abbreviations should be defined in parentheses the first time they appear in the abstract, main text and in figure or table captions and used consistently thereafter. Ensure consistency of abbreviations throughout the article. Use the following abbreviations for measurement units: gram (g), litre (L), milligram (mg), kilogram (kg), seconds (s), minutes (min), and hours (h). Do not add 's' to indicate plural forms of units. Keep abbreviations to a minimum.

SI Units

SI Units (International System of Units) should be used. Imperial, US customary and other units should be converted to SI units whenever possible.