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ORIGINAL PAPER

Dipeptidyl peptidase-4 dysregulation and inflammatory cytokines as dual biomarkers in ulcerative colitis – a case-control study

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ABSTRACT

Introduction and aim. Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) characterized by immune dysregulation and mucosal inflammation. Current UC biomarkers lack specificity, underscoring the need for novel diagnostic tools. Dipeptidyl peptidase-4 (DPP-4), a serine protease implicated in immune modulation and inflammation, and interleukin-7 (IL-7), a cytokine linked to mucosal immune dysregulation, may serve as dual biomarkers. This study aimed to evaluate the diagnostic utility of serum levels of DPP-4, C-reactive protein (CRP), and IL-7 to differentiate UC patients with UC from healthy individuals.

Material and methods. This research conducted a case-control study involving 130 individuals, 65 diagnosed with UC and 65 apparently healthy individuals serving as the control group. Blood samples were obtained to measure DPP-4, IL-7, insulin and C-reactive protein levels.

Results. The study demonstrated a significantly higher level of inflammatory markers compared to the healthy controls with a significantly higher CRP (23.38±17.76 vs. 2.38±0.89 mg/dL, p<0.001) and IL-7 concentrations (62.39±19.7 vs. 22.86±4.73 ng/L, p<0.001). and lower level of DPP-4 activity (1.33±0.19 vs. 2.37±0.35 U/L, p<0.001). ROC curve analysis revealed the excellent diagnostic performance of DPP-4 and IL-7 in identifying UC.

Conclusion. Reduced serum DPP-4 levels and elevated levels of inflammatory markers (CRP and IL-7) highlight their utility as dual biomarkers for the diagnosis and monitoring of UC disease. DPP-4 expression is inversely correlated with inflammation. These biomarkers demonstrate diagnostic potential for UC; however, longitudinal studies are needed to assess their role in monitoring disease progression and response to treatment.

Keywords. dipeptidyl peptidase-4, inflammatory bowel disease, ulcerative colitis

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is caused by chronic intestinal inflammation. These two primary idiopathic conditions differ in their anatomical distribution patterns and the depth of intestinal wall involvement. UC, unlike CD, is characterized by continuous mucosal inflammation confined to the colon.[1] By contrast, CD can affect any part of the gastrointestinal tract and often involves transmural inflammation. Phenotypically, Crohn's is classified using the Vienna and Montreal systems, which categorize patients based on age at diagnosis, disease location, and behavioral manifestations (inflammatory, stricturing, or penetrating).[2] In contrast, UC characteristically involves continuous and symmetric inflammation limited to the colonic mucosa and submucosa, with a sharp demarcation between affected and normal tissues.[3] UC typically begins in the

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rectum and may remain localized (ulcerative proctitis) or extend proximally. The Montreal classification classifies UC as proctitis (E1) with rectal involvement, left-sided colitis (E2) when the disease extends distally to the splenic flexure, and extensive colitis/pancolitis (E3) when the inflammation extends proximally to the splenic flexure.[4]

UC is characterized by chronic inflammation and ulceration of the colon and rectum, with patients typically experiencing gradual-onset symptoms, such as bloody diarrhea that persists for weeks. Although exact etiopathogenesis remains unclear, UC arises from a complex interplay of immune dysfunction, genetic predisposition, and environmental triggers.[5] As a chronic disease with significant morbidity, UC cannot be cured, increasing the risk of colorectal cancer.[6] UC represents a major global health challenge, with an estimated worldwide prevalence of 5 million by 2023. Incidence rates continue to rise in industrialized and developing nations.[7] Canada exemplifies this trend, reporting one of the highest global UC incidence rates at 17.5 / 100,000 person years, alongside an overall inflammatory bowel disease (IBD) of 825 per 100,000 individuals.[8] The United States mirrors this trend, with the prevalence of UC reaching 238 per 100,000 and incidence rates between 19.2–20.2 per 100,000.[9] The European UC epidemiology exhibits bifurcated trends. Northern Europe has the highest incidence (24.3 per 100,000) and prevalence (505 per 100,000).7 In the Middle East, the incidence ranges from 1.37 to 5.0 per 100,000 people, with Saudi Arabia and Qatar showing an annual increase since 2010.[9]

In addition to epidemiological trends, dysregulation of immune mediators such as interleukin-7 (IL-7) has emerged as a key focus, IL-7 is a 25-kDa hematopoietic growth factor produced by stromal cells in the bone marrow, thymus and epithelial cells and plays a crucial role in lymphocyte development and homeostasis.[10] IL-7 signals through a heterodimeric receptor comprising IL-7Rα and a common gamma chain, activating the JAK/STAT and PI3K/Akt pathways to regulate cell survival via modulation of anti-apoptotic factors (Bcl-2, Bcl-xL, and Mcl-1) and pro-apoptotic factors.[11] Compelling evidence demonstrates a significant connection between IL-7 and UC, with IL-7 transgenic mice developing chronic colitis that mimics human UC pathology.[12] Systemic, rather than intestinal, IL-7 has been shown to be essential for colitis persistence.[13] Notably, increased IL-7R expression in the colonic mucosa of UC patients correlates with disease activity and non-responsiveness to anti-TNF therapy, with IL-7 influencing the gut-homing specificity of T cells through α4β7 integrin regulation.[14]

Dipeptidyl peptidase-4 (DPP-4), classified under EC 3.4.14, is a member of the serine peptidase family. DPP was classified into nine categories.[15] In particular, proteins and DPP-4, also known as CD26, are responsible for the degradation of peptides that contain proline or alanine at the penultimate position, an exopeptidase located on the surface of cells.[16] DPP-4 regulates bioactive peptides by cleaving the N-terminal dipeptides from substrates with proline or alanine at the penultimate position.[17] DPP-4 is widely expressed in several organs and cell types, and contributes to immunological control, glucose balance, inflammation, and cancer development. DPP-4 is widely expressed in the gastrointestinal tract, liver, lungs, and kidneys.[18] DPP-4 exerts a variety of pleiotropic effects through enzymatic and non-enzymatic mechanisms when interacting with extracellular matrix proteins.[19] In terms of enzyme activity, DPP-4 cleaved peptides with proline or alanine as second amino acid (NH-Xaa-Pro) were cleaved at the NH2-terminus by DPP-4. The preference for proline was the strongest, followed by a gradually decreased preference for alanine and glycine.[20] DPP-4 has been shown to interact with several proteins in a variety of biological processes, including sodium-hydrogen exchanger 3 (NHE3), mannose-6-phosphatase, insulin-like growth factor II (IGF II), collagen, fibronectin, caveolin I and CD45 tyrosine phosphatase.[21]

DPP-4 plays a complex and controversial role in UC by affecting immune modulation and regulation of incretin. It influences T cell activation and cytokine levels, a protease that has far-reaching effects on endocrine pathways is DPP-4, which is essential to regulate the bioactivity of peptide hormones produced in the gastrointestinal tract.[22] Preventing DPP-4 from breaking down gut hormones helps increase hormone production in the pancreas and improves food processing after meals. This effectively lowers blood sugar levels in individuals with type 2 diabetes.[23]

Aim

The present study was designed to assess the diagnostic potential of serum DPP-4, CRP, and IL-7 as a panel of combined biomarkers to distinguish patients with UC from healthy subjects and to explore their pathophysiological crosstalk in UC-related immune dysregulation and mucosal inflammation. By combining biomarker performance analysis with mechanistic exploration, this study aimed to evaluate the diagnostic utility of serum levels of DPP-4, C-reactive protein (CRP) and IL-7 levels in differentiating patients with UC from healthy individuals.

Material and methods

The present case-control study was conducted at Marjan Medical City Hospital within the Babylon Governorate to investigate factors associated with UC. A

total of 65 patients diagnosed with UC by a gastroenterologist were enrolled based on clinical presentation, endoscopic findings and histopathological examination were enrolled, along with 65 age- and sex-matched healthy controls (HC) recruited from adult volunteers aged 18 to 60 years, consisting of apparently healthy individuals who were selected based on clinical evaluation by a physician. Participants had no prior history or current symptoms suggestive of inflammatory bowel disease, including UC, and had no gastrointestinal complaints at the time of inclusion. Patients were excluded from both groups in the event of active smoking, alcohol consumption, diabetes mellitus, pregnancy or lactation, concomitant chronic diseases, autoimmune disorders, or recent use of antibiotics/probiotics within 3 months before enrollment. The control group. Control group selection was performed to ensure a similar age (±2 years) and sex as the UC cohort. The study protocol was approved by (IRB: 95 -25/11/2024). All cases that did not meet the inclusion criteria or meet the exclusion parameters were systematically excluded using a two-stage evaluation process of evaluation by gastroenterologists and laboratory confirmation. No disease severity stratification (for example, using the Mayo score) was performed, which may have limited the interpretation of biomarker variations in disease stages.

Sample collection

Each participant was given a blood sample (5 mL) drawn from their veins and the patient's sample was rapidly collected. Blood was gradually dispensed into a gel tube (CHAORAN Vacuum, Lot. no. 20231001), coagulated at room temperature for 10-15 min and centrifuged at 3000×g for 10 min. After collection, serum was stored at -40°C for subsequent analysis of DPP-4, insulin, fasting blood sugar (FBS) and C-reactive protein (CRP) levels. Serum levels of DPP-4, Interleukin-7 (IL-7), and insulin were measured using the following ELISA kits provided by (BT LAB, Shanghai, China): DPP-4 ELISA Kit (Cat. No. E0912Hu), Insulin ELISA kit (Cat.No.BTB-E0010Hu), and IL-7 ELISA Kit (Cat. No. E1948Hu). CRP concentrations were analyzed using the ichromaTM fluorescent immunoassay (FIA) platform (Boditech Med- Korea) following the manufacturer's instructions. Calibration curves for each biomarker using kit-provided standards. Quality control procedures included the use of blanks, internal controls, and strict adherence to protocol guidelines. The absorbance for the ELISA assays was measured using a spectrophotometer (Biotech Instruments (USA), and serum fasting blood sugar levels were evaluated using a Simi auto spectrophotometer (Mindray BA-88A, Mindray Bio-Medical, Shenzhen, China).

Statistical analysis

Statistical analyses (SPSS version 25, IBM, Armonk, NY, USA). Both percentages and frequencies were used to illustrate variable groupings. Continuous factors are presented as (mean±standard deviation). The mean values of the two groups were compared using an independent sample t-test. We used a paired t-test to analyze the averages of the two sets of readings.

Results

The average difference between the healthy group and patients with UC, along with the association among the different patient parameters, and the findings of a cohort of 65 patients, with ages ranging from 18 to 60 years, according to demographic data. The findings did not show statistically significant age differences, with the mean age of patients being 31.26±9.35, compared to HCs at 28.98±6.33, with a (p=0.107). Indicated that the change in BMI was not statistically significant, with a (p=0.450) The mean for the healthy group were 23.39±1.17, but for UC patients, the mean was 23.21±1.52. The sex distribution of the analyzed groups included 65 patients with UC, including 26 males (40%) and 39 females (60%), corresponding to controls. As shown in Table 1 comparing biochemical variables, UC patients demonstrated a significantly higher level of inflammatory markers compared to the HCs with a significantly higher CRP (23.38±17.76 vs. 2.38±0.89 mg/dL, p<0.001) and a lower level of the DPP-4 activity (in U/L) (1.33±0.19 vs. 2.37±0.35, p<0.001) and significantly higher levels were determined for IL-7 concentrations (62.39±19.7 vs. 22.86±4.73 ng/L, p<0.001), whereas metabolic parameters such as FBS (90.66±9.88 vs. 95.20±10.90 mg/dL, p=0.014), levels of the insulin (5.49±0.77 vs. 5.36±0.60 mIU/L, p=0.292^NS^) and HOMA-IR (1.21±0.17 vs. 1.25±0.14, p=0.238) were comparable.

Table 1. Comparative analysis of biochemical parameters in patients with ulcerative colitis and healthy controls

Variable	Patients (Mean±SD) (n=65)	Control (Mean±SD) (n=65)	p
FBS (mg/dL)	90.66±9.88	95.20±10.90	0.014
Insulin level (mIU/L)	5.49±0.77	5.36±0.60	0.292
HOMA-IR	1.21±0.17	1.25±0.14	0.238
CRP level (mg/dL)	23.38±17.76	2.38±0.89	<0.001
Interleukin-7 (ng/L)	62.39±19.7	22.86±4.73	<0.001
DPP-4 (U/L)	1.33±0.19	2.37±0.35	<0.001

Table 2 shows that the analysis of the ROC curve for DPP-4 in patients with UC indicated good diagnostic accuracy, with a cut-off value of ≤1.85 U/L for DPP-4 presenting high sensitivity (98%) and specificity (92%) and an overall accuracy of 90%. The AUC was 99% (p35.18 ng / L) and showed perfect sensitivity, specificity, PPV and NPV (100% each), with a accuracy of 100% and an AUC of 100% (p<0.001).

Table 2. ROC curve analysis for DPP-4 in UC patients*

Biomarkers	Cut-off value	Sens %	Spec%	PPV	NPV	Accuracy	AUC%	p
DPP-4 (U/L)	≤ 1.85	98	92	93	98	90	99	<0.001
Interleukin-7 ((ng/L)	> 35.18	100	100	100	100	1.0	100	<0.001

* Sens – sensitivity, Spec – specificity, PPV – positive predictive value, NPV – negative predictive value, AUC – area under the curve

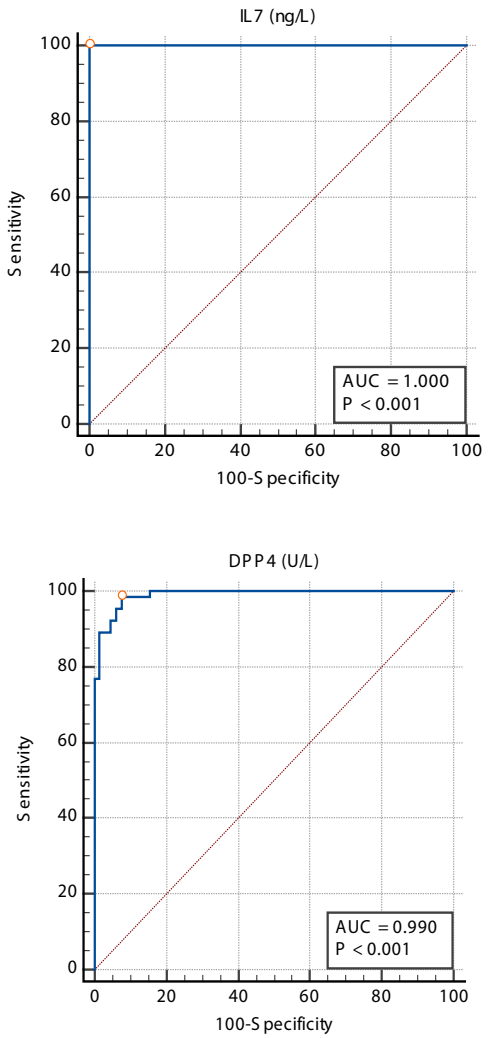

Fig. 1. ROC curve analysis of DPP-4 and interleukin-7

A strong positive correlation was observed between CRP and IL-7 (r=0.872, p<0.0001), whereas both CRP and IL-7 were significantly negatively correlated with DPP-4 (r=-0.533 and r=-0.624, respectively; both p<0.0001). IL-7 showed a weak negative association with HOMA-IR (r=-0.279, p=0.0244). These results highlight notable interactions between the inflammatory markers (CRP and IL-7).

Discussion

UC is often associated with comorbid systemic conditions and environmental factors and involves chronic inflammation of the immune system. Lifestyle factors, such as smoking, obesity, and poor eating habits, may also contribute to the initiation and progression of UC.[24] This study demonstrated that patients with UC had significantly higher CRP levels than those in the control group. This elevation in CRP levels is indicative of its involvement in inflammatory processes and serves as a prognostic marker for the disease. These results are consistent with those of previous studies by Henriksen et al., who reported that CRP levels are frequently used in the follow-up of patients with IBD. Furthermore, CRP levels at diagnosis are correlated with the extent of the disease in patients with UC.[25] CRP is an acute phase reactant that has been identified as a key indicator of IBD. CRP activates the complement cascade, promotes the production of pro-inflammatory cytokines, and assists in the elimination of microbial infections.[26]

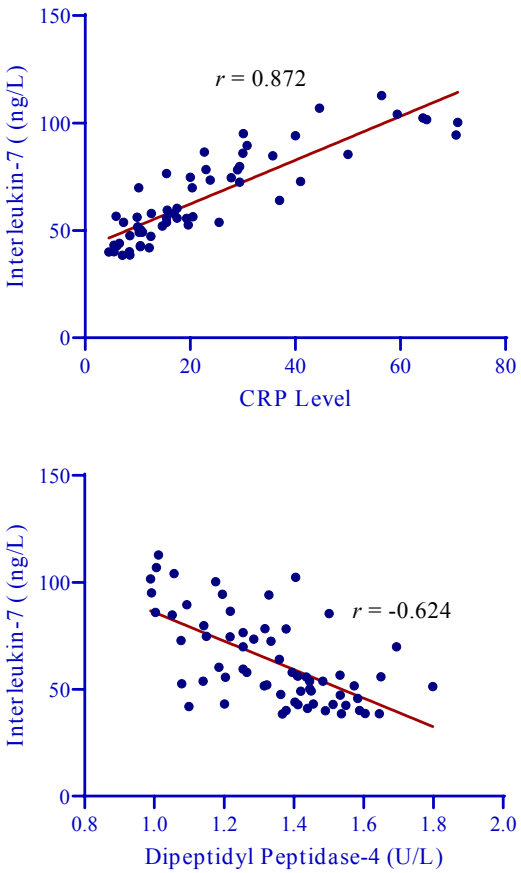

Fig. 2. Correlation charts for IL-7 and different biomarkers

The current study showed significantly higher levels of IL-7 in patients with UC than in HC. The IL-7 signaling pathway has emerged as a critical regulator of mucosal immunity in UC, with increasing evidence linking aberrant IL-7 activity with disease pathogenesis.[27] Belarif et al. demonstrated disrupted IL-7 signaling in UC, with elevated transcripts of the IL-7 receptor pathway observed in inflamed colon tissues of patients with se-

vere UC not responsive to standard therapies, as shown in an analysis of 500 patients with IBD and 100 controls.[14] Barata et al. further demonstrated that patients with UC exhibit elevated systemic and mucosal levels of IL-7 and its receptor subunit IL-7Rα, underscoring the complexity of IL-7 signaling dynamics in different stages of disease stages and tissue compartments. This overexpression was correlated with persistent colonic inflammation and dysregulated immune responses, suggesting a critical role for dysregulation of the IL-7 pathway in UC pathogenesis.[28] The mechanistic role of IL-7 in UC involves complex immune regulation. Studies have revealed that IL7 expression is strongly inversely correlated with regulatory T cell signatures or the regulatory T cell-to-effector T cell ratio in colon tissues of patients with UC before anti-TNF therapy. This suggests that elevated IL-7 signaling may contribute to UC pathogenesis by disrupting immune tolerance mechanisms.[29]

Unlike IL-7, serum DPP-4 levels were significantly reduced in patients with UC compared to those with those in HCs, corroborating the findings of recent studies. Abrahami et al. reported that patients with IBD exhibit lower serum DPP-4 concentrations than healthy individuals, with an inverse correlation between DPP-4 levels and clinical disease activity.[30] While the inverse correlation between serum DPP-4 levels and inflammation suggests a possible role for DPP-4 in disease progression, the causal relationship between reduced DPP-4 levels and inflammation or active disease remains unresolved.[31] In UC, dysregulation of DPP-4-mediated adenosine signaling may exacerbate mucosal inflammation, as adenosine typically attenuates T cell activation.[32] Paradoxically, while circulating DPP-4 levels decrease during active disease, mucosal DPP-4 activity increases, indicating distinct roles in systemic circulation versus localized mucosal inflammation. The apparent contradiction between DPP-4 biomarker behavior (lower levels in active disease) and the enzyme expression patterns in patients showed compartment-specific alterations. While circulating DPP-4 levels decrease during active disease, mucosal DPP-4 activity increases, suggesting a tissue-specific role in inflammation. This dichotomy underscores the involvement of the DPP-4 complex in the pathophysiology of UC, acting as both a biomarker of systemic immune status and a local mediator of gut inflammation.[33] Perry et al. further demonstrated an inverse correlation between serum DPP-4 levels and IBD activity, with lower levels corresponding to higher clinical and endoscopic disease severities.[34] This dichotomy underscores the dual role as a systemic biomarker and a local modulator of gut inflammation.

ROC curve analysis in this study demonstrated that DPP-4 exhibits excellent diagnostic performance with high sensitivity and moderate specificity at a defined cutoff point, underscoring its diagnostic reliability. Lopez et al. evaluated serum DPP-4 levels in patients with IBD, compared active disease to remission, and assessed treatment outcomes. The results revealed significantly lower DPP-4 levels in patients with active IBD than in those in remission and in HCs. DPP-4 effectively distinguished disease activity from remission and predicted the need for escalation of treatment. In particular, higher levels of DPP-4 were observed in patients who responded to biological therapy, and this association was more pronounced in patients with UC than in those with CD. The strongest correlation emerged for UC, underscoring its potential utility in the management of this IBD.[35]

Emerging evidence identifies fecal DPP-4 (fDPP-4) as a novel noninvasive biomarker, with cohort studies reporting significantly lower median levels of fDPP-4 in active UC compared to remission, with a statistically significant difference. Fecal DPP-4 also showed greater accuracy than fecal calprotectin in differentiating endoscopic disease activity. Mechanically, decreased systemic DPP-4 levels during active inflammation may reflect the enzymatic utilization by the inflamed tissues. Immunohistochemical analyses further supported this hypothesis, revealing prominent DPP-4 staining in mucosal biopsies, especially within regenerating epithelial regions and areas infiltrated by lymphocytes.[36]

Study limitations and strengths

The strengths of this study include its structured case–control design with matched controls, which improves internal validity. Their model achieved high diagnostic accuracy and offered insight into potential disease mechanisms, due to the inclusion of diverse biomarkers and comprehensive statistical methods. However, it has limitations, including a small sample size and recruitment from a single center, which may limit its representativeness. The exclusion of comorbidities may limit real-world relevance, and the cross-sectional design restricts causal conclusions. Another limitation is the lack of long-term follow-up data. Notwithstanding these limitations, our findings highlight the diagnostic potential of these biomarkers.

Conclusion

The present findings demonstrate a significant reduction in serum DPP-4 levels and an elevation of inflammatory markers (CRP and IL-7) in patients with UC compared to HC. The inverse correlation between DPP-4 and inflammation underscores its dual utility as a biomarker for the diagnosis and monitoring of UC. ROC analysis confirmed the strong diagnostic accuracy, emphasizing its clinical relevance. Elevated CRP and IL-7 levels are associated with immune dysregulation and mucosal inflammation, reinforcing their role in the pathogenesis of UC. Future studies should incorporate disease stratifica-

tion (eg active vs. remission and treatment response) to refine the clinical utility of these biomarkers.

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Declarations

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Author contributions

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

Conflicts of interest

The author declare that they have no competing interests.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request and pending approval by the College of Science guidelines of the Biomedical Research Ethics Committee.

Ethics approval

A college of medicine at the University of Babylon and hospital ethics committee examined and approved the study protocol, subject information, and authorization form by document number [IRB: 95 -25/11/2024].

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ORIGINAL PAPER

Evaluation of adrenomedullin, growth differentiation factor-15, and atrial natriuretic peptide in patients with rheumatoid arthritis

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ABSTRACT

Introduction and aim. Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by joint inflammation and systemic complications, including cardiovascular involvement. This study evaluated the diagnostic utility of angiotensinogen (AGT), serum amyloid A-4 protein (SAA4) and C-reactive protein (CRP) in RA, along with new cardiac biomarkers adrenomedullin (ADM), growth differentiation factor-15 (GDF-15), and atrial natriuretic peptide (ANP), to assess cardiac complications.

Material and methods. This case-control study included 61 RA patients (divided into newly diagnosed, sDMARD-treated, and sDMARD/bDMARD-treated groups) and 27 healthy controls. Serum levels of AGT, SAA4, CRP, ADM, GDF-15, and ANP were measured using ELISA. Statistical analyses were performed using SPSS software.

Results. RA patients exhibited significantly elevated levels of AGT (40.841±17.285 µg/mL), SAA4 (48.128±17.065 ng/mL), CRP (13.097±18.702 mg/L), ADM (96.295±19.424 pg/mL), GDF-15 (1247.049±854.335 pg/mL), and ANP (334.016±40.874 pg/mL) levels compared to controls ($p \leq 0.001$). No significant differences were observed between the treatment groups for AGT, SAA4, or CRP levels. Cardiac biomarkers (ADM, GDF-15, and ANP) remained elevated in all RA groups, with strong inter-parameter correlations (Spearman $r > 0.6$).

Conclusion. AGT, SAA4, and CRP demonstrate robust diagnostic value for RA. Persistent elevation of ADM, GDF-15, and ANP in all patient groups confirms significant cardiac involvement in RA.

Keywords. adrenomedullin, angiotensinogen, growth differentiation factor-15, rheumatoid arthritis, serum amyloid A-4 protein

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that primarily causes symmetric joint inflammation, pain, and swelling. It typically begins in small joints and progresses to larger ones. RA can also affect multiple systems, including the integumentary, ocular, cardiac, renal, and pulmonary.[1,2] Joint involvement leads to erosion of the bone and cartilage, potentially weakening surrounding muscles and ligaments. Its development involves both genetic and environmental factors.[3,4] Recent studies have focused on innovative biochemical markers to diagnose RA, including angiotensinogen (AGT) and serum amyloid A-4 protein (SAA4).[5] AGT is predominantly synthesized in the liver.[6] The conversion of AGT to angiotensin I is catalyzed by the enzyme renin, which is generated by juxtaglomerular cells in the renal cortex. Several components of inflammatory reactions in various tissues are part of the renin-angiotensin system (RAS). The pathophysiology of RA is influenced by several components of this system, including enzymes and peptides.[7] Two opposing but balanced components, classical and protective, regulate blood pressure, body

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fluid, and electrolyte homeostasis in RAS. Pathological inflammatory disorders such as RA can alter this balance and change the plasma concentrations of its components.[8]

SAA4 is an essential acute phase protein in inflammatory rheumatic diseases (IRD). Recent studies have confirmed the role in IRD pathogenesis. Human SAA4 proteins include 104 amino acids and are conserved throughout the mammalian history.[9] Synthesis is performed in the liver and not just in the liver. SAA Insoluble fibrils are formed by solid aggregates, the "amyloid" of "secondary" amyloid disease. Buildups may impair biological activities and cause organ dysfunction. RA is commonly diagnosed by measuring CRP levels to indicate systemic inflammation.[10]

The atrium produces most of the ANP by processing its precursor into prohormones and conserving them in secretory granules within the atrial cardiomyocytes. Elimination separates them into NT-ProANP and ANP.[11] The release of ANP is initiated by distension of the atrial walls, serving two primary functions: promoting vasodilation and improving renal excretion of sodium and water.[12] There is a substantial association occurs between the mechanical and endocrine activities of the heart in individuals with cardiac disease, perhaps indicating the stage and severity of the condition.[13] Natriuretic peptides are essential for heart physiology and pathology. A thorough understanding of their control in both normal and pathological cardiac states may improve diagnostic precision and treatment strategies, particularly for heart failure (HF) and atrial fibrillation (AF), which are intimately interconnected in a cyclical way. The role of ANP in endocrine activity requires a comprehensive examination to clarify the effects of this peptide on mechanical function.[14] Elevated levels of ANP are often seen in individuals with HF and β-thalassemia major, making them significant indicators for the diagnosis and prognosis of this illness.[15]

A divergent member of the transforming growth factor-β superfamily, growth differentiation factor-15 GDF-15, is alternatively referred to as a cytokine that inhibits macrophage (MIC)-1.[16] This superfamily includes several other proteins. When heart myocytes experience ischemia or increased stress on the ventricular wall, their expression of GDF-15 is dramatically increased, in contrast to the usual, low expression of this factor in most tissues. The regulation of GDF-15, a stress-inducible cytokine, appears to be complicated and tissue specific, as it is controlled by many inflammatory or stress-related proteins such as IL-1 β, TNF-α, interleukin-2, and MCSF-1.[17] The precise role of GDF-15 remains unclear; however, it appears to confer protection against cardiac injury through its anti-hypertrophic, anti-apoptotic, and anti-inflammatory properties, which are potentially mediated by its interaction with an unidentified receptor.[18] GDF-15 is an effective prognostic indicator of cardiovascular complications. Experimental murine ischemia/reperfusion injury models demonstrate a rapid increase in circulating and tissue GDF15 levels after cardiac injury, with elevations that persist for several day.[19] GDF-15 levels are also significantly elevated in patients with β-thalassemia major.[20]

Adrenomedullin (ADM), which was initially discovered in pheochromocytoma tissue in 1993, is currently one of the most significant current cardiac indicators.[21] The peptide consists of 52 amino acids. After translation, pro-adrenomedullin (proADM) is produced and converted into biologically active adrenomedullin (bio-ADM).[22] Various body organs, including the kidneys, lungs, endocrine system and cardiovascular system, produce pro-ADM and bio-ADM in response to volume overload, commonly observed in heart failure.[23] Elevated levels of bio-ADM levels in HF suggest a mechanism to mitigate volume overload by stabilizing the function of the endothelial barrier function.[24] There is a critical need for biomarkers that facilitate precise assessment of congestion, as accurately measuring congestion is notoriously challenging due to significant observer variability. Recent studies have demonstrated a significant correlation between elevated bio-ADM levels and systemic congestion as well as negative outcomes, independent of NT-proBNP. Bio-ADM is a useful biomarker for the diagnosis of HF and the assessment of clinical outcomes.[25]

Aim

This study aimed to evaluate the diagnostic and monitoring efficacy of two novel rheumatoid biomarkers (AGT and SAA4) in patients with RA. Furthermore, we sought to assess cardiac complications associated with RA by quantifying circulating levels of cardiac biomarkers, including ADM, GDF-15, and atrial natriuretic peptide (ANP).

Material and methods

A case-control study was conducted between November 2023 and June 2024 among RA patients who were subjected to different therapeutic regimens. Participants were recruited from rheumatology clinics at the Al-Basrah Teaching Hospital and Al-Mawanah Teaching Hospital in the Basra Governorate, Iraq. Group 1 (Newly diagnosed): n=10, no previous treatment with disease-modifying anti-rheumatic drugs (DMARD). Group 2 (sDMARD-treated): n=22, receiving conventional synthetic DMARD (csDMARD: methotrexate and sulfasalazine) for 3 months. Group 3 (bDMARD-Treated): n=29, receiving biologic DMARDs (bDMARDs: anti-IL6 agents Tocilizumab or Sarilumab) in addition to csDMARDs. Control group: n=27, healthy volunteers of the same age and sex were included as controls. The

study excluded all participants with preexisting chronic diseases, including cardiovascular disease (for example, hypertension, heart failure), diabetes, liver/kidney dysfunction or active infections, as well as individuals outside the 20–66 age range, to focus on patients with RA diagnosed according to the 2010 ACR/EULAR criteria with 6 months of disease duration, willingness to provide informed consent and no recent corticosteroid use (within 3 months). Disease activity in patients with RA was evaluated using DAS28-ESR. The control group consisted of healthy volunteers from the Basra Governorate, matched with RA patients by age and sex, with no history of autoimmune diseases, chronic inflammation, or recent infections. Samples from both groups were collected in a dedicated laboratory within the same geographic region to ensure consistency. The excluded criteria uniformly applied to both groups included barred pregnant individuals and those with acute or chronic comorbidities.

An agreement was signed to conduct this study before the start of the sample collection process. The study design allowed the evaluation of disease progression and treatment efficacy at different stages of RA management, while the inclusion of a control group allowed the exploration of possible biomarkers and clinical parameters that are specific to patients with RA.

A total of 61 RA patients participated in the study, and the sample size was derived from a combination of statistical and practical considerations. Patient availability, resources, time constraints, ethical considerations, and data from the pilot study contributed to the final sample size. Other factors that have limited patient availability include the prevalence of the disease at the study location. Due to time limitations, the number of patients recruited and followed fell within the specified time frame. In making this decision, ethical dimensions around minimizing the burden on participants while retaining scientific rigor were also important considerations. Data from a pilot study could have adjusted the sample size based on the expectation that 61 patients would be sufficiently motivated to discover clinically meaningful differences. As it is pragmatic in the context of real-life clinical research that involves many investigators, statistical power cannot always be achieved when faced with the practical limitations of the real healthcare environment.

Ethical considerations

The present study was fully approved by the Training and Human Development Unit of the Basrah Health Department, which is part of the Ministry of Health in Iraq. This proposal was authorized by the research committee (number 860/2023) on 23 December 2023, in accordance with the Declaration of Helsinki.

Laboratory tests

A sterile gel tube was used to collect whole blood. As a result, the serum was separated for serological testing and ELISA. All kits used were manufactured by Fine Test Company, China. ANG, Cat: EH0550, SAA4, Cat.: EH1155, ANP, Cat. EH2639; GDF-15, Cat.: EH0150, ADM, Cat: EH0779, and C-Reactive Protein (CRP), REF: ACN 210, levels were measured using a human-adaptable ELISA kit from Shanghai Ideal Medical Technology Co., Ltd., and absorbance was measured at a wavelength of 450 nm using a BioTek (USA) 800TS microplate reader.

Statistical analysis

For tabulation and analysis, SPSS spreadsheet version 26.0 was used. Excel was used to display important facts. For each cardiac and rheumatic parameter, the means and standard deviations were calculated for the control, pretreatment, sDMARDs and bDMARDs groups. The Kolmogorov test determined the normality of the variable, which is a prerequisite for parametric and nonparametric analyses. Tables 1 and 2 show how the Mann-Whitney U test was used to compare the control and patient groups. For each biomarker, the Kruskal-Wallis test and post hoc pairwise comparisons were used to compare the four groups (Tables 3 and 4). Spearman's rank correlation coefficient was used to assess the relationships between the biomarkers.

Results

This study compared biochemical markers associated with RA in controls (n=27) and patients with RA (n=61). Demographic and clinical characteristics, including age, sex, and body mass index (BMI), were compared between cohorts. No significant differences were observed in age (patients: 46.57±9.65 years; controls: 43.55±8.06 years; p=0.315) or gender distribution (males: 36% vs 33%, females: 64% vs. 67%; p=0.3). However, RA patients exhibited higher BMI values compared to controls (28.6±2.1 kg/m² vs. 23.6±1.9 kg/m²; p=0.02). Different CRP, SAA4 and AGT values were observed. RA patients had significantly higher levels of all indicators than the control group (p <0.001) (Table 1).

Table 1. Evaluation of biomarker concentrations in control and patient cohorts*

		Patients (n=61)	Control (n= 27)	p
Age		46.57±9.65	43.55±8.06	0.315[a]
Gender	Male, n (%)	22 (36 %)	9 (33 %)	0.3[b]
	Female, n (%)	39 (64 %)	18 (67 %)	
BMI (kg/m²)		28.6±2.1	23.6±1.9	0.02[a]
SAA4 (ng/mL)		48.128±17.065	2.210±0.647	<0.0001[a]
AGT (µg/mL)		40.841±17.285	3.573±1.413	<0.0001[a]
CRP (mg/l)		13.097±18.702	0.756±0.109	<0.0001[a]

* [a] – Mann Whitney's test, [b] – Chi-square

Table 2 indicates substantial changes in all cardiac biomarkers between patients and controls. Patients showed significantly higher serum levels of ADM levels (96.295±19.424 pg/mL) than those of the control group (4.191±0.543 pg/mL) (p<0.001). The patient group had substantially higher levels of GDF15 levels (1247.049±854.335 pg/mL) compared to the control group (41.089±14.884 pg/mL) (p<0.00; the sick group exhibited considerably higher levels of ANP levels (334.016±40.874 pg/mL) than the control group (58.200 ±19.592 pg/mL) (p<0.000).

Table 2. Patients and controls' cardiac parameters

	Patients (n=61)	Control (n=27)	p
Age	46.57±9.65	43.55± 8.06	0.315
ADM)pg/mL(	96.295±19.424	4.191±0.543	<0.0001
GDF15)pg/mL(	1247.049±854.335	41.089±14.884	<0.0001
ANP)pg/mL(	334.016±40.874	58.200±19.592	<0.0001

Table 3 reveals that the rheumatic parameters indicate that the CRP levels exhibited a significant reduction in the control group (0.756 mg/l) compared to the G1 group (45.600 mg/l), third-month sDMARD therapy group (7.045 mg/l), and the G3 therapy G3 group (bDMARD) (6.479 mg/l. No significant differences were observed between the treatment groups. The control group exhibited significantly lower SAA4 levels (2.21±0.647 ng/mL) compared to the other three groups. The G1 (43.28 ng / mL) G2 (50.4 ng/mL), and G3 (53.7 ng/mL) groups did not exhibit significant differences. The control group exhibited AGT levels of 3.57 μg/mL, which were markedly lower compared to the G1 group at 34.71 μg/mL, the G2 group at 43.5 μg/mL, and the G3 group at 41.23 μg/mL.

Table 3. Comparison of rheumatic parameters in patients before and after treatment compared to a control group*

	Control (n=27)	Pretreatment G1 (n=10)	Third month sDMARDs therapy G2 (n=22)	bDMARDs Therapy G3 (n=29)	p
Age	43.55±8.06^{A}	47.90±11.87^{A}	47.09±10.84^{A}	45.72±8.04^{A}	0.4
SAA4 (ng/mL)	2.21±0.647^{A}	43.28±13.46^{B}	50.4±32.7^{B}	53.7±30.6^{B}	<0.0001
AGT (μg/mL)	3.573±1.41^{A}	38.71±16.41^{B}	43.5±20.82^{B}	41.23± 16.74^{B}	<0.0001
CRP (mg/L)	0.756±0.109^{A}	45.600±28.733^{B}	7.045±4.673^{A}	6.479±4.041^{A}	<0.0001

* Capital letters A, B, and C were used to indicate the level of significance following Dunn's multiple comparison test; similar letters indicate no significant difference, whereas different letters indicate significant differences

Table 4 shows that the level of ADM was significantly lower in the control group (4.191 pg/mL) than in the G1 pretreatment G1 group (99.800 pg/mL), the G2 group (95.591 pg/mL), and the bDMARDs) G3 group bDMARDs (95.621 pg/mL), as illustrated in Figure 3. Groups G1, G2 and G3 showed no significant differences. At the GDF15 levels in the control group (41.089 pg/mL) were much lower than in the other three groups. The G1 (2030.1 pg/mL) G2 (1964 pg/mL), and G3 (382.6 pg/mL) groups did not exhibit significant differences. Similarly, ANP levels were markedly reduced in the control group (57.5 pg/mL) compared to those in the other three groups. G1 (305.6 pg/mL), G2 (36.5 pg/mL) and G3 (323.8 pg/mL), with G1 showing a considerable difference from G2.

Table 4. Cardiac parameters in patients before and after treatment relative to the control group*

	Control (n=27)	Pretreatment G1 (n=10)	sDMARDs G2 (n=22)	bDMARDs G3 (n=29)	p
Age	43.55±8.06^{A}	47.90±11.87^{A}	47.09±10.84^{A}	45.72±8.04^{A}	0.4
ADM (pg/mL)	4.19±0.86^{A}	100.9±22.48^{B}	95.6±20.2 ^{B}	92.6±25.6^{B}	<0.0001
GDF15 (pg/mL)	41.89±14.84^{A}	2030.1±261^{B}	1964±470.9^{B}	382.6±107^{C}	<0.0001
ANP (pg/mL)	57.5±18.7^{A}	305.6±29.14^{B}	360.5 ±40.77^{C}	323.8±33.4^{B}	<0.0001

* Capital letters A, B, and C were used to indicate the level of significance following Dunn's multiple comparison test; similar letters indicate no significant difference, whereas different letters indicate significant differences

ADM had a substantial association with all other cardiac and rheumatic indicators, as shown by a Spearman correlation value over 0.6. GDF15 was significantly correlated with the cardiac parameters of ANP and CRP. Among rheumatic markers, ANP has been shown to correlate with SAA4. Rheumatic CRP, which did not show a connection. The relationship between SAA4 and AGT, as indicated by Spearman's correlation coefficient presented in Table 5.

Table 5. Correlation of all parameters

Parameters		GDF15	ANP	CRP	SAA4	AGT
ADM	r	0.660	0.653	0.675	0.612	0.629
	p	0.0001	0.0001	0.0001	0.0001	0.0001
GDF15	r	1.000	0.685	0.724	0.590	0.599
	p		0.0001	0.0001	0.0001	0.0001
ANP	r			0.561	0.600	0.590
	p			0.0001	0.0001	0.0001
CRP	r				0.589	0.591
	p				0.0001	0.0001
SAA4	r					0.755
	p					0.0001

Discussion

CRP levels were significantly elevated in patients with RA (Table 1), with the patient group displaying markedly higher values than the control group. Previous research has demonstrated elevated CRP levels in patients with RA compared to healthy controls, since RA is a chronic inflammatory autoimmune disorder and CRP serve as an inflammation marker.28,29

Consistently elevated levels of SAA4 were identified in patients with RA compared to healthy controls, as indicated in Table 1of the current investigation. This finding supports recent research.[30,31] which characterizes increased SAA4 levels to the systemic inflammatory nature of RA. SAA4, an acute phase protein, is classified as a pro-inflammatory cytokines, which stimulates the liver to produce SAA.[32,33] Previous studies have suggested that SAA4 may serve as a more effective marker of disease activity in RA than conventional inflammatory markers such as CRP and ESR, highlighting the importance of SAA4 in the context of RA pathogenesis.[34] Analysis of SAA4 levels between study groups revealed significant differences. The control group showed significantly lower SAA4 concentrations compared to G1, G2, and G3 (Table 3). Furthermore, no statistically significant changes were detected among the three experimental groups. In certain cases, patients with well-managed RA or remission may show lower SAA4 levels, indicating reduced inflammatory activity. Furthermore, specialized treatments, including biological agents that target specific inflammatory pathways, can effectively modulate SAA4 levels. Fragoulis observed that various therapies have varying effects on SAA4 levels in patients with RA, with certain treatments resulting in a significant drop, which differs from the results of the current study.[35]

This study revealed statistically significant differences in AGT values between the patient and control groups (Table 3). This finding aligns with previous studies that have consistently indicated higher AGT levels in patients compared to healthy controls.[36] Unlike SAA4, AGT levels in RA patients are associated with dysregulated function and activity of the renin-angiotensin-aldosterone system (RAAS)under pathological conditions. In patients with chronic inflammation, such as RA, increased activation of the RAAS system leads to elevated production of AGT in the liver and tissues, which subsequently increases angiotensin II levels, which results in hypertension and vascular damage.[37] In RA, cytokine levels are elevated, which promotes the production of AGT.[38] The current study did not reveal significant differences across G1, G2, and G3. Low levels of AGT in RA are rare or nonexistent, but may linked to liver dysfunction, effective inflammation control, or metabolic variables.[39]

In critically ill patients, elevated serum levels of ADM were observed in comparison to controls. Individuals exhibited significantly higher levels of AM compared to healthy controls, as shown in Table (2), corroboration of extensive research that identifies ADM as a potent biomarker closely linked to heart failure, especially in patients with RA.[40,41] Elevated levels of ADM in RA are correlated with chronic inflammation, hypoxia, and vascular dysfunction within synovial tissue.[42] Despite the anti-inflammatory and protective properties of ADM, its overproduction indicates the body's effort to mitigate the pathological processes associated with RA.[43,44] Elevated ADM levels can function as a biomarker for cardiac disease activity and severity; however, direct investigations of ADM levels in RA patients are limited. Its role as a biomarker in other conditions (eg, sepsis and cardiovascular disease) indicates that it could act as a prognostic marker in RA, especially in patients with severe disease or those who did not respond to treatment.[45,46] However, no significant differences in ADM levels were observed between G1, G2 and G3 (Table 4).

The current study demonstrated statistically significant elevations in RA GDF-15 levels in patients compared to healthy controls, as shown in Table (2). These findings correspond to those of a concurrent study that reported similar results.[47] GDF-15 may function as a biomarker for disease activity and could indicate an elevated risk of cardiovascular complications in patients.[48,49] Groups G1 and G2 did not exhibit significant differences, suggesting that these cohorts demonstrate comparable GDF-15 expression. In particular, G3 had significant differences compared to both G1 and G2 (Table 4), indicating that G3 had lower levels of GDF-15, possibly due to the effects of the biologic therapy. The study emphasized the association between GDF-15 and atherosclerosis, demonstrating a gradual elevation of these levels in patients with RA.[50] GDF-15 levels showed a favorable correlation with markers of disease activity. This indicates that GDF-15 can contribute to the pathophysiology of RA and may function as a biomarker for monitoring disease progression.[51]

The study results revealed statistically significant elevations in ANP levels compared to the control group, as shown in Table (2). These findings are consistent with previous studies that demonstrated a correlation between ANP levels and patients with chronic heart failure and RA.[52,53] The observed elevation of ANP in RA patients is probably due to the synergetic effects of chronic inflammation, fluid retention, and cardiovascular stress. ANP regulates fluid balance, vascular tone, and blood pressure, serving as a compensatory mechanism to protect the cardiovascular system from the adverse effects of chronic inflammation in RA.[54] G1, G2, and G3 exhibited equivalent levels of ANP, with no statistically significant differences identified between them. G2 exhibited significantly higher ANP levels compared to G1 and G3. This discrepancy suggests a possible involvement of ANP in the etiology of the disease or as an indicator of the underlying pathological alterations related to RA. The results indicated a significant difference in ANP concentrations between patients with active RA and those in remission; specifically, patients with active RA demonstrated significantly elevated levels of ANP than

their counterparts in remission.[55,56] ADM exhibited a strong correlation with many cardiac and rheumatic parameters, as shown in Table (5); however, GDF15 and ANP exhibited moderate correlations. CRP levels exhibited a modest correlation with SAA4 and AGT levels.

Conclusion

This study identified notable variations in biomarker levels among RA patients, particularly those with elevated CRP, SAA4, AGT, ADM, GDF-15 and ANP, especially within the pretreatment, chemotherapy and biological treatment groups compared to the control group. SAA4 and GDF-15 are proposed as potential biomarkers of disease activity and progression, with biological treatments linked to a reduction in GDF-15 levels. ADM and ANP levels also exhibited an increase. The findings can help evaluate the severity of the disease, track treatment responses and elucidating the cardiovascular implications of rheumatoid arthritis.

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Declarations

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Author contributions

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

Conflicts of interest

The authors confirm that there are no conflicts of interest to disclose.

Data availability

The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

This study proposal was authorized by the research committee (number 860/2023) on December 23, 2023, in accordance with the Declaration of Helsinki.

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ORIGINAL PAPER

Exploring the role of B complex vitamins in reproductive health – valuable insights and unresolved issues regarding premature ovarian failure

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ABSTRACT

Introduction and aim. Premature ovarian failure (POF) is a condition distinguished by a decline in ovarian follicles in women under 40 leading to a disruption in the normal menstrual cycle and fertility. Vitamin B_6 (pyridoxine) and vitamin B_{12} (cyanocobalamin) play an important role in ovarian health. The aim was to investigate the effects of vitamins B_6 and B_{12}, for predicting POF via the association of these vitamins with other biochemical parameters.

Material and methods. A total of 80 participants with age ≤ 40 years, 40 POF women, and 40 fertile control women were recruited in this study from November 2023 to March 2024. Serum levels of prolactin (PRO), thyroid hormones, estradiol (E-2), progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), 17-alpha hydroxylase, and inhibin alpha (INH-α) were measured using the ELISA technique while vitamins B_6 and B_{12} were measured by HPLC.

Results. Serum levels of reproductive hormones (LH, E2, progesterone, and testosterone), vitamin B_6, B_{12}, T_3, T_4, 17-alpha hydroxylase, and INH-α were markedly reduced in POF patients, while levels of FSH, TSH, and PRO were significantly elevated compared to healthy controls.

Conclusion. This study highlights the critical role of vitamins B_6 and B_{12} in the pathophysiology of POF, suggesting their potential as biomarkers for diagnosis and prognosis.

Keywords. 17-alpha-hydroxylase, inhibin-alpha, premature ovarian failure, vitamin B_6, vitamin B_{12}

Introduction

Premature ovarian failure (POF) is a condition that affects women's reproductive health, with its occurrence becoming more prevalent in recent years.[1] POF, often referred to as premature menopause, occurs when a woman experiences cessation of ovarian activity and amenorrhea before turning 40, along with the serum FSH level that rise to the menopausal range. This condition can result in infertility and can cause both physical and emotional challenges.[2-4] Women with POF face a higher risk of psychological disorders, cardiovascular problems, osteoporosis, autoimmune diseases, cognitive impairment, and infection of the urinary and reproductive systems compared to those without the condition.[5-7]

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Ovarian follicles are composed of three types of cells: granulosa cells, oocytes, and theca cell. Granulosa cells and oocytes contain follicle-stimulating hormone (FSH) and luteinizing hormone (LH) receptors, which play a critical role in follicular growth and development.[8-10] Folliculogenesis is a structured process involving the maturation of follicles in preparation for ovulation.[11-13] However, this process is disrupted in cases of POF.[1,14,15] Biochemically, POF is defined by reduced levels of gonadal hormones, such as estrogens and inhibins, accompanied by elevated gonadotropins (LH and FSH), a state referred to as hypergonadotropic amenorrhea. This increase in FSH is typically more pronounced than that of LH, with FSH levels exceeding 30 IU/L serving as a diagnostic indicator of ovarian failure.[16-19]

Genetic, immunological, metabolic, viral, and medicinal factors all contribute to the etiology of POF.[2,20-23] B complex, as water-soluble vitamin, is a critical compound for overall cellular metabolism.[24] Pyridoxine ($B\text{-}_6$) plays an essential role in the regulation of thyroid and sex hormones, the promotion of ovarian maturation, and the production of vital female hormones such as estrogen and progesterone. It is believed to regulate the activity of various steroid hormone receptors at the transcriptional level.[25] Cobalamin ($B\text{-}_{12}$) is a corrin-based compound containing a corrin ring and plays a critical role in human somatic cell metabolism.[26] $B\text{-}_{12}$ is essential for nervous and reproductive systems, supporting normal RBC formation, growth, synthesis of DNA, as well as amino acid, fatty acid, and cellular energy metabolism.[27-29] Once inside the cell, $B\text{-}_{12}$ is converted into two active forms: adenosylcobalamin and methylcobalamin. Methylcobalamin functions as a coenzyme for methionine synthase, which plays vital role for metabolism of methionine-homocysteine, commonly referred to as the cycle of methionine. $B\text{-}_{12}$, a key player in one carbon metabolism, is a complex metabolic factor essential for human reproductive health.[30] Vitamins, particularly $B\text{-}_{12}$, are crucial for reproductive health, promoting organ function and mitigating oxidative stress, pro-inflammatory cytokines, and circulating homocysteine levels.[31-33] Homocysteine acts as a metabolic linker that hinders the synthesis of intracellular antioxidants. It inhibits key antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase.[34] A subclinical deficiency of $B\text{-}_{12}$ impairs the conversion of excess homocysteine to methionine, leading to an increase in homocysteine levels. This, in turn, promotes the production of H_2O_2, which contributes to the accumulation of reactive oxygen species (ROS) accumulation.[35-37] Oxidative stress, caused by an imbalance between the generation of free radicals and antioxidant levels, can cause detrimental transformation of mitochondrial DNA and protein damage, inevitably provoking cell death.[38-41] Methionine serves as a key precursor for S-adenosyl-methionine, an avital compound required for the methylation of phospholipid, neurotransmitters, amines, DNA, RNA and myelin basic protein.[42,43] A reduction in S-adenosyl-methionine levels can disrupt DNA methylation, potentially altering fetal metabolic programming and increasing susceptibility to non-communicable diseases later in life.[44] Consequently, $B\text{-}_{12}$ deficiency may lead to homocysteine buildup and reduced circulation methionine, impacting reproduction, as well as protein and DNA methylation.[45,46] The disruptions in redox balance, DNA methylation, and mitochondrial function are particularly detrimental to ovarian tissue, which is highly sensitive to oxidative stress. Granulosa cells, essential for follicle maturation, are especially vulnerable. Experimental studies have shown that elevated homocysteine levels promote apoptosis in granulosa cells, contributing to follicular atresia. Kaplan and Türk showed that B-12 administration reduced histological markers of tissue damage in rat ovaries by reducing oxidative stress.[34] Additionally, Deniz et al. revealed that treatment with $B\text{-}_{12}$ was effective in decreasing inflammation and oxidative stress caused by ovarian ischemia-reperfusion by downregulating the TLR-4/NF-kB signaling pathway, which is associated with inflammation.[47]

The enzyme 17-alpha hydroxylase is expressed in the adrenal glands and gonads, serving as a key branching point in the synthesis of cortisol and sex hormones. This enzyme facilitates two distinct steps in steroidogenesis. Individuals with deficiency in this enzyme commonly present with hypertension, hypokalemia, amenorrhea and absent of secondary sexual characteristics.[48,49] Inhibin-alpha (INH-α)Plays a role in regulating follicle development by responding to increased levels of FSH in gonads, which in turn triggers negative feedback to the anterior pituitary, reducing circulating FSH levels.[50-52] The interaction between vitamins of the B complex, particularly $B\text{-}_6$ and Cobalamin ($B\text{-}_{12}$), and the risk of POF remain intricate and not yet fully understood.

Aim

This study aims to investigate the correlation between vitamin $B\text{-}_6$ and $B\text{-}_{12}$ levels and POF. More research is required to elucidate their roles and potential therapeutic applications.

Material and methods

Subjects

In this study, 80 participants aged 40 years or under were split into two groups: group one consisted of 40 women with POF, whereas group two included forty healthy fertile women as a control group. From November 2023 to March 2024, POF patients were collected from the Higher Institute of Infertility Diagnosis and Assisted Reproductive Technologies located in Baghdad, Iraq. Women with an unclear etiology of POF, autoimmune

diseases, and pelvic surgery were excluded. The research was approved by the University of Technology-Iraq Bioethics Committee on Scientific Research, approval no. BCSR11, on March 24, 2024.

Methods

Blood samples were collected from two groups: the first group (healthy control) on the second day of their menstrual cycle and the second group (POF group) under conditions appropriate to their health status. For each participant, 5 mL of peripheral blood was drawn and centrifuged for 5 minutes at room temperature.

ELISA methods technique

The serum obtained was analyzed for hormone levels including FSH, LH, thyroid hormones, prolactin (PRO), progesterone, estradiol (E-2) and testosterone, 17-alpha hydroxylase and INH-α using the ELISA technique (CUSABIO BIOTECH COM), E-2, INH-α, and progesterone measured used competitive inhibition ELAZA kits, the microtiter plate included in the kits is pre-coated with goat anti-rabbit antibody, standards and samples were placed in designated wells along with specific antibody for these hormones and horseradish peroxidase (HRP) conjugated antigen.[53-55] A competitive reaction occurs between HRP labeled to target hormones and the unlabeled form of hormone for antibody binding. A substrate solution is then introduced, leading to color development inversely proportional to hormone concentration in the sample.[56-59] The reaction halted and the color intensity is measured, while the other tests was measured by used quantitative sandwich ELAZA kits, the microplate included in the kits is pre-coated with antibody specific for target hormone, Standards and samples were added to wells along horseradish peroxidase (HRP)-conjugated antibody targeting hormone. After washing to remove unbound reagents, a substrate solution is introduced, resulting in color development inversely proportional to the hormone concentration bound in the initial step.[60-62] The reaction is stopped and the color intensity is measured.

HPLC technique

Vitamin B_6 and B_{12} concentrations were measured using the HPLC technique, the HPLC Autosampler SyKNM from Germany S5200 pump model S1122 Column oven S4011, and a UV detector S3240 set at wavelength 230 nm with a C-18 column., using acetonitrile (A) and NaH2PO4 (B), with a gradient of 100% (B) at 3 minutes, 80% (B) at 6 minutes, and 60% (B) at 15 minutes. The flow rate remained constant at 1.0 mL/min.[40,63,64] Standard vitamins B_6, B_{12} were sourced from Sigma. Creating a standard solution began with dissolving 10 mg of each of vitamins B_6 and B_{12} in methanol to form a 100 parts per million (ppm) stock solution, diluted to 100 mL, then the stock solution was further diluted to prepare other standard solutions by subsequent dilution of the stock solutions.[65-67]

Post hoc power analysis

The results of the post hoc power analysis indicate that the study had a very high ability to detect differences in testosterone levels between the control and POF groups. The mean testosterone level in the control group was 0.47 with a standard deviation of 0.22, while the mean in the POF group was 0.09 with a standard deviation of 0.02. The effect size, calculated using Cohen's d, showed a very large difference between the two groups, reaching 2.55, which reflects a significant variation in testosterone levels. The sample size consisted of 40 individuals in the control group and 40 in the POF group, further strengthening the reliability of the study. The post hoc power reached 1.0 (100%), which means that the study was fully capable of detecting any real effect if it existed, which is a strong indicator of the statistical reliability of the results.

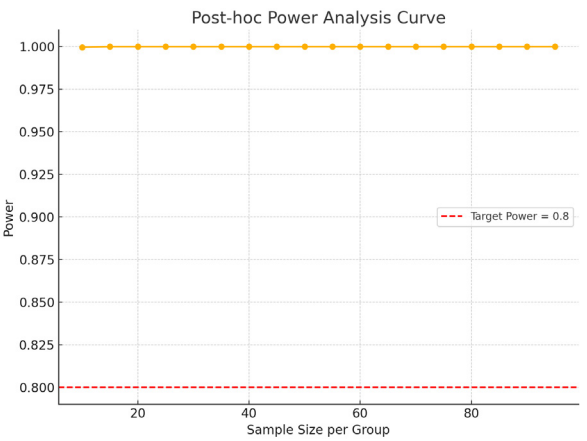

Fig. 1. Post hoc power analysis curve: effect of sample size on statistical power

The post hoc power analysis Figure 1 shows that the study has a very high power to detect differences between the two groups, with the statistical power remaining close to 1.0 (100%) even with small sample sizes. The horizontal axis represents the sample size per group, while the vertical axis reflects the level of statistical power. The orange line indicates how the power remains consistently high across different sample sizes. On the contrary, the red dashed line represents the minimum acceptable power level of 0.8, commonly used as a benchmark for detecting real effects in scientific studies. This result means that the survey can detect any actual differences between the groups, due to the enormous effect size (Cohen's d = 2.55), making the likelihood of a Type II error (failing to detect an actual effect) almost nonexistent.

Statistical analysis

All statistical analyses in this study were conducted using statistical Package for Social Science (SPSS) version 29.0 windows (SPSS, Inc., Chicago, IL USA). Descriptive statistics were expressed as mean±standard deviation (SD). Differences between mean values were evaluated using Student's t test, with significance threshold set at $p< 0.05$, while $p>0.05$ was considered nonsignificant.

Results

Table 1 presents the biochemical profiles of the studied groups, highlighting highly significant differences ($p<0.001$) between the POF group and the control. Serum levels of reproductive hormones LH, E2, progesterone, and testosterone were markedly reduced ($p<0.001$) in POF patients, while levels of FSH and PRO were significantly elevated compared to healthy controls. Furthermore, thyroid hormones (T3 and T4) showed a significant decrease ($p<0.001$) in the POF group, accompanied by an increase in TSH levels compared to the control group. Table 2 and Figure 1 highlight significant differences in levels of $B\text{-}_{12}$, $B\text{-}_{6}$, and INH-α between the two studied groups: POF and healthy controls. These levels were markedly lower in POF patients compared to the control group.

Table 1. The mean (±SD) of age and hormones for the control and POF groups

Characteristic	Mean±SD		95% CI	p
	Control group (n=40)	POF group (n=40)		
Age (year)	32.07±5.370	32.92±4.26	-3.00–1.30	0.43
FSH (mIU/mL)	3.40±1.10	38.64±11.59	-38.95– -31.51	<0.001
LH (mIU/mL)	3.67±1.64	0.83±0.40	2.30–3.38	<0.001
E-2 (pg/mL)	160.31±38.76	10.93±2.31	136.9–161.76	<0.001
Progesterone (ng/mL)	2.27 ±2.03	0.06±0.062	1.56–2.86	<0.001
PRO (ng/mL)	14.62±4.09	38.25±7.25	-26.26– -20.99	<0.001
Testosterone (ng/mL)	0.47±0.22	0.09 ±0.022	0.31–0.45	<0.001
TSH (mIU/L)	2.75±1.11	8.18±1.87	0.34–6.1	<0.001
T_3 (nmol/L)	1.46±0.36	0.68±0.09	0.05–0.66	<0.001
T_4 (nmol/L)	122.99±23.82	40.49±15.62	4.50–73.50	<0.001

Table 2. The mean (±SD) of vitamin $B\text{-}_{6}$, vitamin $B\text{-}_{12}$, 17-alpha hydroxylase, and inhibin-α for the control and POF groups

Characteristic	(Mean±SD)		95 % CI	p
	Control group (n=40)	POF group (n=40)		
Vitamin $B\text{-}_{6}$ (ng/mL)	40.68±5.75	15.17±3.81	23.32–27.68	<0.001
Vitamin $B\text{-}_{12}$ (ng/mL)	30.68±5.75	5.45±3.42	23.11–27.37	<0.001
17-alpha hydroxylase (ng/dL)	50.68±47.17	0.032±0.018	35.56–65.73	<0.001
INH-α (ng/mL)	29.12±9.11	3.26±1.28	22.91–28.80	<0.001

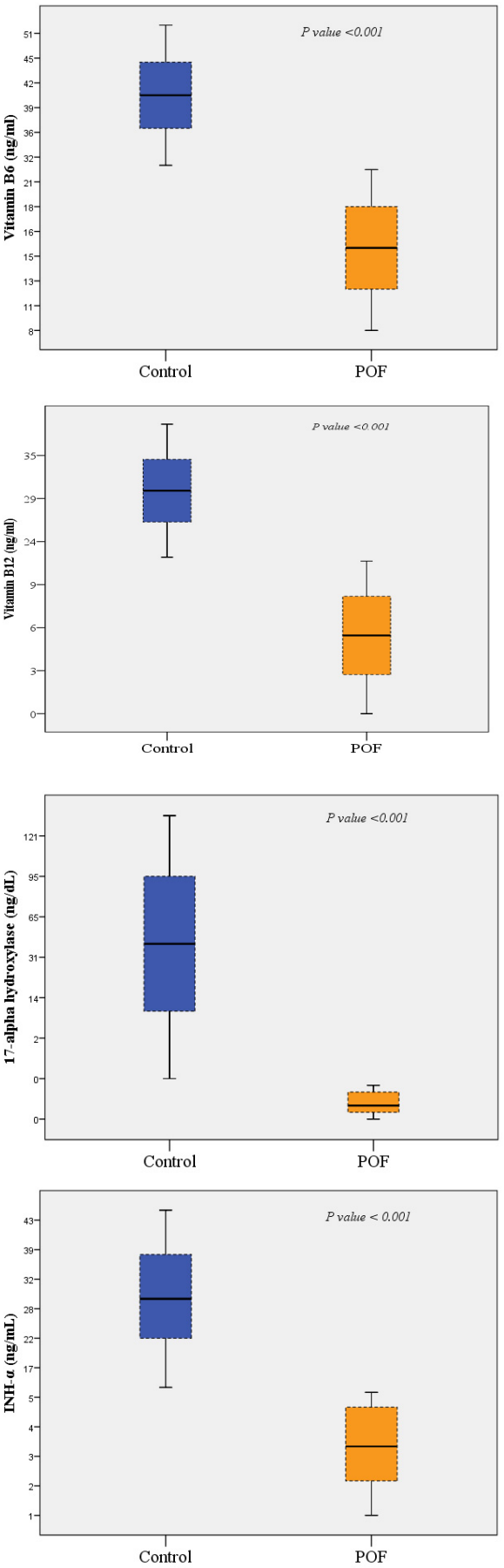

Fig. 2. Mean distribution of biochemical parameters in POF patients group and control group

Discussion

The eight water-soluble vitamins that make up the B-vitamin complex cooperate as coenzymes in an array of anabolic and catabolic enzyme activities to play vital roles in cellular activity.[24,32] Vitamin B_6 is necessary for the synthesis and metabolism of various hormones, including estrogen and progesterone, which is crucial for menstrual regularity, fertility, and overall reproductive health.[68] Several studies evaluated B-vitamin circulating levels in the context of neuroimmune diseases.[69-71] The present study revealed the evaluation of the effect of B_6 and B_{12} vitamins in POF patients and suggests that they are related to POF as well as hormonal dysregulation that occurs with ovarian disorders.

The current study demonstrated that B_6 and B_{12} vitamins were significantly lower in POF patients compared to healthy control. These results align with studies conducted by Brilliant et al.[72] and Kamrul-Hasan et al.[73], which also reported deficiencies of these vitamins in female reproductive disease, highlighting their potential role in ovarian health.

We did not account for dietary intake, supplementation, or lifestyle factors that could influence these levels. However, it is important to note that none of the participants in our study took vitamin supplements minimizing potential confounding effects for supplementation.

Antioxidant micronutrients, such as vitamin and minerals, are vital for female reproductive health as they help manage oxidative stress and facilitate essential cellular function.[74-76] Recent studies indicate that B vitamins could influence ovarian health and function. Specifically, B_{12} can help mitigate ovarian ischemia-reperfusion injury by reducing inflammation and oxidative stress.[47] Similarly, B_6, which is critical for reproduction, affects sulfur and seleno amino acid metabolism and may influence ovarian health and embryo development under oxidative stress.[77] Although not explicitly related to POF, vitamin B_6 has been shown to lower serum PRO levels in hyperprolactinemic patients, a condition relevant to reproductive health.[78] These findings hint at a relationship between vitamins and ovarian function, but more research is required to confirm these links and explore their therapeutic potential in POF.

17-alpha hydroxylase enzyme was significantly lower in POF group compared to the control group which agrees with certain theory reported that a lower level of 17-alpha hydroxylase enzyme alters ovarian functions and the production of sex hormones, as this enzyme is responsible for converting progesterone into cortisol.[79] Women with 17-alpha hydroxylase deficiency often experience infertility and related complications due to disruptions in the steroidogenic pathway. These abnormalities lead to impaired levels of androstenediones, 17-hydroxyprogesterone, and E-2.[80] Weak estrogen feedback to the pituitary may result in mildly elevated FSH and LH levels, increasing the risk of ovarian cysts, particularly multiple luteinizing ovarian cysts. Furthermore, increasing PRO levels may be due to increased GnRH activity. Another common reproductive endocrine manifestation in women with 17-alpha hydroxylase deficiency is deduced testosterone levels. However, in females, low testosterone has minimal impact on reproductive development and fertility. However, this characteristic can serve as a clinical indicator, emphasizing the need to consider 17-alpha hydroxylase deficiency in cases where pubic hair is absent or absent during gynecological examination. In general, partial 17-alpha hydroxylase deficiency presents with endocrine abnormalities that correspond to the degree of enzymatic dysfunction.[81]

The current study demonstrated a significant decrease in the INH-α ($p<0.001$) in POF group compared to the control group. Ovarian follicle granulosa cells are the main producers of INH-α. Blocks the ability of the pituitary gland to secrete FSH, which is its primary role. INH-α is produced by mature and developing follicles during a normal menstrual cycle. This hormone works with the pituitary gland to decrease FSH levels.[82] In accordance with this theory, the reduced level of INH- α level in patients with POF increases the release of FSH by eliminating the inhibitory feedback of the pituitary gland. Several studies have reported a lower serum inhibin in individuals with POF.[83,84] This deficiency is believed to contribute to disease development, as it leads to increased activation activity. In turn, activation stimulates FSH secretion, accelerating folliculogenesis and speeding up ovarian follicle depletion, which ultimately results in early menopause.[85] Genetic variation in genes responsible for inhibin production has been associated with its reduced serum levels, which may amplify activation effects due to diminished inhibin secretion.[85] Previous study identified INH-α (rs12720062 G / A) and showed genotype GG and allele G as potential risk factors for POF, while genotype GA and allele A appear productive. However, genotype AA does not seem to have a significant influence on disease susceptibility.[86] The reduced levels of 17-alpha hydroxylase and INH-α observed in POF patients' highlights their critical roles in maintaining ovarian function l. While direct evidence linking vitamin B_6 and B_{12} to the synthesis of these proteins is limited l, their fundamental roles in enzymatic reactions and gene expression suggest that deficiency could adversely affect ovarian health. More research is necessary to elucidate these relationships and determine whether supplementation could serve as a therapeutic strategy for POF. Given the observed association between vitamin B_{12} and B_6 levels and POF, it is essential to explore whether correcting such deficiencies by targeted supplementation might influence disease progression, severity of symptoms, or fertility

outcomes of affected women. Randomized controlled trials are recommended to evaluate this hypothesis.

Reduced PRO, thyroid hormones (T_3, T_4), LH, E2, and progesterone in patients with POF due to ovarian dysfunction and altered hormonal functions can all contribute to the development of POF or exacerbate its symptoms. Support this theory; a study reported that infertility in women is indicated by the significant positive correlation TSH has with PRO, FSH, and LH. When diagnosing infertility, these hormonal assessments enable a standard etiological approach.[87,88]

Our participants were recruited from Baghdad, which may limit the generalizability of the findings. Future studies should consider the possible impact of ethnic and genetic diversity on vitamin metabolism and ovarian health.

Study limitations and recommendations

This study provides a cross-sectional initial snapshot of biochemical parameters in POF patients and does not establish causality. A key limitation of this study is the relatively small sample size (80 participants), which may affect the statistical power and generalizability of the results. Although our findings provide valuable information on the possible association between B-$_6$ and B-$_{12}$ and POF, larger-scale longitudinal studies involving more diverse populations to validate these observations. Further research should incorporate dietary assessment, supplement intake, physical activity, and genetic analysis to provide a more comprehensive understanding of the factors that influence vitamin levels in POF. These unmeasured variables could introduce bias and limit the generalizability of the findings. Another major drawback is the lack of population diversity; more research involving participants from multiple centers is necessary to improve external validity in examining possible environment-gene interactions that can affect POF risk. However, the existing literature suggests that adequate levels of vitamin B are necessary for women's health, especially ovarian function, but also that further investigated studies are required to determine their therapeutic role in POF management.

Conclusion

The study findings revealed a significant deficiency in vitamin B-6, B-12, INH-α, and 17-alpha hydroxylase levels in patients with POF. These results suggest that POF is associated with disturbances in vitamin B-6 and B-12, as well as dysregulation of INH-α and 17-alpha hydroxylase production. These biochemical abnormalities may contribute to the pathogenesis and clinical manifestations of POF. More research is needed to elucidate the underlying mechanisms and explore potential therapeutic interventions that target these deficiencies, aiming to improve the diagnosis and management of POF patients.

Declarations

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Author contributions

Conceptualization, D.A.H. and A.S.A.; Methodology, N.M.A., A.H.J.A. and A.R.A.; Software, H.R.-S.; Validation, H.R.-S., A.R.A. and A.H.J.A.; Formal Analysis, D.A.H.; Investigation, N.M.A. and A.H.J.A.; Resources, D.A.H. and A.S.A.; Data Curation, A.H.J.A.; Writing – Original Draft Preparation, A.S.A.; Writing – Review & Editing, A.R.A. and H.R.-S.; Visualization, N.M.A.; Supervision, A.R.A. and H.R.-S.; Project Administration, N.M.A.; Funding Acquisition, D.A.H. and A.S.A.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

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

Ethics approval

The study protocol was approved by the University of Technology-Iraq Bioethics Committee (approval no.: BCSR11) on 24 March 2024. All procedures were in accordance with internationally recognized, ethical standards, particularly those in the Declaration of Helsinki.

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ORIGINAL PAPER

Frailty in liver cirrhosis – insights into prevalence, determinants, and clinical impact from a tertiary center experience

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ABSTRACT

Introduction and aim. Frailty has emerged as a critical determinant of poor outcomes in patients with liver cirrhosis. This study aims to determine the prevalence and predictors of frailty in patients with liver cirrhosis and assess its clinical impact on disease severity.

Material and methods. This cross-sectional study included 460 patients with liver cirrhosis were classified into frail Clinical Frailty Scale (CFS) >4 and non-frail (CFS≤4) groups.

Results. The prevalence of frailty among the studied patients was 45.7%. Frail patients were significantly older (60.61±9.06 years), without gender predilection (43.7%, 47.9%). Patients with frailty exhibited significantly worse liver function; higher bilirubin (1.30 (0.90–2.0) vs 0.90 (0.66–1.30) mg/dL, p<0.001) and low albumin (3.9 (3.50–4.10) vs 3.2 (2.60–3.70) g/dL, p<0.001). Multivariate analysis identified age (OR=1.055, 95%CI: 1.023–1.088, p<0.001), body mass index (OR=9.803, 95%CI: 5.067–18.963, p<0.001), and high nutritional risk (OR=20.186, 95%CI: 8.456–48.191, p<0.001) as independent predictors of frailty.

Conclusion. Frailty is a significant concern in patients with liver cirrhosis, particularly those with advanced age, diabetes, and severe hepatic dysfunction. Optimizing outcomes for this patient population requires a multi-faceted approach that considers liver disease management, routine frailty assessment, and interventions to enhance physical resilience and address co-morbidities.

Keywords. cirrhosis, frailty, prevalence

Introduction

Frailty is a multidimensional syndrome characterized by diminished physiological reserves, increased vulnerability to stressors, and impaired ability to maintain homeostasis following minor health perturbations.[1] Originally studied in geriatric populations, frailty is now recognized as a pivotal determinant of clinical outcomes in various chronic diseases, including liver cirrhosis.[2]

In the context of cirrhosis, frailty arises from a complex interplay of sarcopenia, malnutrition, systemic inflammation, and hepatic encephalopathy, all of which compromise functional capacity.[3] Cirrhotic patients with frailty are at heightened risk for complications such as falls, infections, prolonged hospitalizations, and increased mortality.[4] Moreover, frailty adversely impacts candidacy and outcomes for liver transplantation, leading to higher delisting rates and poorer post-transplant survival.[5]

Recent studies report that frailty affects nearly 40–50% of patients with cirrhosis, even among those with compensated disease.[4-7] Despite this growing recogni-

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tion, frailty remains under-assessed in routine hepatology practice, particularly in low-resource settings. Standard liver disease severity scores such as Model for End-Stage Liver Disease (MELD) or Child-Pugh do not capture the functional and nutritional decline that defines frailty, underscoring the need for integrated prognostic models.

Multiple tools have been developed to evaluate frailty in cirrhosis, including performance-based measures [e.g., Liver Frailty Index (LFI)], self-reported scales, and clinician-administered tools such as the Clinical Frailty Scale (CFS).[8] The CFS offers the advantages of simplicity, feasibility, and reproducibility, making it suitable for real-world clinical settings.[9]

In Egypt and other Middle Eastern countries, limited data are available on the prevalence and implications of frailty in cirrhosis, despite high burdens of viral hepatitis and metabolic liver diseases. This gap hinders evidence-based interventions for risk stratification and patient optimization.

Aim

This study aims to determine the prevalence of frailty among patients with liver cirrhosis at a tertiary care center in Egypt, identify its demographic, clinical, and nutritional predictors, and explore its relationship with disease severity and transplant eligibility. By highlighting frailty's burden and correlates, we hope to inform better clinical decision-making and encourage routine frailty assessment in hepatology practice

Material and methods

This prospective cross-sectional study was conducted at the National Liver Institute (NLI), Menoufia University, Egypt, between September 15, 2023, and June 15, 2024. The study protocol was approved by the Institutional Review Board of NLI (IRB approval number: 0014014FWA00034015), and written informed consent was obtained from all participants.

Eligible participants were adults aged 18 years or older with a confirmed diagnosis of liver cirrhosis based on clinical, biochemical, and radiologic criteria, and/or liver biopsy when available.[10] Patients were required to be physically and cognitively able to participate in clinical and nutritional assessments.

Exclusion criteria included the presence of hepatocellular carcinoma or any other active malignancy, recent gastrointestinal bleeding (within the past two weeks), overt hepatic encephalopathy (Grade ≥2 based on West Haven criteria), neurological or musculoskeletal disorders impairing mobility, recent major surgery (within four weeks), advanced cardiopulmonary disease, prior liver transplantation, or life expectancy of less than six months due to non-hepatic causes.

The sample size was calculated using the normal approximation method for estimating a population proportion, assuming a 50% prevalence of frailty among patients with cirrhosis, consistent with prior studies such as Tandon et al.[3] and Padhi et al.[7] a 95% confidence level, and a ±5% margin of error. A minimum of 410 patients was required to achieve 80% power to detect a 10% difference. To account for potential exclusions or missing data, the target sample size was increased by 10–15%, resulting in a final enrollment of 460 patients.

Frailty was assessed using the Clinical Frailty Scale (CFS), a validated 9-point clinician-rated scale evaluating physical function, independence, and comorbidity burden. Patients were classified as frail (CFS >4) or non-frail (CFS ≤4).[11-13] Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared and classified according to WHO criteria.[14]

Nutritional status was evaluated using the Royal Free Hospital Nutritional Prioritizing Tool (RFHNPT), which categorizes patients into low (score 0), moderate (score 1), or high (score 2–7) nutritional risk.[15]

Liver disease severity was assessed using established scoring systems including the MELD, Child-Pugh score, and albumin-bilirubin (ALBI) score. Laboratory parameters used in these calculations were obtained on the same day as the frailty assessment.[10]

Data were collected through structured interviews and clinical examinations, including demographic data (age, sex), cirrhosis etiology, presence of comorbidities (diabetes mellitus, hypertension), and clinical features such as ascites, lower limb edema, and hepatic encephalopathy. The primary outcome was the prevalence of frailty (CFS >4). Secondary outcomes included associations between frailty and liver disease severity (MELD, ALBI, Child-Pugh scores), nutritional status (RFHNPT), BMI categories, and decompensating features.

This study is reported in accordance with the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines.

Statistical analysis

Data were analyzed using SPSS software, version 26.0 (IBM Corp., Armonk, NY, USA; 2019). Categorical variables were expressed as frequencies and percentages, and continuous variables as means ± standard deviations or medians with interquartile ranges, depending on distribution. Comparisons between groups (e.g., MELD <15 vs. ≥15; transplant-eligible vs. ineligible) were conducted using Chi-square or Fisher's exact test for categorical variables, and Student's t-test or Mann–Whitney U test for continuous variables. Univariate and multivariate logistic regression were performed to identify predictors of transplant eligibility, including CFS, age, MELD, diabetes, hypertension, and ascites. Spearman's rank correlation was used to examine the association between CFS and MELD score. A p-value <0.05 was considered statistically significant.

Results

A total of 460 patients with liver cirrhosis were enrolled. Based on the Clinical Frailty Scale (CFS), 210 patients (45.7%) were classified as frail (CFS >4), while 250 (54.3%) were non-frail. Demographic analysis revealed a median age of 61.0 years (IQR: 55.0–66.0) in frail patients and 56.0 years (IQR: 50.0–62.0) in non-frail patients (p<0.001). There was no significant sex difference between groups. Comorbid conditions such as diabetes mellitus (45.2% vs. 25.2%; p<0.001) and hypertension (42% vs. 21.7%; p=0.007) were more prevalent in the frail group. Ascites and lower limb edema were also significantly more common (82.1% vs. 17.9%; p<0.001) (Table 1).

Frail patients had significantly worse liver disease severity scores. Median MELD score was higher in frail vs. non-frail patients (11.0 vs. 8.0; p<0.001), and a greater proportion were classified as Child-Pugh Class C (17.6% vs. 1.2%; p<0.001). ALBI scores also correlated positively with frailty (rs=0.426, p<0.001) (Table 2).

Nutritional risk, assessed by the RFHNPT, was strongly associated with frailty. The RFHNPT score showed a robust correlation with CFS (rs=0.753, p<0.001) (Table 2, Figure 1).

BMI analysis revealed a U-shaped association with frailty. Among underweight individuals (BMI <18.5 kg/m²), 96.1% were classified as frail. Similarly, 95.5% of patients with obesity (BMI ≥30 kg/m²) were frail. In contrast, most patients with normal BMI (18.5–24.9 kg/m²) were non-frail, comprising 84.8% of that category. Overweight individuals (BMI 25–29.9 kg/m²) also showed a high prevalence of frailty (76.1%).

In multivariate logistic regression, independent predictors of frailty included: older age (OR=1.055; p=0.001), high nutritional risk (OR=20.186; p<0.001), and overweight/obese BMI categories (OR=9.803; p<0.001) (Table 3).

Table 1. Demographic and clinical characteristics of the two patients' groups

Variable	Non-frail (CFS≤4) n=250	Frail (CFS>4) n=210	Test	p
Age, years	56.0 (50.0–62.0)	61.0 (55.0–66.0)	18236.0	<0.001
Sex (Male)	138 (56.3%)	107 (43.7%)	0.827	0.363
Diabetes mellitus	63 (25.2%)	95 (45.2%)	20.322	<0.001
Ascites and lower limb edema	17 (6.8%)	78 (37.1%)	64.123	<0.001
Total bilirubin (mg/dL)	0.90 (0.66–1.30)	1.30 (0.90–2.00)	17119.5	<0.001
Albumin (g/dL)	3.90 (3.50–4.10)	3.20 (2.60–3.70)	14032.0	<0.001
MELD Score	8.0 (6.0–11.0)	11.0 (8.0–16.0)	16101.5	<0.001

Table 2. Correlation of frailty with liver disease severity and nutritional scores (Spearman's rank correlation)

Score	Test	p
MELD score	0.437	<0.001
ALBI score	0.426	<0.001
Child-Pugh score	0.433	<0.001
RFHNPT score	0.753	<0.001

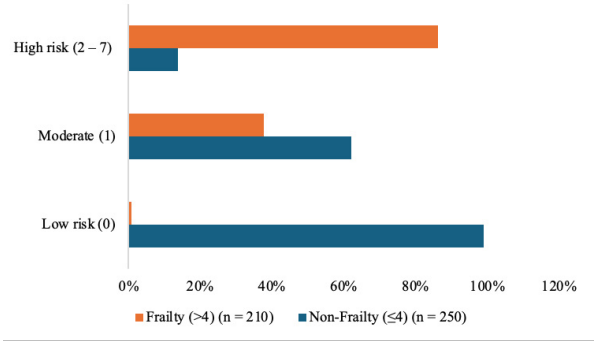

Fig. 1. Nutritional risk in frail vs. non-frail cirrhotic patients

Table 3. Logistic regression analysis for predictors of frailty (CFS >4)

Variable	Univariate OR (95%CI)	p	Multivariate OR (95%CI)	p
Age (per year increase)	1.061 (1.038–1.084)	<0.001	1.055 (1.023–1.088)	0.001
MELD Score	1.172 (1.118–1.228)	<0.001	1.153 (0.946–1.404)	0.158
Albumin	0.298 (0.221–0.402)	<0.001	0.517 (0.293–0.912)	0.023
RFHNPT (High risk)	19.864(11.78–33.48)	<0.001	20.186 (8.456–48.191)	<0.001
BMI (Overweight/ Obese)	10.238(5.96–17.58)	<0.001	9.803 (5.067–18.963)	<0.001

Discussion

This study highlights the high prevalence and clinical relevance of frailty in patients with liver cirrhosis and is associated with distinct demographic and clinical patterns. Older age and comorbidities such as diabetes and hypertension were significantly more common in the frail cohort, underscoring the interplay between systemic health and liver-related frailty. Our findings reinforce that frailty, as assessed using the CFS, is not merely a reflection of chronological aging but rather a multidimensional syndrome that correlates with liver disease severity, nutritional compromise, and reduced transplant eligibility. The Clinical Frailty Scale identified 45.7% of patients as frail, aligning with previous studies reporting frailty prevalence in cirrhosis ranging from 40% to 50%, even among patients with relatively preserved liver function.[7,11-13]

The CFS, adopted in our study due to its ease of implementation and real-world applicability, offers a pragmatic alternative as a validated simple screening tool to more complex tools such as the LFI.[12-13] While the LFI incorporates performance-based assessments (grip strength, chair-stands, balance), which enhances its predictive value for waitlist mortality and hospitalization[16], its routine use remains limited in many clinical settings due to time, training, and equipment constraints. Conversely, the CFS, being a clinician-judgment-based tool, enables rapid bedside assessment with proven prognostic relevance in liver disease populations.[12-13] However, the subjective nature of the CFS may render it less sensitive to subtle declines in physical function and more

vulnerable to inter-observer variability, thus potentially underestimating frailty in early stages.[17] Future research should explore the complementarity of both tools, especially in settings where resource limitations preclude objective measures.

Our study also underscores the intricate relationship between frailty and liver dysfunction. Frailty was more frequently observed in patients with features of hepatic decompensation, including ascites and hypoalbuminemia, and in those categorized within higher Child-Pugh and MELD score classes. This suggests a bidirectional interplay in which progressive liver dysfunction contributes to physiological decline and, conversely, frailty may exacerbate vulnerability to liver-related complications.[18] These findings support integrating frailty assessments into routine cirrhosis management to enhance risk stratification and inform decisions regarding transplant candidacy, inpatient care needs, and palliative approaches.

Importantly, the association between frailty and nutritional status emerged as particularly robust. The observed overlap between high frailty scores and elevated nutritional risk scores is consistent with the literature, where malnutrition is identified as both a contributor to and consequence of frailty in cirrhosis.[4,19] This highlights the importance of combining frailty screening with structured nutritional assessments. Early nutritional interventions – including adequate protein intake, branched-chain amino acid supplementation, and late evening snacks – have been shown to mitigate sarcopenia and improve physical reserve.[20-22]

Our findings also reveal a U-shaped relationship between body mass index (BMI) and frailty, where both underweight and obese individuals demonstrated elevated frailty prevalence. While under nutrition has long been recognized as a frailty determinant, obesity – particularly sarcopenic obesity – has emerged as a critical phenotype characterized by excess adiposity with reduced muscle mass and function.[14,23] This paradox may lead to misclassification of nutritional status if BMI alone is used and emphasizes the necessity of body composition analysis in cirrhotic populations.

Although sarcopenia and frailty are distinct entities, their overlap is considerable. Sarcopenia represents the physical dimension of frailty and shares pathophysiological underpinnings, including chronic inflammation, hormonal dysregulation, and metabolic imbalance.[24] While our study did not employ direct measures of sarcopenia, such as muscle mass or strength, the strong associations between frailty, nutritional risk, and BMI serve as surrogate indicators. Future studies incorporating imaging-based assessments or bioimpedance analysis may better delineate the interaction between these conditions.

The multivariate model identified age, advanced liver disease, and ascites as independent predictors of frailty. These findings mirror previous research and reinforce the notion that liver-specific factors outweigh traditional cardiovascular comorbidities, such as diabetes and hypertension, in determining frailty risk in cirrhosis.[4,7] This insight has important clinical implications, suggesting that even in patients without significant cardiometabolic burden, frailty may develop as a direct consequence of hepatic insufficiency and associated systemic effects.

A notable strength of this study is its relatively large sample size and comprehensive assessment of frailty using validated tools in conjunction with nutritional and hepatic parameters. However, several limitations should be acknowledged. The cross-sectional design precludes conclusions about the temporal evolution of frailty or its reversibility. Additionally, the exclusive use of the CFS may limit granularity in functional assessment compared to objective frailty indices. Our findings also reflect a single-center experience and may not be generalizable across diverse healthcare systems or populations.

Future research should focus on longitudinal frailty trajectories and the impact of targeted interventions, including pre-habilitation programs that integrate exercise, nutrition, and psychosocial support, especially for transplant candidates. Comparative studies of the CFS and LFI in diverse clinical settings would also help define optimal frailty assessment strategies

Conclusion

Frailty is a prevalent and clinically impactful condition among patients with liver cirrhosis, reflecting a convergence of physiological decline, malnutrition, and hepatic dysfunction. The Clinical Frailty Scale, owing to its practicality and validated utility, offers a feasible screening tool in routine hepatology practice, particularly in resource-limited settings. Our findings advocate for the systematic integration of frailty assessment into the management of cirrhotic patients to improve risk stratification, guide nutritional and rehabilitative interventions, and inform transplant eligibility decisions. Future multicenter and longitudinal studies incorporating objective frailty and sarcopenia measures are warranted to deepen our understanding of frailty's trajectory and its modifiability in liver disease.

Declarations

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Author contributions

Conceptualization, E.M. and E.M; Methodology, A.A.; Software, A.A.; Validation, E.M., E.M. and A.A.; Formal Analysis, E.M.; Investigation, A.A.; Resources, A.A.; Data Curation, E.M.; Writing – Original Draft Prepara-

tion, E.M.; Writing – Review & Editing, A.O. and E.M.; Visualization, A.O.; Supervision, A.O. and B.G.; Project Administration, A.O. and B.G.; Funding Acquisition, E.M. and A.A.

Conflicts of interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability

Data is available upon request from the corresponding author.

Ethics approval

The study protocol was approved by the Institutional Review Board of NLI (IRB approval number: 0014014FWA00034015).

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ORIGINAL PAPER

Baicalein's neuroprotective effects in a lansoprazole-induced Alzheimer's model in Wistar rats

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ABSTRACT

Introduction and aim. Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most frequently diagnosed type of dementia. Baicalein, a flavonoid found in the roots of *Scutellaria baicalensis*, was useful in preventing neuronal injury in various neurodegenerative models. The present study was designed to determine the neuroprotective effect of baicalein in lansoprazole-induced AD in Wistar rats.

Material and methods. We used male Wistar rats randomly divided into six groups: control, vehicle, lansoprazole-induced, and baicalein+lansoprazole or baicalein treated. The lowest dose, lansoprazole (30 mg/kg), was administered orally for 28 weeks to induce AD-like pathology. Baicalein (10 mg/kg) was coadministered with lansoprazole in the treatment group. Cognitive functions were evaluated using a Morris water maze (MWM) and a novel object recognition test. Acetylcholinesterase (AChE) activity, determination of oxidative stress, including malondialdehyde, superoxide dismutas, and reduced glutathione levels, and inflammation-related cytokines such as tumor necrosis factor α were biochemically analyzed. We collected hippocampal sections to assess amyloid beta deposition and neuronal integrity histochemically.

Results. Lansoprazole-induced rats showed marked cognitive decline accompanied by enhanced AChE activity, increased oxidative stress, and levels of pro-inflammatory cytokines compared to controls ($p<0.01$). Co-administration of Baicalein significantly improved cognitive performance, AChE activity was reduced to control levels, oxidative stress markers decreased to near-normal values in the brain and blood, and inflammatory cytokines were significantly lower compared to rats treated with the lansoprazole-treated rats ($p<0.01$). Baicalein treatment attenuated plaque deposition and neuronal injury, as revealed by histopathological analysis in vivo.

Conclusion. Baicalein has a protective effect on lansoprazole-induced AD in Wistar rats. These appear to be due to the flavonoid's antioxidative, anti-inflammatory, and enzyme-inhibiting activities on AChE. These results propose baicalein as a potential therapeutic agent for further study with respect to prevention and treatment, especially in patients under long-term administration of proton pump inhibitors.

Keywords. Alzheimer disease, anti-inflammatory, baicalein, lansoprazole, oxidative stress

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Introduction

Alzheimer's disease (AD) is the most common type of dementia that affects the elderly population. It is marked by behavioral changes, cognitive decline, and a decline in social functioning.[1] The defining characteristic of AD is the gradual deterioration of cognitive functions, with recent fact memory, spatial orientation, attention, and executive functions among the first to be compromised.[2] Subsequently, there are issues with speech and behavior that impact daily life.[2] Several elements potentially play a role in the onset of AD, including genetic anomalies, mitochondrial dysfunction, modified processing of amyloid precursor protein, the emergence of neurofibrillary tangles, insufficiency in neurotrophic factors, as well as heightened oxidative stress.[3] The accumulation of extracellular amyloid in the brain stimulates microglia activation, leading to the release of various proinflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor α (TNF-α), and IL-6. Furthermore, amyloid accumulation induces mitochondrial dysfunction by hindering oxidative phosphorylation, increasing reactive oxygen species (ROS) production, disrupting mitochondrial energy, and interacting with various mitochondrial proteins.[4]

The potential link between proton pump inhibitors (PPIs) and AD has been a subject of growing interest in recent years. PPIs such as omeprazole, lansoprazole, and pantoprazole are commonly prescribed for acid-related diseases such as gastroesophageal reflux disease and peptic ulcers.[5] However, recent findings suggest that their long-term use may have unintended effects on cognitive function and neurodegeneration. Other research indicates that PPIs can encourage the buildup of beta-amyloid (Aβ), a characteristic of AD.[6] Some studies show that some PPIs can modify lysosomal function and decrease Aβ clearance, which could be a factor in plaque development in the brain.[7-8] PPIs such as omeprazole and lansoprazole have been found to modulate gamma-secretase activity, an enzyme involved in Aβ generation, and result in increased amyloidogenic processing.[9] PPIs can modulate acetylcholine concentration by inhibiting choline acetyltransferase, an enzyme responsible for acetylcholine synthesis. Acetylcholine deficiency is a characteristic of cognitive impairment in AD.[10]

According to their hypothesis, PPIs penetrate the blood-brain barrier and block vacuolar-type ATPase proton pumps. Due to this suppression of V-ATPases, microglial lysosomes have a higher pH and microglial phagocytosis breaks down less Aβ.[11] It is known that amyloid precursor protein (A-42 and A-42) and amyloid beta (A-40) are localized in mitochondrial membranes, interact with mitochondrial proteins, obstruct nuclear-encoded mitochondrial proteins from entering mitochondria, disrupt the electron transport chain, enhance ROS, damage mitochondria, and affect neuronal function.[12]

In addition, buildup of amyloid beta in the synaptic terminals in AD patients may contribute to synaptic degeneration and cognitive loss.[13] Therefore, the production of reactive oxygen species is caused by the release of electrons and the formation of free radicals. However, inhibition of the electron transport chain does not ensure a direct increase in ROS production of ROS within the mitochondria.[14] An excess of reactive oxygen species in the brain can lead to oxidative stress together with neuroinflammatory release, which can harm the central nervous system as well as result in neurodegenerative diseases such as AD.[15]

The current investigation was carried out to establish lansoprazole-induced neurological modification as an animal model of AD in rats.[16] Furthermore, the model was verified by modulation of various neurobehavioral and neurochemical parameters.[17] Baicalein is a flavonoid compound isolated from the roots of *Scutellaria baicalensis*, known as Baikal skullcap or Chinese skullcap, a Chinese herbal medicine.[18] Baicalein is a traditional Chinese medicine used for centuries to treat inflammatory disorders, infections, and even cancer. Baicalein has been supported by modern scientific research that reveals its numerous pharmacological activities suggesting that it may also be of significant interest in modern medicine.[19] Baicalein, on the other hand, is highly effective in reducing inflammation, which is a complex biological response necessary for recovery but when prolonged is associated with diseases such as arthritis, cardiovascular diseases, and neurodegenerative disorders to name a few.[20] Modulation of multiple signaling pathways and molecular targets is done by inhibiting cyclooxygenase-2 (COX-2) and lipoxygenase (LOX), the two enzymes that are mandatory in the process of inflammation. This reduces the production of pro-inflammatory mediators such as prostaglandins and leukotrienes and relieves inflammation.[21]

In addition to its anti-inflammatory activity, baicalein also demonstrates considerable antioxidant action. Oxidative stress caused by an imbalance of free radical and antioxidant levels is known to be associated with many chronic diseases, such as cancer, diabetes, or heart diseases.[22] Baicalein possesses a shielding activity against oxidative damage to the cells and helps to restore cell levels, thereby reducing the incidence of oxidative stress-related diseases.[21] It achieves this by scavenging free radicals and boosting the activity of natural antioxidant enzymes, two oxidative stress prevention techniques.[22] For the central nervous system, the effects of associations with baicalein are however worth noting. Baicalein confers neuroprotective actions along several pathways such as antioxidant protection, anti-inflammation, synaptic modulation, inhibition of apoptosis, induction of autophagy, and stabilization of the blood-brain barrier. Through these mechanisms, it

is considered a promising candidate for the therapeutic treatment of Alzheimer's disease, Parkinson's disease, stroke, epilepsy, and other neurodegenerative conditions. More scientific and clinical work is needed to confirm its efficacy against human neurological conditions.[23] It has also been shown in animal models of AD that baicalein is capable of preventing amnesia as well as improving their cognitive abilities.[24]

Aim

The aim was to investigate the neuroprotective effects of baicalein in a lansoprazole-induced Alzheimer and model in Wistar rats.

Material and methods

Animals

After approval of the study by the Institutional Animal Ethics Committee (IAEC) Reg. No.711/PO/ReRc/S/02/CPCSEA and date of registration: 29 October 2002, Meerut Institute of Engineering and Technology, Meerut, 36 female Wistar rats weighing 200-300 g were raised at the Animal House Facility (Meerut Institute of Engineering and Technology, Meerut U.P., India). The animals were confined in polypropylene cages measuring 49x34x26 cm, with unrestricted access to food and water. All animals were housed under the same standard conditions, including a 12-hour cycle of light and darkness, a quiet chamber, and twice-weekly bedding changes. The temperature was maintained at 23°C±2°C, and a relative humidity of 65±5%. All behavioral experiments were conducted between 9:00 am and 5:00 pm. The study protocol complied with the institutional policies and the Institutional Animal Ethics Committee for the care and use of experimental animals. Everyone worked together to minimize the amount of animals and any discomfort they might have had. According to the laws of the Government of India, the Institutional Animal Ethics Committee (IAEC) authorized the study protocol. It was registered with 711/PO/ReRc/S/02/CPCSEA 29/10/2002 as protocol number IAEC/MIET/CPCSEA/Meeting No: 04/2023/Protocol No.192. The rodents were acclimatized to the laboratory environment before the experimentation phase.

Experimental design and procedure

For the purpose of this study, 36 female Wistar rats, weighing between 200 and 300 g, were used. The rats used in the study were divided into sex groups each with 6 rats (n=6) that are detailed in the (Table 1). The study lasted 42 days, while the detailed experimental design is given in (Fig. 1). The rats were placed in individual cages that had 6 rats per cage before getting used to behavioral tests. Group I (vehicle group): rats were administered normal saline orally for 42 days. Group II (per se group): rats were given 10 mL/kg of test drug (baicalein) orally dissolved in distilled water for 14 days. Group III (toxin group): rats received 30 mL/kg (orally) lansoprazole dissolved in distilled water to induce AD up to 28 days. Group IV (toxin+low dose): rats received 30 mL/kg (orally) of lansoprazole dissolved in distilled water to induce AD for up to 28 days and then administered baicalein 5 mg/kg orally for 14 days. Group V (toxin+high dose): rats received lansoprazole 30 mL/kg (orally) dissolved in distilled water to induce AD for up to 28 days

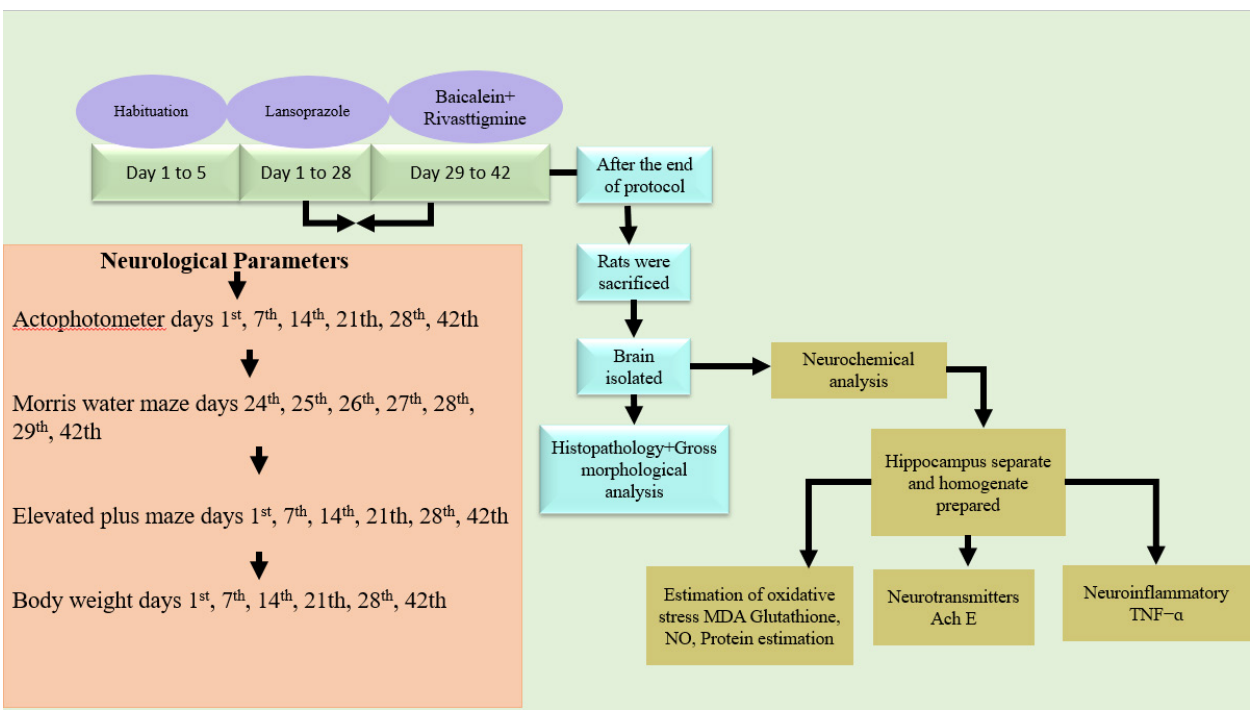

Fig. 1. Representation of the experimental procedure

and then baicalein 10 mg/kg orally for 14 days. Group VI (toxin+dose of std): rats received lansoprazole 30 mL/kg (orally) dissolved in distilled water to induce AD up to 28 days and then administered Rivastigmine 0.3 mg/kg intraperitoneally for 14 days**.**

Table 1. Representation of experimental grouping

S. No.	Groups	Animal species	Number of animals
1	Normal group (normal saline 10 mg/kg orally)	Albino Wistar rats	6
2	Perse group		6
3	Toxin group (lansoprazole 30 mg/kg)		6
4	Toxin group (lansoprazole 30 mg/kg)+test drug (baicalein 5 mg/kg)		6
5	Toxin group (lansoprazole 30 mg/kg)+test drug (baicalein 10 mg/kg)		6
6	Toxin group (lansoprazole 30 mg/kg)+standard drug (rivastigmine 0.3 mg/kg)		6

Drugs and chemicals

The following substances were used: Sigma Aldrich co., an NMDA receptor antagonist at 50 mg/mL *i.p.;* lansoprazole PPI (proton pump inhibitor) and administered orally at 30 mg/kg. dimethyl sulfoxide (DMSO), and EDTA from CDH; baicalein was obtained from Shipra Herbal (An ISO and GMP Certified Company), and administered orally at 5 mg/kg and 10mg/kg.[25] The selective baicalein antagonist Rivastigmine (API) was purchased from Sigma Aldrich Co. and administered intraperitoneally at 0.3 mg/kg. Sodium carbonate, sodium hydroxide, sodium bicarbonate, hydroxylamine, and CDH hydrochloric acid. All medications and chemicals were of analytical quality and were prepared immediately before injection.

Induction of AD

Lansoprazole, a widely used PPI drug for treating disorders related to acids, has also been involved in cognitive impairment and AD pathology. AD-like pathology due to the administration of lansoprazole 30 mg/kg (for 28 days p.o.) was established in rodent models by modifying Aβ metabolism, oxidative stress, neuroinflammation and neurotransmitter systems.[26]

Body weight

The experiment included the measurement of body weight on days 1, 7, 14, 21, 28, 35, and day 42 as a major predictive variable in the logistic regression study for AD due to lansoprazole. The test drug, baicalein, was administered to evaluate its therapeutic effect in the prevention of neurodegenerative effects. Changes in body weight were correlated with disease progression, and in fact, flight time is suggested to be induced by baicalein-mediated prevention of lansoprazole-induced Alzheimer's pathology- associated with overall loss of body weight.[27]

Neurobehavioral parameters

Morris Water Maze assessment

The Morris Water Maze (MWM) is a standard behavioral test to measure spatial memory and learning in rodents. It involves a circular swimming pool with opaque water and a submerged hidden escape platform just below the surface. Rodents learn to use extra-maze visual cues to swim and find the platform during training trials. Escape latency, path length, and time in the target quadrant are measured to assess learning and memory retention. The test trial, which is performed without the platform, assesses the capacity to recall. Reversal learning can be tested by moving the platform to examine cognitive flexibility. MWM is widely applied in neuroscience studies to investigate cognitive impairment, neurodegenerative disorders, and the impact of pharmacological treatments. Its sensitivity and reliability to hippocampal function make it an important tool for evaluating memory-related disorders and possible therapeutic interventions.[28]

Locomotor activity

Using a digital actophotometer, the impulsive locomotion was measured on days 1, 7, 14, 21, 28, and 42. Mental activity was also observed. Rats were used to measure mental move activity after training for 180 seconds in the actophotometer chamber. A digital actophotometer prepared with infrared light-sensitive photocells was used to measure locomotor activity, and the counts per minute were noted for the intervals of five minutes.[29]

Elevated plus maze

Rats were placed individually at the end of each open arm in an exploratory situation (new situation), facing away from the central platform; thus, we used this situational test to demonstrate a level of new situations with the possibility of fear faced by animal so-called elevated plus maze (EPM) on days 1, 7, 14, 21, 28 and 42. The latency to cross from the open arm into either enclosed arm was recorded for each rat. Once this delay was noted, the rat was allowed to explore the plus-maze for the following 20 s freely spared of restraints or researcher interference in selecting open vs. closed arms. Shorter transfer latency values were associated with better long-term memory.[30]

Biochemical parameters

Brain homogenate preparation

Rats in each group were individually anesthetized at a low dosage of ether, killed, and given PBS perfusion when the 10-week experiment was complete. The brains were immediately washed in cold water and removed from the skull. The hippocampal were separated in separate containers, and after cooling PBS at pH 7.4, phosphate buffer (0.1 M) was used to homogenize the tissues

at 800×g for five minutes at 4°C, or ten times their volume. The resulting supernatant was centrifuged for 10 minutes at 10,000×g, at 4°C. To perform additional biochemical calculations, the supernatant was separated and stored at -80°C.[31]

Estimation of AChE level

Ellman's techniques are used to measure the amount of AChE in the hippocampus of the brain. A mixture of three milliliters (mL) of PBS (25 mM, pH 7.4), 0.1 mL of DTNB (5,5-dithio-bis-(2-nitrobenzoic acid, 10 mM in 15 mM $NaHCO_3$) and 0.2 mL of 75 mM acetyl-thiocholine iodide are used for reaction. The mixture was incubated for ten minutes at a temperature of 25°C. Following evolution, 0.2 mL of the hippocampus homogenates' supernatants were combined. From Perkin Elmer, the twenty spectrophotometers measured the optical density at 412 nm in five minutes, recording variations in absorbance every thirty seconds. Nano moles of hydrolysis of acetylthiocholine iodide per milligram of proteins were detected.[32]

Estimation of lipid peroxidation (LPO)

The hippocampal region of the brain was using TBARS as a lipid peroxidation indicator. Using Will's method, the concentration of MDA was determined. A total of 0.5 mL of Tris-HCL and 0.5 mL of supernatant were mixed and incubated at 37°C for two hours. One mL of 10% trichloroacetic acid was added to the reaction mixture after incubation, and the mixture was centrifuged for ten minutes at 1000×g. Therefore, thiobarbituric acid was diluted to 0.67% using 1 mL of quiet supernatant. Boiling water was poured over the reaction tube for ten minutes. One ml of distilled water was added once it had cooled. At a wavelength of 532 nm, the absorbance was measured. Millimoles of TBARS were recorded for each milligram of protein.[33]

Assessment of catalase (CAT)

CAT amount determined using Calibre's (1985) method. A reaction mixture comprising 0.05 ml of supernatant, 1 mL of hydrogen peroxide (H_2O_2, 0.019 M), and 1.95 mL of supernatant was combined with phosphate buffer (0.5 M; pH 7.0) to modify the final product's volume to 3 mL. The PerkinElmer Lambda 20 spectrophotometer was used to record changes in absorbance at 240 nm every 30 seconds. Measurement of CAT activity was expressed in nmol of H_2O_2 / min / mg protein.[35]

Estimation glutathione (GSH)

To conduct the GSH test, 1 mL of the 4% w/v sulfosalicylic acid precipitate and 100 mL of the tissue homogenate supernatant were combined. Then store the reaction mixture in a refrigerator between 2 and 8°C. Samples were centrifuged at 1200×g for 15 minutes at 4°C after an hour. The particle was removed once the supernatants were removed. For the purpose of obtaining a faint yellow tint, 100 µL of this supernatant was mixed with 200 µL of 5,5-dithiobis-2-nitrobenzoic acid, 2.7 mL of 0.1 M phosphate buffer (pH 8), and 2.7 mL of this supernatant. It was discovered that the generated color was 412 nm using a UV-visible spectrophotometer (Perkin Elmer, USA). To calculate this, the molar extinct coefficient was used.[34]

Estimation of superoxide dismutase (SOD)

The Marklund-developed approach was used to assess the mobility of the SOD (Turf) protein for particular changes. The capacity of the Grass protein to prevent pyrogallol from autoxidizing serves as a gauge for the efficacy. Ten microliters of hippocampi homogenate were rapidly combined with nine hundred microliters of Tris-HCl cushion (1 mL of EDTA and one millimoles of Tris, at a pH of 8.2). Subsequently, 20 µL of room temperature pyrogallol (13 mm) was combined with the previously indicated response mixture. Spectrophotometry was used to measure changes in absorbance at 420 nm during a 30-second rest interval. The amount of material projected to restrict 50% of the total pyrogalol autooxidation per minute is known as one Grass action unit (U/min/mg protein).[35]

Estimation of the nitrite level

Hippocampal nitrite levels were not entirely restored by the Griess reagent. The Griess reagent, which contained 0.1%N-(-naphthyl ethylenediamine dihydrochloride, 1% sulphanilamide, and 5% phosphoric acid), was mixed 1:1 with the supernatant. The cakes were then cooked for 10 minutes at room temperature. A Perkin Elmer Lambda 20 spectrophotometer was one of the instruments used to measure the absorbance at 540 nm. The protein nitrite concentration was expressed in milligrams per milligram using the sodium nitrite average curve.[36]

Estimation of neuroinflammatory marker

Neuroprotective effects of baicalein in the model of the TNF-α alterations in lansoprazole-induced AD rat. The outcomes were evaluated by one-way ANOVA followed by the post hoc Tukey multiple comparison test and demonstrated that lansoprazole-induced AD decreased the level of TNF-α in the rat hippocampus and the whole brain. Data are obtainable as mean±SD (n=6).[37]

Gross morphological and histopathological examination

Following the completion of the experimental protocol, rats were sacrificed by deep anesthesia induction and intraperitoneal injection of sodium phenobarbitone at a

concentration of 270 mg/mL. After that, the rat brain is removed and stored at a pH of 7.4 in 4% paraformaldehyde for later. The rats' brains were sectioned into 5-mm thick sections after the 42-day experimental schedule, arranged coronally (from the cerebral cortex anterior to the posterior poles) on a white background and captured on camera using a high-end digital camera to allow clear visualization of the various regions of the brain and damaged areas.[38] After this, the hippocampus was separated and preserved in saline phosphate buffer. saline. Each slide in the histological investigation was kept in a variable ethanol concentration. The molds were created with the aid of a tissue processor. Hematoxylin and eosin staining was then used to help them stand out from each other and imaging was done with fluorescence microscope.[39]

Statistical analysis

The recorded values from several tests were statistically analyzed using Prism software (GraphPad Prism 8.0.1, San Diego, CA, USA). To assess the neurochemical results, Tukey's Post-Hoc Test for multiple comparisons and the One-Way ANOVA with repeated measures were used. Additionally, to evaluate differences in neurobehavioral evaluation across treatment groups, a two-way analysis of variance (ANOVA) was used, followed by a post-hoc Bonferroni's test. A graphic representation of the experimental data was created using mean and standard deviation (SD). The probability threshold of $p<0.01$ was established as the significance criterion for the findings.

Results

Body weight

On days 1, 7, 14, 21, 28, and 42 of the investigation, the weight changes of the six groups were compared. On the first day of the experiment, there were no significant differences in weight between the groups. The body weight of the animals that received continuous lansoprazole-induced lansoprazole decreased from day 1 to day 28. On days 21 and 28, rats in the lansoprazole-induced groups weighed significantly less than rats in the vehicle group treated, perse group-treated. from day 29-42, rats were treated with baicalein and rivastigmine, that is, lansoprazole+5 mg/kg baicalein and lansoprazole+10 mg/kg baicalein and lansoprazole+0.3 mg/kg rivastigmine, resulting in significant restoration in body weight compared to lansoprazole-induced rats. Upon completion of the protocol, it was determined that there was a dose-related increase in the restoration of body weight of rats in the 10 mg/kg treatment group compared to the lansoprazole+baicalein 5 mg/kg treatment group of lansoprazole+baicalein (Fig. 2, F value: 5.40).

Neuroprotective effects of baicalein on neurobehavior alterations in AD rats

Morris water maze

Lansoprazole-induced AD rats had significantly lower TSTQ levels compared to the vehicle group-treated, and those treated with the perse group. Furthermore, treatment with Baicalein by oral route at doses of 5 mg/kg significantly and 10 mg/kg improved TSTQ significantly in rodents with lansoprazole-induced AD. From day 29-42 rats were treated with baicalein and rivastigmine, that is, lansoprazole+5 mg/kg baicalein and lansoprazole+10 mg/kg baicalein and lansoprazole+0.3 mg/kg rivastigmine, which results in a significant increase in TSTQ compared to lansoprazole-induced rats. Upon completion of the protocol, it was determined that there was a dose-related increase in TSTQ of rats in the lansoprazole+baicalein 10 mg/kg treatment group of 10 mg/kg compared to the treatment group (Fig. 3, F value: 6.35).

Locomotor

Actophotometer measurements of impulsive locomotion were conducted on days 1, 7, 21, 28 and 42 of the experimental protocol. On the first and seventh day of the investigation, there was no noticeable distinction between the locomotions of the groups. On day 14, 21 and 28, AD-induced rats with AD exhibited significantly distinct locomotion compared to the vehicle group treated, per se group-treated from day 29-42 rats were treated with baicalein and rivastigmine, i.e., lansoprazole+5 mg/kg baicalein and lansoprazole+10 mg/kg baicalein and lansoprazole+0.3 mg/kg rivastigmine, which results of significant restoration in locomotor activity as compared to lansoprazole-induced rats. At the end of the protocol, it was determined that there was a dose-related increase in the locomotion of rats in the lansoprazole+bacalein 10 mg/kg treatment group compared to the lansoprazole+bacalein 5 mg/kg treatment group (Fig. 4, F value: 1.8).

EPM

On days 1, 7, 14, 21, 28, and 42, inactivity (immobility) was assessed in rats against baicalein. On days 14, 21 and 28 the lansoprazole group exhibited distressing behavior and had a significantly prolonged period of immobility increased as compared to the vehicle group treated and perse group-treated. from day 29–42 rats were treated with baicalein and rivastigmine, that is, lansoprazole+5 mg/kg baicalein and lansoprazole+10 mg/kg baicalein and lansoprazole+0.3 mg/kg rivastigmine, which results in significant restoration in immobility time compared to lansoprazole-induced rats. Upon completion of the protocol, it was determined that there was a dose-related increase in immobility of rats in the lansoprazole+bacalein 10 mg/kg treatment group as compared to the

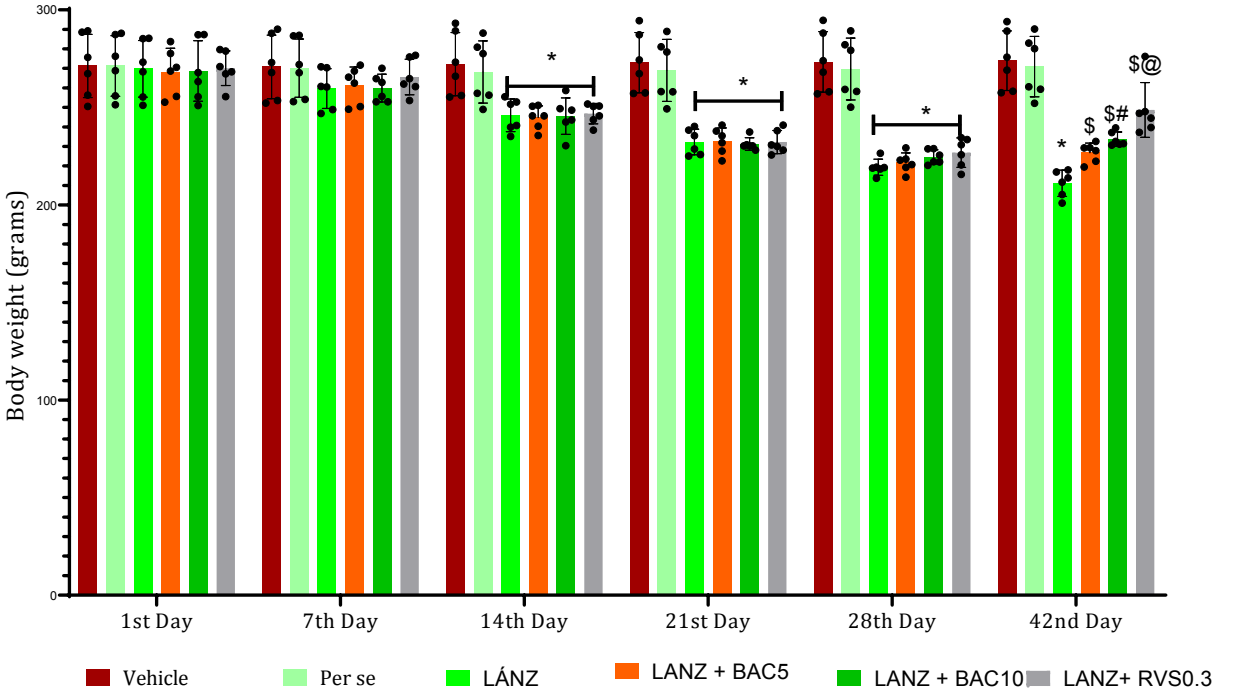

Fig. 2. Baicalein improves body weight alterations in AD rats, the statistical data analysis presented by two-way ANOVA using the Bonferroni post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; $# versus lansoprazole (LANZ), lansoprazole+bacalein (BAC) 10; $ versus lansoprazole, lansoprazole+bacalein 5, lansoprazole+bacalein 10 and lansoprazole+RVS 0.3; $@ versus lansoprazole, lansoprazole+bacalein 5 and lansoprazole+bacalein 10

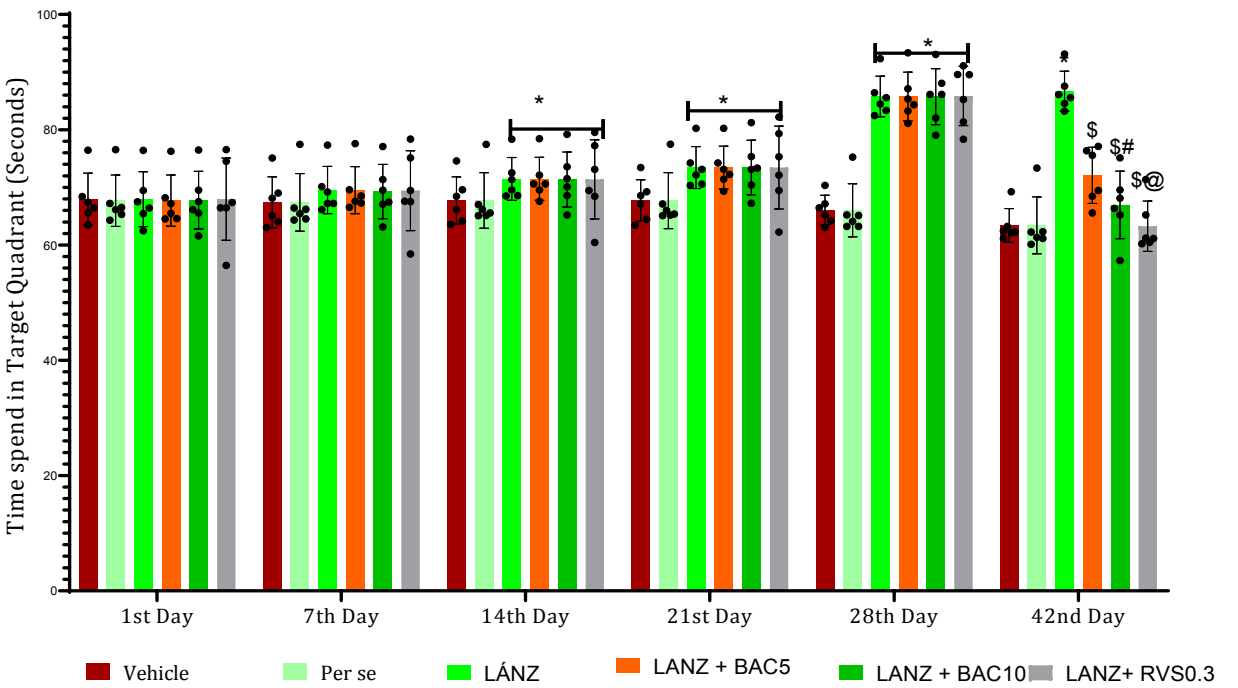

Fig. 3. Baicalein improves memory and cognition in AD rats induced by experimental lansoprazole, statistical data analysis presented by two-way ANOVA employing the Bonferroni post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+baicalein (BAC) 10; $ versus lansoprazole, lansoprazole+baicalein 5, lansoprazole+baicalein 10 and lansoprazole+RVS 0.3; $@ versus lansoprazole, lansoprazole+baicalein 5 and lansoprazole+baicalein 10

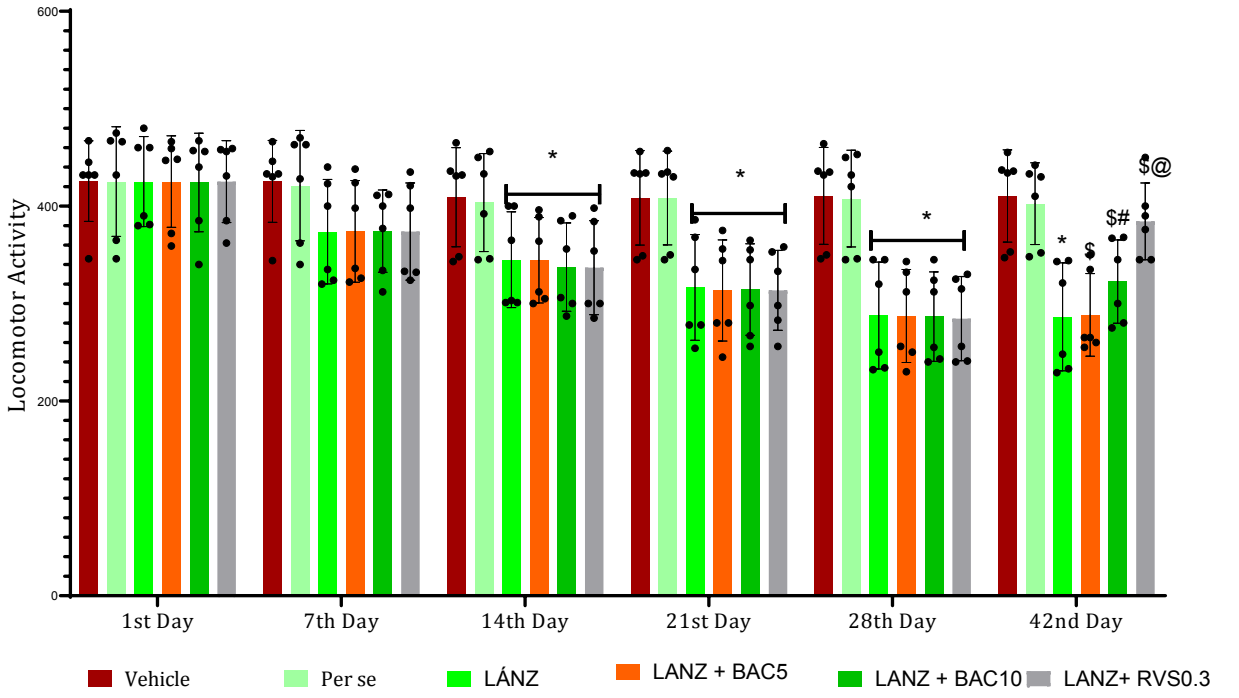

Fig. 4. Baicalein improves locomotion behavior in AD rats induced by experimental lansoprazole, statistical data analysis presented by two-way ANOVA employing the Bonferroni post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+baicalein (BAC) 10; $ versus lansoprazole, lansoprazole+baicalein 5, lansoprazole+baicalein 10 and lansoprazole+RVS 0.3; $@ versus lansoprazole, lansoprazole+baicalein 5 and lansoprazole+baicalein 10

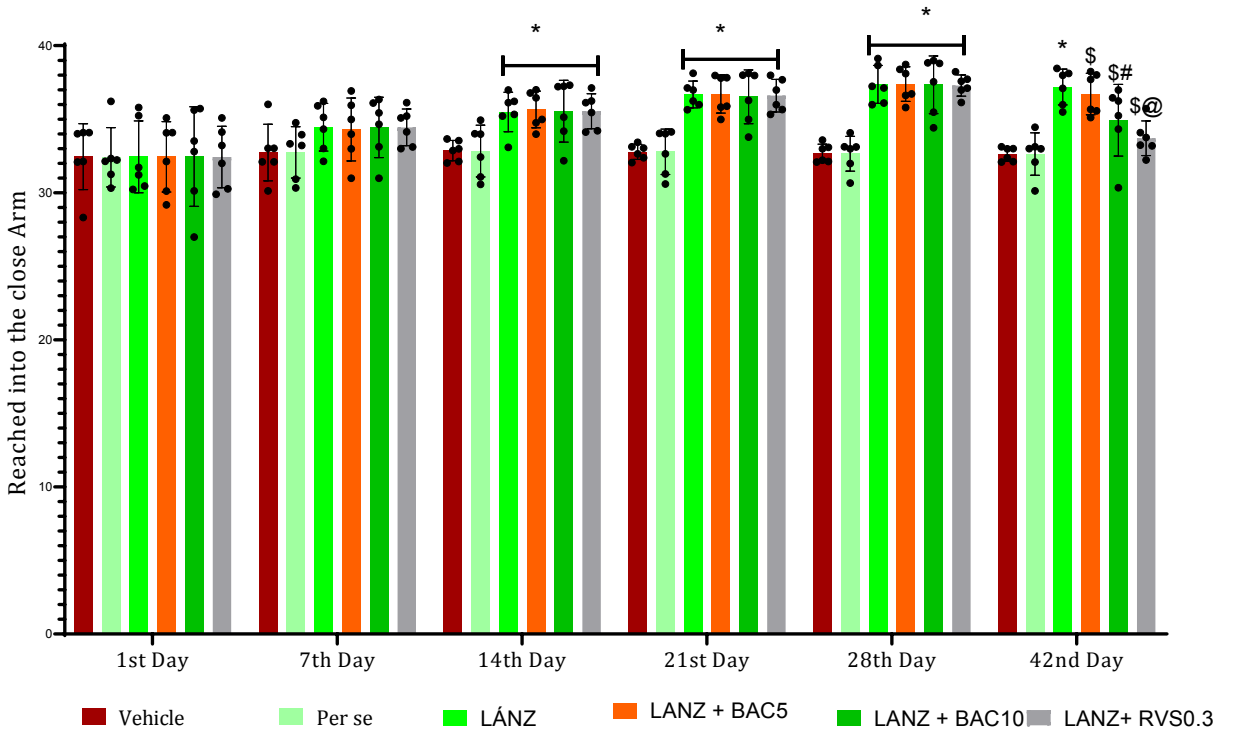

Fig. 5. Baicalein reduces immobility time in AD rats induced by experimental lansoprazole, statistical data analysis presented by two-way ANOVA employing post-hoc test Bonferroni, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+baicalein (BAC) 10; $ versus lansoprazole, lansoprazole+baicalein 5, lansoprazole+baicalein 10 and lansoprazole+RVS 0.3; $@ versus lansoprazole, lansoprazole+baicalein 5 and lansoprazole+baicalein 10

lansoprazole+baicalein 5 mg/kg treatment group (Fig. 5, F value: 2.1).

Neuroprotective effects of baicalein on neurotransmitter alterations in lansoprazole-induced AD rats

Figure 6 represents the result of baicalein in the AChE in lansoprazole-induced AD rats. The outcomes were evaluated by one-way ANOVA followed by the post hoc Tukey Kramer multiple comparisons test and showed that baicalein decreased the level of AChE in the rat hippocampus. Data are obtainable as mean ± SD (n=6). The signs indicate a difference compared to the Lansoprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10mg/kg) were administered to rats from day 29 to 42nd days (F value: 4.11).

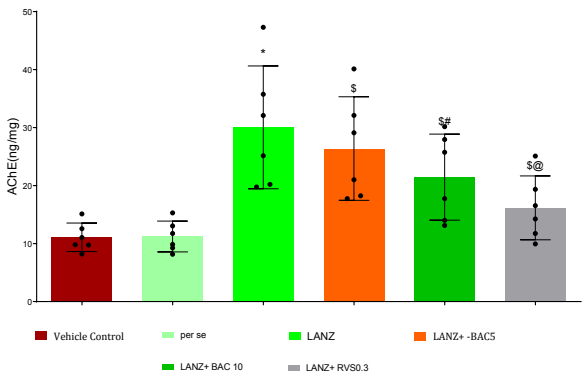

Fig. 6. Baicalein decreases the AchE level in AD rats induced by experimental lansoprazole, statistical data analysis presented by one-way ANOVA employing post-hoc test Tukey, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+baicalein (BAC) 10; $ versus lansoprazole, lansoprazole+baicalein 5, lansoprazole+baicalein 10 and lansoprazole+rvs 0.3; $@ versus lansoprazole, lansoprazole+Baicalein 5 and lansoprazole+baicalein 10

Neuroprotective effects of baicalein on neurochemical alterations in lansoprazole-induced AD rats

Neuroprotective effects of baicalein on LPO alterations in lansoprazole-induced AD rats

Figure 7 represents the outcome of baicalein on the level of LPO in the Lansoprazole-induced AD rats. The results were evaluated by one-way analysis of variance followed by the Tukey multiple comparison post hoc test and demonstrated that baicalein decreased the level of LPO in the rat hippocampus. Data are obtainable as mean±SD (n=6). Signs indicate a difference compared to the lansoprazole-induced AD group. Two different doses of baicalein (5mg/kg and 10mg/kg) were administered to rats from 29 to 42nd days (F value: 0.95).

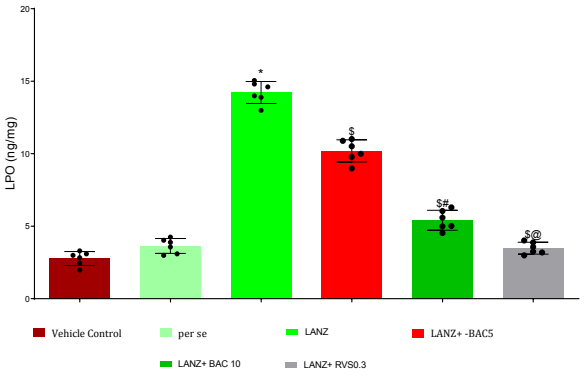

Fig. 7. Baicalein decreases the level of LPO in experimental lansoprazole-induced AD rats, the statistical data analysis presented by one-way ANOVA using the Tukey post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; $# versus lansoprazole (LANZ), lansoprazole+Baicalein (BAC) 10; $ versus lansoprazole, lansoprazole+Baicalein 5, lansoprazole+baicalein 10 and lansoprazole+rvs 0.3; $@ versus lansoprazole, lansoprazole+Baicalein 5 and lansoprazole+baicalein 10

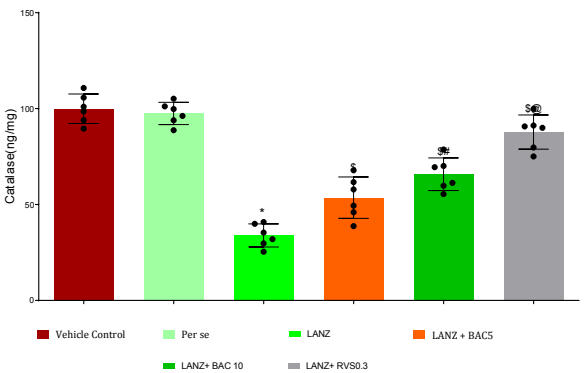

Fig. 8. Baicalein restores the catalase level in experimental lansoprazole-induced AD rats, the statistical data analysis presented by one-way ANOVA employing Tukey post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+bacalein (BAC) 10; $ versus lansoprazole, lansoprazole+bacalein 5, lansoprazole+baicalein 10 and lansoprazole+rvs 0.3; $@ versus lansoprazole, lansoprazole+baicalein 5 and lansoprazole+baicalein 10

Neuroprotective effects of baicalein on CAT alterations in lansoprazole-induced AD rats

Figure 8 represents the outcome of baicalein on CAT level in lansoprazole-induced AD rats. The results were evaluated by one-way analysis of variance (ANOVA) followed by the post hoc Tukey multiple comparisons test and showed that baicalein decreased the level of CAT in the rat hippocampus. Data are obtainable as mean±SD (n= 6). Signs indicate a difference compared to the lan-

soprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10 mg/kg) were administered to rats from day 29 to 42nd days (F value: 63.43).

Neuroprotective effects of baicalein on GSH alterations in lansoprazole-induced AD rats

Figure 9 represents the result of baicalein on GSH level in lansoprazole-induced AD rats. The outcomes were evaluated by one-way ANOVA followed by the post hoc Tukey multiple comparison test and demonstrated that baicalein decreased the level of GSH in the rat hippocampus. Data are obtainable as mean±SD (n=6). Signs indicate a difference compared to the lansoprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10 mg/kg) were administered to rats from 29 to 42nd days (F value: 2.12).

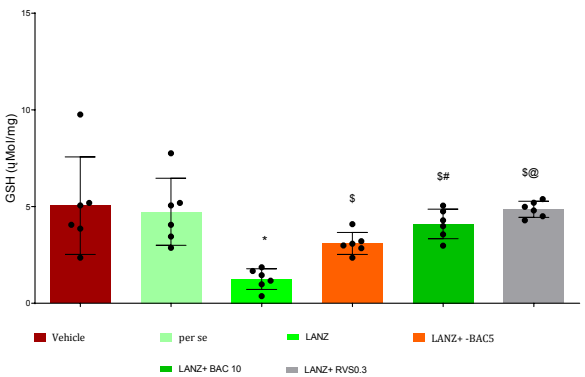

Fig. 9. Baicalein restores the level of GSH in experimental lansoprazole-induced AD rats experimental, the statistical data analysis presented by one-way analysis of variance (ANOVA) employing Tukey post hoc test, values presented as mean±SD (p<0.01); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ), lansoprazole+bailein (BAC) 10; $ versus lansoprazole, lansoprazole+baicalein 5, lansoprazole+baicalein 10 and lansoprazole+rvs 0.3; $@ versus lansoprazole, lansoprazole+baicalein 5 and lansoprazole+baicalein 10

Neuroprotective effects of baicalein on SOD alterations in lansoprazole-induced AD rats

Figure 10 represents the outcome of baicalein on SOD level in the lansoprazole-induced AD rats. The results were evaluated by one-way ANOVA followed by the post hoc Tukey multiple comparison test and demonstrated that baicalein decreased the level of SOD in the rat hippocampus. Data are obtainable as mean±SD (n=6). Signs indicate a difference compared to the lansoprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10 mg/kg) were administered to rats from 29 to 42nd days (F value: 0.38).

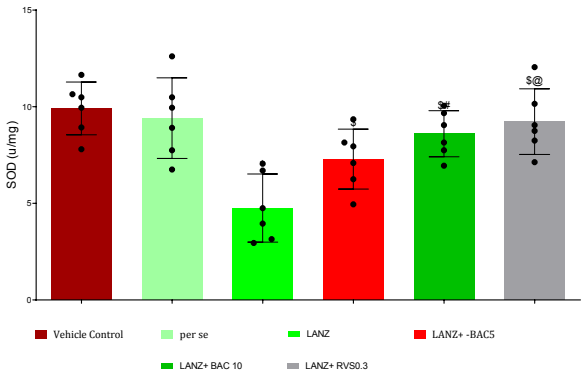

Fig. 10. Baicalein restores the level of SOD in experimental lansoprazole-induced AD rats, the statistical data analysis presented by one-way ANOVA employing the Tukey post hoc test, values presented as mean±SD (p<0.01); [n=six Wistar rats in each experimental group], * versus vehicle and perse; $# versus lansoprazole (LANZ); $ versus lansoprazole, lansoprazole+bacalein (BAC) 5 and lansoprazole+baicalein 10

Neuroprotective effects of baicalein on nitrite alterations in lansoprazole-induced AD rats

Figure 11 represents the outcome of baicalein on the level of nitrite in lansoprazole-induced AD rats induced by lansoprazole. The results were evaluated by one-way analysis of variance followed by the Tukey multiple comparisons test and demonstrated that baicalein decreased the level of nitrite in the rat hippocampus. Data are obtainable as mean±SD (n=6). Signs indicate a difference compared to the lansoprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10 mg/kg) were administered to rats from 29 to 42nd days (F value: 2.20).

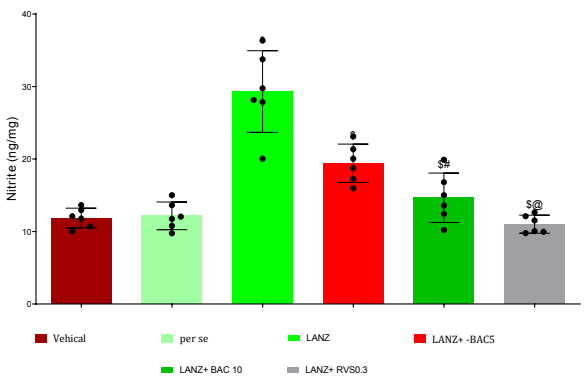

Fig. 11. Baicalein decreases the level of nitrite in experimental lansoprazole-induced AD rats, the statistical data analysis presented by one-way ANOVA using Tukey post hoc test, values presented as mean±SD (p<0.01); [n=six Wistar rats in each experimental group], * versus vehicle and per se; # versus lansoprazole (LANZ); $ versus lansoprazole, lansoprazole+baicalein (BAC) 5 and lansoprazole+baicalein 10

Neuroprotective effects of baicalein on neuroinflammatory markers in AD rats

Figure 12 represents the outcome of baicalein on the TNF- level in AD rats induced AD rats. The results were evaluated by one-way analysis of variance followed by the post hoc Tukey multiple comparisons test and demonstrated that baicalein decreased the level of TNF-α in the rat hippocampus. Data are obtainable as mean±SD (n=6). The signs indicate differences compared to the lansoprazole-induced AD group. Two different doses of baicalein (5 mg/kg and 10 mg/kg) were administered to the rats from the 29th to 42nd day (F value: 3.00).

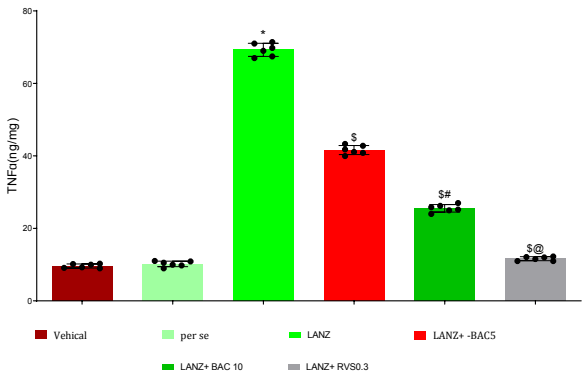

Fig. 12. Baicalein decreases TNF-α in AD rats induced by experimental lansoprazole, statistical data analysis presented by one-way ANOVA employing post-hoc test Tukey, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], * versus vehicle and perse; $# versus lansoprazole (LANZ); $ versus lansoprazole, lansoprazole+baicalein (BAC) 5 and lansoprazole+baicalein 10

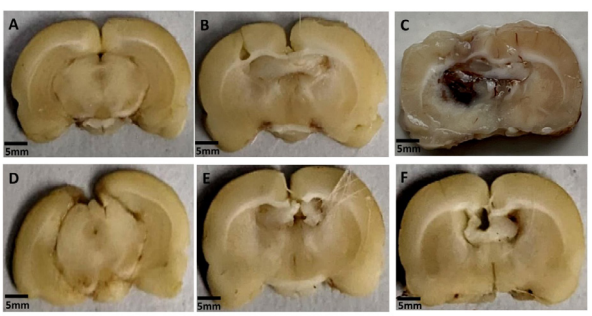

Fig. 13. Baicalein effects on gross pathological deficiencies in experimental lansoprazole-induced AD rats, the statistical data analysis presented by one-way ANOVA using Tukey post hoc test, values presented as mean±SD ($p<0.01$); [n=six Wistar rats in each experimental group], A: vehicle, B: per se, C: lansoprazole, D: lansoprazole+baicalein 5, E: lansoprazole+baicalein 10, F: lansoprazole+rsv 0.3 mg/kg

Neuroprotective effects of baicalein on gross pathological deficits in lansoprazole-induced AD rats

Figure 13 shows the gross morphological changes in the rat brain from day 1 to day 42 in different groups, which shows degeneration in the lansoprazole treated group as compared to vehicle control and perse control group. In the following Figures D and E show restored degeneration in the rat brain compared to the lansoprazole-induced AD rat brain, which shows potential effects of baicalein on degeneration caused by toxin-induced rats. Figure F shows standard drug-treated rats as compared to toxin control and baicalein-treated groups.

Neuroprotective effects of baicalein on histopathological deficits in lansoprazole-induced AD rats

Upon examination of each slide under a microscope, it was noted that the group treated with lansoprazole exhibited more pronounced signs of plaque formation and irregular tangle formation compared to the control group. In addition to reducing the number of unfolded tangles inside the brain, baicalein, as well as the entire brain, which was employed as a treatment group, showed some improvement in the hippocampal region (Fig. 14).

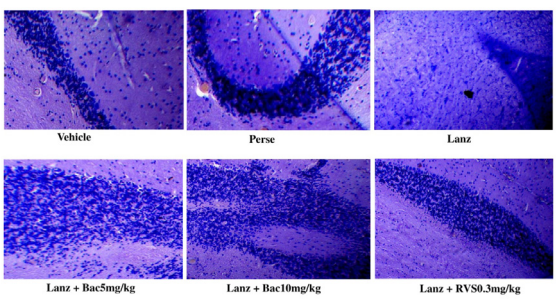

Fig. 14. Effects of baicalein on histopathological deficits in AD rats

Discussion

AD poses a major and increasing challenge within the global healthcare system, affecting cognitive function, memory, and behavior progressively.[40] The main pathological features of AD are extracellular amyloid-beta plaques and intracellular hyperphosphorylated tau-associated neurofibrillary tangles NFT.[41] Oxidative stress, neuroinflammation, and synaptic dysfunction are exceedingly involved in the pathogenic processes leading to the pattern of progressive neuronal loss seen in AD.[42] Conventional medical management is inadequate at preventing, or reversing the progression of this disease and as a result, new therapeutic pathways need to be explored.[43]

Recently, natural compounds like flavonoids have gained attention due to their potential ability to protect neurons. Baicalein, a flavonoid obtained from the roots of *S. baicalensis*, has emerged as one of the effective

neuroprotective agents in several models of neurodegenerative diseases, which is attributed to its antioxidant, anti-inflammatory, and other beneficial effects.[44] A model of this type is the use of lansoprazole, a proton pump inhibitor commonly used to treat gastrointestinal diseases, but in experimental animal studies linked with worsening of AD-like pathology.[45] If anything, Lansoprazole treatment has been shown to drive amyloid-beta accumulation and tau hyperphosphorylation mimicking two of the major features seen in AD pathology, providing a consistent model that is amenable to the evaluation of potential therapeutic interventions.[46]

The present study was undertaken to determine the neuroprotective property of baicalein in lansoprazole-induced AD model in Wistar rats. From day 29 to 42nd of the study, all the animals were administered two different doses of baicalein i.e. 5mg/kg and 10 mg / kg, and standard drug used for Alzheimer's disease, ie rivastigmine 0.3mg/kg for 14 days. The study used 28 days for the introduction of a lansoprazole, whereas the introduction of Baicalein lasted 14 days. Cognitive function was periodically assessed during the study by performing behavioral assessments such as the Morris water maze, the actophotometer, and EPM.[47] The biochemical evaluations compared brain tissue of treatment rats with controls and focused on molecular and cellular markers of oxidative stress, neuroinflammation, and neurodegeneration.[48] During the lansoprazole induction phase, rats showed clear deficits in spatial learning and memory retention indicated by longer escape latency from the Morris water maze and lower discrimination index in the novel object recognition task. These behavioral deficits were accompanied by biochemical changes consistent with neurodegeneration, particularly ROS and lipid peroxidation products as well as pro-inflammatory cytokines in the brain of rats treated with lansoprazole[49] All these facts confirm the negative influence of lansoprazole on neuron functioning and prove the model to successfully depict AD-like pathological characteristics.

In contrast, treatment with baicalein throughout the 14-day intervention period proved to be significantly more beneficial for neuroprotection during the 14-day intervention period. Rats supplemented with baicalein had better outcomes in both of the behavioral tests compared to rats who received lansoprazole alone. For example, baicalein supplementation decreased the escape latency in the Morris water maze, which implied improved spatial learning and memory retention.[50] Similarly, in the object recognition paradigm, baicalein-treated rats achieved better discrimination indices, thus indicating maintained recognition memory and cognitive faculties as well as processing. Aggressive behavioral assessments led to the corresponding decrease in markers of oxidative stress and neuroinflammatory mediators in the brains of baicalein-treated rats in comparison to controls.

The potent neuroprotective properties of baicalein were more specifically observed at the molecular level through mechanistic studies. Free radical scavenging and lipid-peroxidation inhibited baicalein extended to the neuronal membranes, demonstrating baicalein was an antioxidant. Baicalein was able to shield such neurons from oxidative damage and mitochondrial dysfunction attributed to cell-based neurodegenerative processes, particularly in AD by alleviating the oxidative stress. Furthermore, baicalein suppressed the release of TNF-α. As a result, the neuronal integrity and synaptic functions of treated rats, compared to control rats, were preserved in part by these anti-inflammatory effects.

Considering the fact that baicalein has so many positive effects for the preservation of neurons, it seems quite promising in the treatment of AD. In addition to antioxidant and anti-inflammatory actions, baicalein is believed to modulate further signal transduction pathways that promote the survival of neurons and the plasticity of synapses. In addition, baicalein may also alter the expression and processing of APP, thereby decreasing amyloid-beta peptide synthesis and deposition in the brains of AD patients. These molecular mechanisms support the multifaceted effects of baicalein in the treatment of AD by ameliorating the pathological conditions that compromise cognitive function.

Conclusion

The research investigated the neuroprotective effects of baicalein in wistar rats with AD induced by lansoprazole, a proton pump inhibitor associated with an increased risk of AD. Baicalein, a flavonoid of *S. baicalensis*, is recognized for its antioxidant and anti-inflammatory properties. In this study, the rats were divided into groups and treated with lansoprazole to induce AD, followed by the administration of baicalein. Cognitive functions, oxidative stress markers, and neuronal integrity were evaluated using behavioral tests, biochemical assays, and histopathological analyzes. The findings indicated that baicalein markedly improved cognitive performance, improving memory and learning capabilities on maze tests. Additionally, it reduced oxidative stress markers, such as MDA, and elevated levels of antioxidant enzyme levels like SOD and catalase. Histopathological analysis revealed a reduction in amyloid-beta plaques and neuronal loss in the hippocampus, indicating a neuroprotective effect. These results suggest that baicalein offers neuroprotection through its potent antioxidant properties, which reduce amyloid beta aggregation and help preserve neuronal integrity. Consequently, baicalein shows promise as a therapeutic agent for mitigating the symptoms and progression of Alzheimer's disease, particularly in cases triggered by external fac-

tors such as lansoprazole. More studies, including clinical trials, are necessary to validate these protective effects in humans and to elucidate the underlying molecular mechanisms.

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Declarations

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Author contributions

Conceptualization, A.G. and P.N.T.; Methodology, A.G.; Software, F.G.; Validation, P.N.T., A.G. and S.N.T.; Formal Analysis, F.G.; Investigation, P.S.; Resources, A.G.; Data Curation, P.S.; Writing – Original Draft Preparation, P.S.; Writing – Review & Editing, F.G.; Visualization, S.N.R.; Supervision, P.N.T.; Project Administration, P.S.

Conflicts of interest

All authors declared the absence of any personal conflicts related to this work.

Data availability

Data will be made available according to the Journal policy.

Ethics approval

The protocol number is IAEC/MIET/CPCSEA/Meeting No: 04/2023/Protocol No.192.

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ORIGINAL PAPER

Comparison of clinical profile and outcomes in patients with carbapenem resistant and carbapenem sensitive gram-negative bacteraemia

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ABSTRACT

Introduction and aim. Carbapenem-resistant Gram-negative bacteraemia (CR-GNB), an emerging public health concern due to limited treatment options and high mortality rates. Carbapenems, face reduced efficacy against resistant strains, posing a significant challenge. The aim was to compare clinical profiles and outcomes between CR-GNB and carbapenem-sensitive (CS-GNB) and to identify factors influencing mortality among these patients.

Material and methods. This prospective study was conducted at the tertiary care teaching hospital, enrolling 115 patients with GNB (55 CR-GNB and 60 CS-GNB). Following institutional approval and informed consent, patients underwent standardized testing (blood culture and susceptibility testing) with the VITEK method.

Results. CR-GNB patients had significantly longer hospital stays (12.88 vs. 8.87 days, p=0.001), higher ICU admissions (90% vs 49.3%), and prolonged antibiotic use (8.7 vs 6.04 days, p=0.001). Pneumonia was more prevalent in CR-GNB (42.5%) while UTIs dominated in CS-GNB cases (64%). Kaplan-Meier analysis showed increased mortality risk in CR-GNB, with hazard ratios of 1.82 (day-14) and 2.12 (day-28).

Conclusion. Thus, in our study CR-GNB posed a significant hazard for mortality risk. Thus, early identification, stringent infection control measures, and antimicrobial stewardship are crucial and to develop effective treatment strategies tailored to high-risk populations can enhance patient survival and limit the resistance.

Keywords. antimicrobial stewardship, beta-lactam antibiotics, carbapenem, gram-negative bacteraemia

Introduction

Gram-negative bacteremia (GNB) is a formidable clinical challenge, often associated with severe systemic infections requiring prompt intervention due to the inherent risks of elevated mortality and morbidity.[1] Recently, there has been a discernible shift in the bloodstream infections epidemiology, with GN bacteria, particularly *Enterobacteriaceae* family, eclipsing Gram-positive (GP) counterparts.[1–3] This transition coincides with an alarming rise in multidrug-resistant (MDR) GN infections, posing a global health threat.[2–4]

Carbapenems, a key class of beta-lactam antibiotics, have long been regarded as the cornerstone of treatment for a diverse spectrum of GNB infections.[5] These antibiotics are classified into group-1 (ertapenem) which exhibits limited activity against non-fermentative

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GNB (NF-GNB), and group-2 (imipenem, meropenem, doripenem) were active against NF-GNB, particularly in nosocomial infections.[5–7] Due to their broad-spectrum activity against both GP and GN bacteria, they are last-resort agents for MDR infections.[8–10] However, rising incidence of carbapenem resistance (CR), mediated by the intricate web of mechanisms by carbapenemases enzymes such as KPC, NDM-1, and OXA48, as well as efflux pump mechanisms, has significantly compromised their clinical utility.[11,12]

Among CR-GNB particularly *Enterobacteriaceae* (CRE), *Pseudomonas aeruginosa* (CRPA), and *Acinetobacter baumannii* (CRAB), which evolved into a critical concern, in nosocomial bloodstream infections.[13] The increasing prevalence of CR-GNB isolates has prompted global authoritative bodies including the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), emphasizing the imperative need for surveillance, antimicrobial stewardship, and novel therapeutic approaches.[9] Thus, responding to the global escalation of MDR among GN bacilli, there exists an urgent need for meticulous examination of in-vitro susceptibilities to carbapenems.[1,3,14] Given the geographic variability in CR rates, disparities observed between Western and Asian countries, including India, emphasizing the need for region-specific investigations for effective containment strategies.[3,14,15]

Despite ongoing advancement in antimicrobial therapy, the clinical management of CR-GNB infections remains challenging. Understanding the clinical characteristics and outcomes of patients with CR-GNB is essential to guiding treatment decisions and optimizing therapeutic strategies on a global scale.[2,11,16,17] However, exciting literature on the clinical profiles and treatment outcomes of patients with CR-GNB remains limited, despite its critical relevance to patient care, and understanding it can contribute nuanced insights that extend beyond the healthcare setting.[2,11,18] In essence, this will seek to illuminate not only the immediate clinical challenges posed by CR but also to contribute to the broader discourse on global antimicrobial resistance strategies.[6]

The rising incidence of CR-GNB underscores the need for further research to bridge current knowledge gaps and develop effective treatment algorithms,[3,9,14] With this background, the present study aims to compare clinical profiles, microbiological spectrum, treatment approaches, and outcome of patients with CR-GNB and carbapenem sensitive (CS-GNB). Additionally, this study seeks to identify factors influencing mortality among these patients. With the WHO designating CRAB, CRPA, and CRE as critical priority pathogens,[3,19] this study aligns with global efforts to combat antimicrobial resistance. Furthermore, it evaluates group-2 carbapenems' efficacy against *Enterobacteriaceae* resistant to group-1 agents.

Aim

To compare clinical profiles, microbiological spectrum, treatment approaches, and outcome of patients with CR-GNB and CS-GNB.

Material and methods

It was a hospital-based prospective analytical cross-sectional study was conducted by General Medicine department at tertiary-care teaching hospital, in Puducherry from the period of 2022 to 2024. Following Institutional Human Ethics Committee (IHEC) approval (MG-MCRI/Res/01/2021/38/IHEC/78), patients admitted to various departments including wards, intensive and cardiac care units (ICU and CCU), and cardio-thoracic vascular surgery units (CTVS), with positive blood cultures presented with CR-GNB and CS-GNB were considered for the study.

Eligibility criteria for the study participants include patients aged more than 18 years diagnosed with suspected sepsis confirmed by positive blood cultures with CR-GNB and CS-GNB by antibiotic susceptibility testing and who completed at least seven days of treatment from day one of the admission. Pregnant women, those diagnosed with polymicrobial bacteraemia, and cases deemed contaminants were excluded from the study.

Considering the prevalence of the CR-GNB especially Enterobacteriaceae was 18.5% in a study done by Thomas et al, in India.[20] Taking the confidence level at 95%, power at 80%, and 7 as precision, the sample size was calculated to be 115. Using the formula,

$$n = \frac{Z_{\alpha/2}{}^2 * p\,(1-p)}{d^2} = \frac{1.96^2 \times 0.18\,(1-0.18)}{0.07^2} = 113.4$$

(rounded to the nearest higher figure of 115) ($Z_{\alpha/2}$ – 1.96; prevalence (p) – 0.18; precision (d)) – 0.07) the sample size calculated. Consecutive sampling technique was used to include all patients with inclusion criteria until the desired sample size was achieved.

Data were collected using a semi-structured proforma which included demographic details along with relevant clinical and laboratory data. Patients admitted with suspected sepsis underwent routine investigations including blood culture and antibiotic susceptibility testing (AST). Initial treatment for suspected sepsis was empirical, with definitive treatment based on blood culture results with either CR-GNB and CS-GNB.

Blood samples were collected aseptically from patients with suspected sepsis and inoculated into standard aerobic and anaerobic blood culture bottles, which were then incubated in an automated system (BacT/ALERT) for continuous monitoring. Once the cultures flagged positive, samples were sub-cultured onto appropriate solid media (MacConkey agar) for isolation. Following isolation, bacterial identification and AST

were performed using the VITEK 2 compact system (bioMérieux, France). Identification of GNB was done using GN-ID cards and AST was conducted using AST-GN cards (GN83) according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[21]

Data analysis

The collected data were entered in Microsoft Excel and analyzed using Epi-info (ver. 7.2.2.6; Centers for Disease Control and Prevention, Atlanta, GA, USA, and World Health Organization) and IBM Statistical Package for Social Sciences for Windows (SPSS Inc. version 23.0, Chicago, IL, USA). Normality of the variables were measured using Shapiro-Wilk test and Q-Q plot. The data was presented in the form of numbers and percentages for qualitative variables and mean ± standard deviation (SD)/median with interquartile range (IQR) for quantitative variables. Appropriate tests of significance i.e., Chi-square test or Fisher exact test, and Student t-test was used to test the significance. Values of p <0.05 was statistically significance. Kaplan-Meier survival analysis was used to evaluate the mortality outcomes.

Results

In this study, among 115 patients, 40 (34.8%) were diagnosed with CR-GNB, while 75 (65.2%) had CS-GNB. The demographic details and comorbidity status of the study participants were presented in

Table 1. The odds of developing CR-GNB among the patients with diabetes and hypertension were 1.0 and 2.24 times higher when compared to the patients with no comorbidity*

Variables		CR-GNB (n=40)	CS-GNB (n=75)	p
Age (in years)		57.45±17.26	56.43±11.65	0.730[a]
Gender	Female	11 (27.5)	33 (44.0)	0.080[b]
	Male	29 (72.5)	42 (56.0)	
Diabetes mellitus	Present	24 (60.0)	11 (60.0)	1.000[b]
	Absent	16 (40.0)	30 (40.0)	
Hypertension	Present	15 (37.5)	43 (57.3)	0.043[b]
	Absent	25 (62.5)	32 (42.7)	
CVA	Present	9 (22.5)	17 (22.7)	0.980[b]
	Absent	31 (77.5)	58 (77.3)	
CKD	Present	7 (17.5)	11 (14.7)	0.690[b]
	Absent	33 (82.5)	64 (85.3)	
Other comorbidities	Present	7 (17.5)	7 (9.3)	0.200[b]
	Absent	33 (82.5)	68 (90.7)	

* [a] – independent t-test; [b] – Pearson's Chi-square test, categorical variables were presented in number (percentage); while continuous variables were presented in mean± SD, CS – carbapenem sensitive, CR – carbapenem resistant, GNB – gram negative bacterium, CVA – cerebrovascular accident, CKD – chronic kidney disease

The mean SOFA score was significantly elevated in the CR-GNB group (8.1 ± 2.4) when compared to CS-GNB group (6.5 ± 2.0), indicating greater severity in CR-GNB cases. The outcome of the patients admitted in ICU and ward were presented in Table 2. The odds of admitting the patients in ICU with CR-GNB was 9.24 times higher than the CS-GNB in our study.

Table 2. Outcomes of the patients admitted in the hospital (n=115)*

Variables		CR-GNB (n=40)	CS-GNB (n=75)	p
Admission	ICU	36 (90.0)	37 (49.3)	<0.01
	Ward	4 (10.0)	38 (50.7)	
Indwelling catheter	No	8 (20.0)	40 (53.3)	<0.01
	Yes	32 (80.0)	35 (46.7)	
Mortality in 14 days	Alive	21 (52.5)	64 (85.3)	<0.01
	Death	19 (47.5)	11 (14.7)	
Mortality in 28 days	Alive	9 (22.5)	63 (84.0)	<0.01
	Death	31 (77.5)	12 (16.0)	

* Pearson's Chi-square test, CS – carbapenem sensitive; CR – carbapenem resistant; GNB – gram negative bacterium; ICU – intensive care units

Table 3. Disease distribution in patients with CR- and CS-GNB (n=115)

Disease	CR-GNB (n=40) n (%)	CS-GNB (n=75) n (%)
Abdominal wall cellulitis	1 (2.5)	0 (0)
Catheter related blood stream infection (CRBSI)	2 (5.0)	3 (4.0)
Cellulitis	4 (10.0)	1 (1.3)
Cholecystitis	0 (0)	1 (1.3)
Cholelithiasis	0 (0)	1 (1.3)
Colangitis	0 (0)	1 (1.3)
Corrosive Poisoning	0 (0)	1 (1.3)
Diabetic foot ulcer	1 (2.5)	0 (0)
Infective endocarditis (IE)	1 (2.5)	0 (0)
Melioidosis	1 (2.5)	1 (1.3)
Necrotizing fascitis	2 (5.0)	2 (2.7)
Peritonitis	4 (10.0)	0 (0)
Pneumonia	17 (42.5)	11 (14.7)
Spontaneous bacterial peritonitis (SBP)	1 (2.5)	1 (1.3)
Typhoid	0 (0)	3 (4.0)
Urinary tract infection (UTI)	6 (15.0)	48 (64.0)
UTI+ pneumonia	0 (0)	1 (1.3)

In Table 3, distribution of the disease for both CR- and CS-GNB were presented, where 42.5% and 15% of the patients in CR-GNB and 14.7% and 64% of patients in CS-GNB were presented with pneumonia and urinary tract infection (UTI). *Klebsiella* (37.5%), and *E. coli* (25%) were the most common pathogens presented in CR-GNB group, while in CS-GNB E. coli (56%) was most common followed by *Pseudomonas* (14.7%) (Fig. 1). In CS-GNB group the duration of antibiotics was 6.04±2.57 days while in CR-GNB group it was 8.7±4.28 days and found to be statistically significant. Similarly, the duration of hospital stay was found to be 8.87±2.29 days and 12.88±5.67 days in CS-GNB and CR-GNB and

were statistically significant (p 0.001) shows that patients with CR-GNB higher hospital stay when compared to CS-GNB patients. The type of antibiotics used were presented in Table 4.

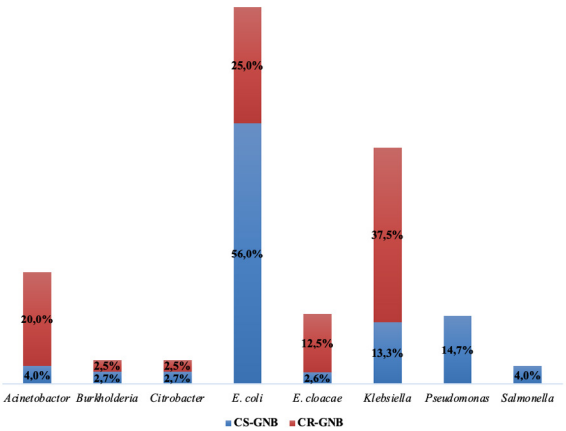

Fig. 1. Distribution of pathogens in patients with CR-GNB and CS-GNB

Table 4. Antibiotics used in patients with CR- and CS-GNB after the culture report (n=115)

Antibiotics	CR-GNB (n=40) n (%)	CS-GNB (n=75) n (%)
Cefoperazone sulbactam and vancomycin	1 (2.5)	0 (0)
Meropenem	1 (2.5)	22
Amikacin	1 (2.5)	0 (0)
Cefixime	0 (0)	1 (1.3)
Cefoperazone sulbactam	0 (0)	15 (20.0)
Cefotaxime	0 (0)	1 (1.3)
Ceftazidime	0 (0)	1 (1.3)
Ceftazidime avibactam	6 (15.0)	1 (1.3)
Ceftazidime avibactam, aztreonam	1 (2.5)	0 (0)
Ceftazidime avibactam polymyxin B	1 (2.5)	0 (0)
Ceftriaxone	0 (0)	11 (14.7)
Ciprofloxacin	0 (0)	2 (2.7)
Colistin meropenem	1 (2.5)	1 (1.3)
Colistin teicoplanin	1 (2.5)	0 (0)
Cotrimoxazole	0 (0)	1 (1.3)
Norfloxacin	0 (0)	3 (4.0)
Piperacillin tazobactam	2 (5.0)	15 (20.0)
Polymyxin B	17 (42.5)	0 (0)
Polymyxin B teicoplanin	5 (12.5)	0 (0)
Polymyxin B tigecycline	1 (2.5)	0 (0)
Teicoplanin	1 (2.5)	1 (1.3)
Teicoplanin linezolid	1 (2.5)	0 (0)

Survival of the patients on day 14 (Fig. 2) and day 28 (Fig. 3) were plotted using the Kaplan-Meier curve among CR-GNB and CS-GNB. It showed a clear trend of poorer survival outcomes among patients with CR-GNB compared to CS-GNB. On day 14, hazard ratio (HR) was 1.82 (0.85–3.91, p=0.122) indicating that CR-GNB patients had an 82% higher risk of mortality at any given time compared to CS-GNB patients, though this was not statistically significant. By day 28, the HR increased to 2.12 (0.35–12.93, p=0.416) for CR-GNB group indication 2.12 times the risk of mortality compared to CS-GNB patients, but due to the wide confidence interval suggests variability in outcomes (Table 5).

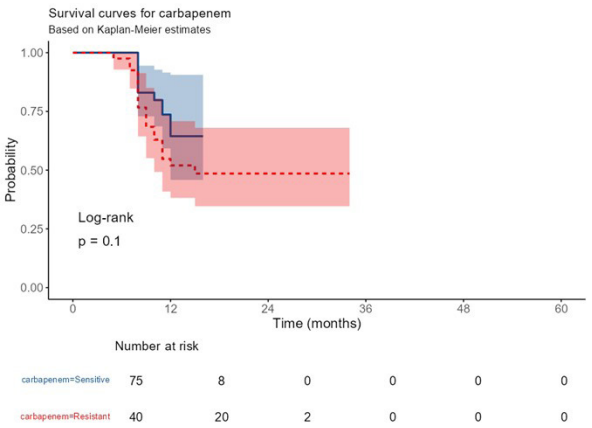

Fig. 2. Kaplan-Meier curve for the survival of the patients (day 14)

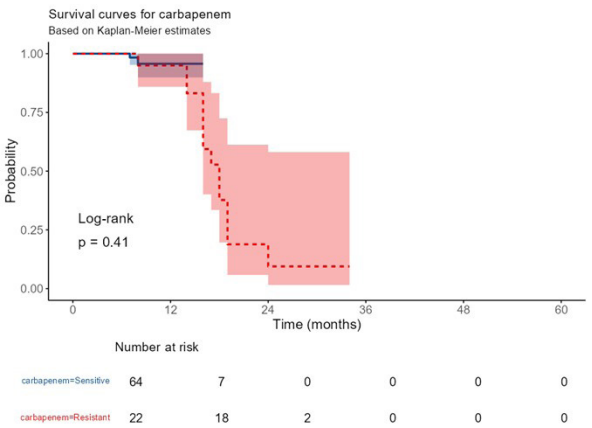

Fig. 3. Kaplan-Meier curve for the survival of the patients (day 28)

Table 5. Hazard ratio of the carbapenem*

Carbapenem	n (%)	HR (95% CI); p
Day 14		
Sensitive (ref)	75 (65.2)	1.82 (0.85–3.91); 0.122
Resistant	40 (34.8)	
Day 28		
Sensitive (ref)	64 (74.4)	2.12 (0.35–12.93); 0.416
Resistant	22 (25.6)	

* Cox-regression analysis, ref – reference group, HR – hazard ratio, CI – confidence interval, a HR greater than 1 indicates increased risk, while less than 1 indicates decreased risk compared to the reference group

Discussion

This hospital-based prospective study which was conducted by General Medicine department at tertiary-care teaching hospital, in Puducherry which aimed to compare the clinical profile and outcomes of the patients with CR-GNB and CS-GNB. Our findings indicate that CR-GNB infections are associated with greater disease severity, a higher likelihood of ICU admission, and an increased mortality risk, particularly in high-risk populations with underlying comorbidities.

The gender distribution in our study revealed that a higher percentage of male patients were present in the CR-GNB (72.5%) compared to the CS-GNB group (56%). Conversely, the percentage of female patients was higher in the CS-GNB group (44%) than in the CR-GNB group (27.5%). Research by Doi et al., highlighted similar trends, where male patients were more frequently observed with CR-GNB infections due higher exposure rates to healthcare settings and invasive procedures, increasing their risk for resistant infection.[18]

Additionally, diabetes was equally prevalent in both groups (60%), reinforcing the findings by Cruz-López et al., indication that diabetes acts as a well-known risk factor for infections due to weekend immune dysfunction.[22] Study by Aon et al., showed that 30-day hospital mortality was significantly higher among the diabetes group when compared to non-diabetic patients (48.9% and 28.2%, respectively) demonstrated that there is an association between the diabetes and CR among the patients.[23] Similarly, another study by Ghareeb et al., also resulted that patient with diabetes had increased CR when compared to non-diabetes patients.[24] On the other hand, hypertension was significantly more common in CS-GNB group (57.3%) compared to CR-GNB (37.5%) group, a trend was also observed in studies by Tängdén et al., and Paul et al. suggested that while comorbidities predispose patients to infections, they do not necessarily correlate directly with resistance patterns.[16,25] As for the patients with CKD, is common in CR-GNB patients in our study (17.5%), aligns with the study by Theuretzbacher et al. noted that CKD patients, often requiring dialysis, have higher exposure to invasive procedures, increasing the risk of multidrug-resistant infections.[17]

A higher percentage of CR-GNB patients were admitted to the ICU (90%) compared to the CS-GNB group (49.3%). Study by Tängdén et al., and Muteeb et al. reported that CR-GNB infections are more severe and require more intensive management.[16,26] This shows that increased severity is attributed to the limited treatment options and the high virulence of the pathogens involved, necessitating critical care interventions.[10,11,16,26] Similarly, the prolonged hospital stay was associated with CR-GNB (mean 12.88 days) ally with the study done by Doi et al. who attributed that extended hospitalization to treatment failure and higher rates of complication.[18] Hassoun-Kheir et al. conducted the study resulted that *CR-Enterobacterales* (CRE) colonized patients among the 1-year survivor had increased length of hospital stay than the controls (odds ratio (OR) 1.52; p <0.001).[27] Likewise, study by Sharma et al. resulted that the median hospital stay was 17 days in CRE patients than non-CRE patients and another study by Priyendu et al. had the median days of 12 in *CR-Klebsiella pneumonia* (CRKp) patients, align with our study results where the patients with CR-GNB had the median days of 12 than CS-GNB patients.[27,29] These indicated that CR colonization had longer hospital stay than CS-GNB patients.

As for the antibiotic duration we found that it was significantly longer in CR-GNB patients (8.7 vs 6.04 days, p=0.001) reflecting that the difficulty in achieving successful treatment outcomes. As per Infectious Disease Society of America (IDSA) 2024 guideline for the treatment of antimicrobial resistant GNB,[30] showed that treatment duration varies as per the type of antibiotic and the patient's status. Studies by Paul et al,[25] highlighted that prolonged antibiotic therapy is both a cause and a consequence of antibiotic resistant, as patients with resistant infections often require multiple treatment modifications. Study by Soto et al,[31] showed that CRE patients required prolonged courses of active therapy of about 14 days while the short course required 9 days which was similar to our study findings. Similarly, the guideline by Park et al,[32] also implied that CRE required longer duration of treatment when compared to CS patients.

A study by Paul et al,[25] highlighted that the use of indwelling catheters is a significant risk factor for acquiring CR-GNB. The study found that patients with such devices are more likely to develop severe infections, which often require more intensive and prolonged treatment, further increasing the risk of antibiotic resistance.[25] Our study's findings align with these observations, demonstrating a significantly higher prevalence of indwelling catheters in patients with CR-GNB infections.

Disease distribution varied significantly between the two groups. The most common disease in the CR-GNB group was pneumonia (42.5%), followed by urinary tract infections (UTIs) (15%) and cellulitis (10%). In contrast, the CS-GNB group showed a different pattern. UTIs were the most prevalent disease in this group (64.0%), followed by pneumonia (14.7%) and typhoid (4.0%). According to Cruz-López et al. CR-GNB infections are frequently linked to pneumonia, particularly ventilator-associated pneumonia (VAP), due to the critical nature of the patients, frequent use of invasive devices and prolonged hospital stays.[22] This is consistent with our finding that pneumonia is the most common disease in the CR-GNB group.

Klebsiella pneumoniae (37.5%) and *E. coli* (25%) were the most common pathogens in CR-GNB cases, whereas in CS-GNB, E. coli was the predominant isolate

(56%). This pattern is in consistent with the global surveillance studies by Nordmann and Poirel also reported that *K. pneumoniae* and *E. coli* are among the leading the CR pathogens worldwide.[33] While study by Afify et al. also found that *K. pneumoniae* were resistant to colistin similar to our study findings.[34] This explains the increased rate of pneumonia in patients with CR-GNB where *K. pneumoniae* plays a major role in causation of CR in the patients.

Polymyxin B (42.5%) was the most frequently used antibiotic in CR-GNB group, followed by the ceftazidime-avibactam (15%). In contrast, meropenem was the primary agent in CS-GNB cases (28%). The increased use of combination therapies such as polymyxins with other agents reflects current treatment guidelines that emphasize the synergy for overcoming resistant, as suggested by IDS guidelines, and Doi et al.[18,30] Another study by Nordmann and Poirel showed that use of ceftazidime-avibactum had gained traction as an alternative in treating CRE.[32]

Survival analysis showed an increased risk of mortality in the CR-GNB group. The HR on day 14 was 1.82 (p 0.122) while on day 28 it was 2.12 (p 0.416), suggesting a trend towards higher mortality in CR-GNB patients, although statistical significance was not reached. Studies by Tsachouridou et al. and Kitaya et al. reported similar findings, indicating that CR-GNB infections significantly impact patient survival, especially in critically ill individuals.[1,2] Study by Cienfuegos-Gallet et al. showed that CRKp had lower survival time when compared to CSKp patients (relative time is 0.44).[35] Similarly, study by Kulkova et al. also provided that significant difference in 28-days survival in patients with CR-GNB and CS-GNB (log rank p=0.033) which was similar to our findings but found to be significant.[36] Thus, high mortality rates were found to be occurred in patient with CR-GNB than CS-GNB. Although these results in our study did not reach statistically significant, likely due to the sample size, they reflect a clinically meaningful trend, as patients with CR-GNB infections experience prolonged illness and a higher likelihood of mortality over time. These findings align with global data, emphasizing the urgent need for early intervention, aggressive infection control, and targeted antimicrobial therapy to mitigate the impact of CR-GNB on patient survival.

Conclusion

This study highlights the substantial healthcare burden of CR-GNB infections, which lead to longer hospital stays, extended antibiotic treatment, higher ICU admissions, and increased mortality. Key strategies include early identification, optimized empirical therapy, and strong infection control to improve outcomes. Antimicrobial stewardship is critical in managing antibiotic use and curbing resistance spread, especially given the link between comorbidities (like hypertension, diabetes, and CKD) and CR-GNB. The frequent use of indwelling catheters in resistant cases underscores the need for strict infection control. Global surveillance, targeted interventions for high-risk groups, and continued research are essential for combating antimicrobial resistance effectively.

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Declarations

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Author contributions

Conceptualization, V.R. and K.S.C.; Methodology, V.R. and S.S.; Software, A.K.; Validation, V.R. and S.S.; Formal Analysis, A.K.; Investigation, AK; Resources, AK; Data Curation, AK; Writing – Original Draft Preparation, A.K. and V.R.; Writing – Review & Editing, S.S. and K.S.C.; Visualization, SS; Supervision, K.S.C.; Project Administration, V.R. and S.S.

Conflicts of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

All data generated or analyzed in this study are included in this published article.

Ethics approval

The Institutional Ethical Committee (MGMCRI/Res/01/2021/38/IHEC/78) approved this study.

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ORIGINAL PAPER

Ezetimibe mitigates DNCB-induced mouse model of eczematous dermatitis

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ABSTRACT

Introduction and aim. Atopic dermatitis (AD) is a life-long inflammatory dermatosis that features dry, erythematous skin. Ezetimibe is a lipid-lowering agent with enhanced anti-inflammatory and anti-oxidative capacities. This work attempted to evaluate the anti-eczematous action of topically administered ezetimibe in a mouse prototype of 1-chloro-2,4-dinitrobenzene (DNCB)-evoked AD. To our knowledge, this is the first study to investigate the topical use of ezetimibe in an experimental model of AD.

Material and methods. Thirty male Swiss albino mice were randomly allocated into five groups: healthy control, DNCB-induced model, vehicle, tacrolimus (0.1% ointment), and ezetimibe (2% ointment). Treatments were applied daily for 21 days. Clinical severity scores, total and differential leukocyte counts, histopathological changes, immunohistochemical expression of interleukin (IL)-4 and IL-13, and tissue levels of IgE, malondialdehyde (MDA), IL-17, IL-31, transforming growth factor-β (TGF-β), and tumor necrosis factor-α (TNF-α) were assessed.

Results. DNCB increased dermatitis severity (EASI score 9.8±0.7 vs. 0.5±0.1 in controls, $p<0.001$), total leukocytes (14.2±1.6 $\times10^3$/mL vs. 3.9±0.6 $\times10^3$/mL, $p<0.001$), and IgE (356±42 ng/mL vs. 92±15 ng/mL, $p<0.001$). Ezetimibe treatment significantly reduced EASI scores (2.1±0.4, $p<0.01$ vs. DNCB), leukocytes (5.9±0.3 $\times10^3$/mL, $p<0.01$ vs. DNCB), IgE (128±18 ng/mL, $p<0.01$ vs. DNCB), and MDA (2.3±0.4 µmol/L vs. 5.9±0.7 µmol/L in DNCB, $p<0.001$). Pro-inflammatory cytokines IL-4, IL-13, IL-17, IL-31, TGF-β, and TNF-α were also markedly decreased (all $p<0.05$), with effects comparable to tacrolimus.

Conclusion. Topical ezetimibe significantly alleviated DNCB-induced AD-like lesions by reducing histopathological changes, leukocyte infiltration, IgE, oxidative stress, and key inflammatory cytokines. These findings support ezetimibe as a potential adjunctive topical therapy for immune-mediated dermatoses, warranting future clinical evaluation.

Keywords. atopic dermatitis, DNCB, eczema, ezetimibe, inflammatory dermatosis, immune-mediated skin diseases

Introduction

Atopic dermatitis (AD) is a recurring and remitting auto-inflammatory skin disease evoked by the immune system that often begins during the early stages of life. It is often known as eczematous dermatitis and is the most common skin condition that involves a rapid development of itchy patches on the skin.[1-3] Dermatosis is marked by atopic inflammation, lichen formation,

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itchiness, greater vulnerability to infection, and rashes, frequently followed by acute pruritus.[1,4,5] Inheritance variables, immune disruption, compromised skin barriers, and external stimuli are believed to be among the fundamental reasons.[6] AD collapses beneath the category of atopic ailments that encompass food intolerance, pulmonary asthma, rashes and hay fever, many of which are relevant pathologies associated with elevated immunoglobulin E (IgE) concentrations and diminished filaggrin concentrations.[7,8] Furthermore, AD can occur concurrently with other immune-driven dermatoses, such as psoriasis.[9-13] Furthermore, a possible correlation between ulcerative colitis and AD is shown in the literature study.[14-17] Meanwhile, an immune system mismatch in the Th1/Th2 reactions is a distinguishing prosperity of AD pathogenesis, with higher rates of released cytokines, including interleukin (IL)-4 and IL-13, which drive Th2 replication and B cell IgE liberation[1]. IgE value is a specified indicator of AD intensity, which is corroborated by the efficiency of IgE-focused therapy to reduce AD symptoms among sufferers.[18-20] Tumor necrosis factor (TNF)-α is a significant inflammatory cytokine that modulates immunological responses and cellular division.[21-23] It also facilitates the relocation of NF-κB into keratinocytes, which assists in transcriptional signaling and the liberation of cytokines, particularly IL-6 and IL-8.[24-28] Moreover, IL-17 is a pivotal target for treatment, since it fosters the secretion of TNF-α, exacerbating the effects of atopic inflammation.[8] IL-17 amplification elevates IL-6 and IL-1β concentrations in keratinocytes, which are associated with the pathogenesis of AD.[2,5] Additionally, transforming growth factor (TGF)-β is an anti-inflammatory cytokine that facilitates angiogenesis while simultaneously promoting differentiation and influencing the expansion of keratinocytes; yet, excessive production of TGF-β1 in skin cells induces significant inflammation.[29-31] However, oxidative damage has a role in the etiology of AD. Environmental factors, such as contaminants, ultraviolet radiation, illness, and mental illness, may elevate reactive oxygen species (ROS) outputs, finally exceeding the protective capacity of the anti-oxidative barrier.[32-36]

Nevertheless, topical medicines, including calcineurin blockers, glucocorticoids, and, lastly, phosphodiesterase 4 (PDE4) antagonists such as crisaborole, are the bedrock of AD management.[37-39] Yet, repeated usage of these medicines might cause an array of unwanted effects. Thus, there is a pressing demand for the identification of innovative and secure methods for treating AD.[40,41] The approval of targeted biologics such as dupilumab, tralokinumab, nemolizumab, baricitinib, upadacitinib, and abrocitinib suggests subsequent best alternatives for controlling moderate to severe forms of AD.[42,43]

Ezetimibe is a lipid-diminishing medication that specifically hinders the cholesterol transporter Niemann-Pick C1-like protein 1 (NPC1L1) in the jejunum brush borders, restricting the digestion of cholesterol in the stomach and bile systems. Ezetimibe was demonstrated to be safe and efficient in decreasing the contents of cholesterol regularly accessible to hepatocytes. As a result of diminished cholesterol supply, the liver raises LDL receptor generation and LDL removal from the circulation.[44-47] Ezetimibe was established to stop atherosclerotic events when combined with statin, making it useful in the treatment of dyslipidemia and related atherosclerotic complications.[48,49] Ezetimibe's impact on many inflammatory indicators was also studied. Ezetimibe was revealed to lower atherosclerotic plaques, limit macrophage accumulations, and prevent liberation of chemotactic cytokines like MCP-1 and TNF-α.[50,51] Other observation demonstrate that combining ezetimibe with simvastatin can effectively alleviate autoimmune alopecia totalis and alopecia universalis, making it a promising treatment option for resistant alopecia areata. [52] This combination also considerably lowered amounts of IL-1β and IL-18 in obese individuals.[53] Beyond that, this medicine exerted protective actions on IL-1β-exacerbated matrix collagen breakdown in murine chondrocytes by modifying NF-κB and Nrf2/HO-1 molecular parameters.[54] In parallel, ezetimibe enhanced endothelial functions and decreased inflammation indicators, disease progression, and aortic stiffening among individuals with rheumatoid arthritis.[55] In comparable vein, ezetimibe shows inhibitory impacts on acetic acid-exacerbated rat prototype of ulcerated colitis by downregulating inflammatory and oxidative indicators, particularly the Akt/NF-κB/STAT3/CXCL10 signaling network.[56]

Aim

Previous studies suggest that ezetimibe possesses anti-inflammatory, antioxidant, and immunomodulatory properties; however, its anti-eczematous efficacy has not been investigated. This study aimed to evaluate, for the first time, the anti-atopic effects of topical ezetimibe in a DNCB-induced murine model of AD, providing novel preclinical evidence for its potential repositioning as a topical therapy in dermatological inflammatory disorders.

Material and methods

Drugs and chemicals

The suppliers of 1-chloro-2,4-dinitrobenzene (DNCB) was the Sigma Aldrich, Germany. Ezetimibe was purchased from Hangzhou Hyper Chemicals Limited. The 0.1% tacrolimus product was supplied by Cleveland Clinic, USA. The extra chemicals utilized in this investigation had been procured from several sources who met specific standards.

Preparation of ezetimibe ointment

2.5 g of white wax (beeswax), 1.25 g of hard paraffin, 2.5 g of lanolin, polyethylene glycol, and a sufficient quan-

tity of soft paraffin were melted together in a saucepan on a hot plate at 70 °C.[57] 2g of ezetimibe powder was placed to the melted mixture while being continuously stirred.[58,59] The entire mixture was blended while transported on ice to produce 2% ezetimibe ointment.[60-64]

Vehicle components formulation

2.5 g of white wax, 1.25 g of hard paraffin, 2.5 g of lanolin, polyethylene glycol, and an adequate amount of white soft paraffin were combined and heated in a container on a heated surface at 70°C. The whole solution was constantly agitated and mixed.[65,66]

Study design

This work was licensed by the institutional review board (IRB) of the College of Medicine at Al-Nahrain University (approval no. UNCOMIRB20241037 on 14/10/2021). The experiments were carried out from November 2021 to July 2022. The trial consisted of thirty male Swiss albino-kind mice, with weight about 20 to 30 g and age about 8 to 9 weeks. Mice were placed in a sterile, isolated, and properly designated room with a humidity level of 49±2%. All investigations had been performed in conformity with rules of ethics for the handling of animals.

Animal preparation and sampling

Mice had been randomly separated into 5 different categories of 6 mice each: a healthy untreated control group, an induction non-treated group, a vehicle group, an ezetimibe group, and a tacrolimus group. Following a 14-day acclimation period, mice underwent rigorous hair extraction from their back areas (about 4 cm^2) with a precision shaving device. The residual hair strands were subsequently extracted with a depilatory lotion. To develop AD-mimicking skin irritation, DNCB was dispersed in an acetone-based olive oil mixture at a 3:1 proportion and watered down to 0.5 and 1% strengths. Mice developed a sensitivity with 200 µL of 1% DNCB applied to their back skin-folds for 3 straight days, whereas the healthy controls did not obtain any medications. Mice with AD-like symptoms were treated with 200 µL of 0.5% DNCB locally each other day for 21 days to prevent unexpected resurgence. The vehicle group was given a topical vehicle formulation daily for 21 days, the tacrolimus group obtained tacrolimus 0.1% ointment daily for 21 days, and the ezetimibe group administered ezetimibe 2% ointment product daily for 21 days as indicated in Figure 1. At the completion of the tests, all animals underwent the intraperitoneal analgesic medicines xylazine and ketamine (80 mg/kg). Upon achieving complete anesthesia, the animals were put to death by exsanguinations, which are the optimal means of harvesting and preserving.[67-70] Skin biopsies were subsequently processed to develop tissue homogenates for bio-indicator and histopathology analyses.[71-74]

Measurement of total and differential WBC counts

The complete blood counts involves the overall amount of leukocytes/white blood cells (WBC) alongside the individual percentages for each WBC subgroup, such as neutrophils, lymphocytes, monocytes, and eosinophils. Blood specimens were taken in EDTA-containers and scrutinized with a small 5-part hematologic tester, namely the Mindray BC-5000 version.[75,76] The methodology is based on triangular laser scattering, flow-cytometry, and biochemical dye processing.[77-81]

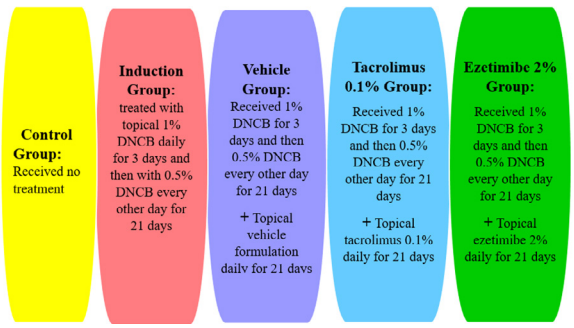

Fig 1. Tabular form of study groups

Estimation of severity grading of AD

The intensity of DNCB-evoked AD-mimicking cutaneous patches was estimated by 4 clinical manifestations: erythema, erosion/excoriation, epidermal thickenings, and lichenification, based on the Eczema Area and Degree Index (EASI) scoring technique.[82] The metrics were evaluated on a scale of 0 (none), 1 (mild), 2 (moderate), and 3 (severe) based on their severity, with total dermatitis levels determined by summing all analyzed data, achieving a highest possible score of 12.[83] The back skin-fold thickening was calculated using a vernier calibrator measuring instrument on the central line of the back skin. [84-86] Dermatitis severity was assessed by two independent raters. Dermatitis scoring and treatment administration were conducted in a blinded fashion to minimize observer bias. The experimenters were unaware of the treatment groups during scoring.

Histopathological estimation

The dorsal skins of mice were obtained from various categories and preserved in 10% neutralizing-buffer formaldehyde employing recognized guidelines.[87-90] Fixed paraffin was then applied to the skin samples. The paraffin-soaked tissues were sliced into slices and prepared for staining with hematoxylin and eosin. The derived specimens were viewed utilizing a light microscopy, and histological changes were documented.[91-95] Histological scoring relied on an amended edition of the scoring system described by Jeong et al., commonly used for DNCB-induced dermatitis models. Parameters encompassed skin thickness/acanthosis, hyperkeratosis, parakeratosis, erosion, inflammatory cell invasion, and

edema.[96] The grading mechanisms varied from 0, signifying no irregularity, to 1+, denoting mild irregularity; 2+, representing modest irregularity; and 3+, signifying substantial irregularities.

Immunohistochemistry (IHC) examination

The immunohistochemistry approach was used to assess the severity of AD lesions by determining the scores of IL-4 and IL-13. It is technique for detecting antigens or haptens in biological tissues. The immunohistochemical assessment in this study employs specific antibodies for identifying the protein product of gene expression in the tissues of the investigated animal groups. Following the application of the main antibody (rabbit monoclonal anti-IL-4 and anti-IL-13), the corresponding secondary antibody and a chromogen agent (3,30-diaminobenzidine) are then incorporated into paraffin-fixed segments of the skin tissues.[31,97-99] The skin portions have been treated with hematoxylin. The immunohistochemistry investigation is scored via the following method using semi-quantitative scoring (positive stained cells): "Score 0=no stain; score 1=25%; score 2=26–50%; score 3=51–75%; score 4=67–100%.[30,100,101]

Biochemical marker measurement

Using sandwich enzyme-linked immunosorbent-assays (ELISA), the amounts of IgE, malondialdehyde (MDA), IL-17, IL-31, TGF-β, and TNF-α in mouse skin tissues were determined by the supplier's rules. IgE Invitrogen: catalogue no.: 88-50460-88, sensitivity: 4 ng/mL; MDA Invitrogen: catalogue no.: EEA015, sensitivity: 1.13 μmol/L; IL-17 Sino biological: catalogue no.: KIT51065, sensitivity: 8 pg/mL; IL-31 Invitrogen: catalogue no.: BMS6030, sensitivity: 9.1 pg/mL; TGF-β Invitrogen: catalogue no.: 88-8350-88, sensitivity: 8 pg/mL; TNF-α Sino biological: catalogue no.: KIT50349, sensitivity: 15.69 pg/mL. To ensure statistical validation, ELISA measurements were done in duplicate. Following a cleansing with washing buffer, the pores were treated with a biotin-based-specific antibody.[102-106] Upon extracting the disengaged biotin-linked antibody, streptavidin/horseradish peroxidases (HRP) were gently transmitted into the wells. Tetramethylbenzidine (TMB) substrate solution was added once all unbound components were eliminated. The amounts of various variables in every experimental group were determined spectro-photometrically by matching their optical density to regular curves. The HRP-combined antibodies and indicators switched to yellow upon the addition of the stop solution The levels of various variables in each experimental group were determined spectro-photometrically by comparing their optical densities to standard curves. Following the addition of the stop solution, the HRP-conjugated antibodies and substrates produced a yellow color, which was measured using an absorbance microplate reader at a wavelength of 450 nm. [107-109]

Statistical analysis

The statistical evaluation was conducted using the program Excel 2013 and the SPSS statistical program edition 24 (IBM, Armonk, NY, USA). The numerical parameters are presented in terms of means with standard deviation (±SD), and the level of significance is estimated at a p value of less than 0.05. Means were contrasted

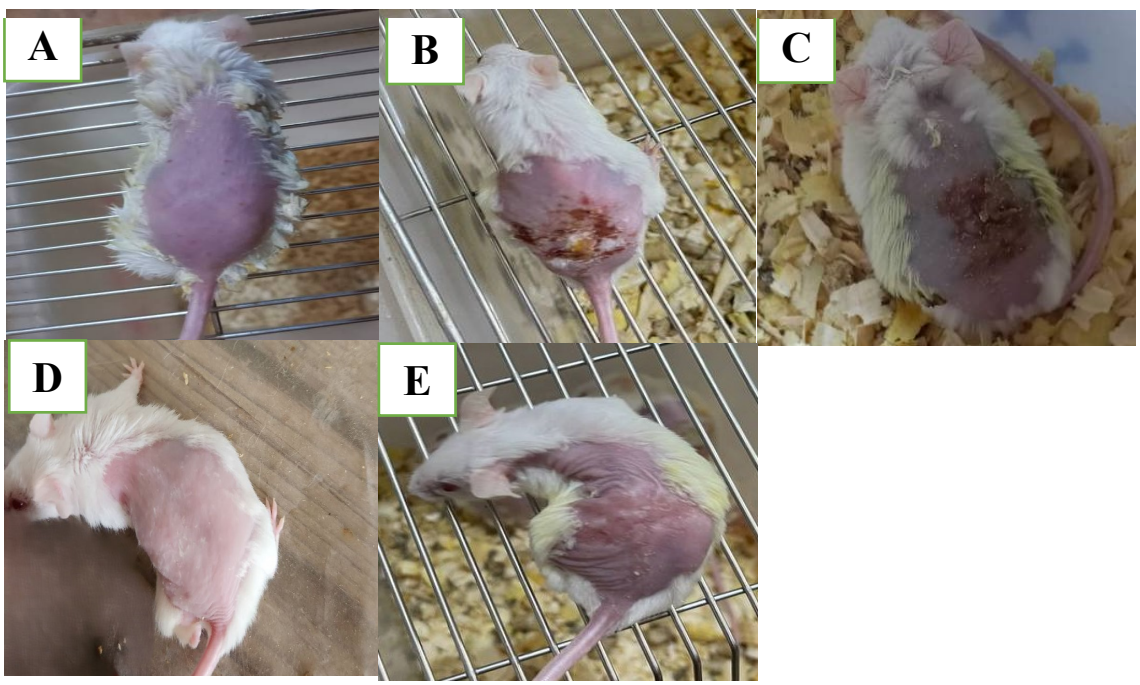

Fig. 2. Images displaying the degree of severity of eczematous lesions in study groups: A – control group, B – induction group, C – vehicle group, D – tacrolimus group, E – ezetimibe

with the t-test for independence and an assessment of variance (ANOVA). Pearson's correlation is adopted to determine linear relationships among the groups under study.[110,111]

Results

Impact of researched agents on observational severity scores

The induction and vehicle groups had significantly elevated observational severity levels than normal controls ($p<0.05$). Also, there was no considerable variance among induction/model and vehicle groups with respect to observational dermatitis levels ($p > 0.05$). Nonetheless, the dermatitis severity ratings got significantly reduced in the tacrolimus and ezetimibe-treated groups when matched with the induction and vehicle groups ($p<0.05$). No noteworthy variances were detected across the tacrolimus and ezetimibe groups with respect to observational dermatitis levels ($p>0.05$) as observed in Figure 2 and Figure 3.

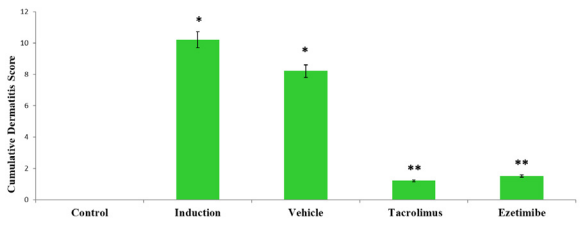

Fig 3. Impact of researched agents on the observational severity scores, data are illustrated as mean±SD; *denote considerable variation ($p<0.05$) vs. control group; # denote considerable variation ($p<0.05$) vs. induction and vehicle groups

Table 1. Impact of researched agents on total WBC, neutrophils, lymphocytes, monocytes, and eosinophils counts[a]

Variables ($x10^3$/mL)	Groups					p
	Control	Induction	Vehicle	Tacrolimus	Ezetimibe	
WBC	3916±558	14180±1856*	14120±1625*	6640±614#	5850±290#	<0.05
Neutrophils	1465±221	4934±1434*	4910±1522*	2880±198#	2519±250#	<0.05
Lymphocyte	2260±638	5940±1086*	5890±982*	3220±580#	2995±442#	<0.05
Monocytes	81±12	1325±352*	1294±416*	543±118#	404±44#	<0.05
Eosinophils	111±28	1939±512*	1888±448*	725±124#	577±93#	<0.05

[a] data were expressed as mean±SD, * – denotes remarkable change ($p<0.05$) contrasted to the control group, # – denotes remarkable change ($p<0.05$) contrasted to the induction and vehicle groups

Effect of tested agents on total and differential WBC counts

The percentages of total WBC, neutrophils, lymphocytes, monocytes, and eosinophils were substantially increased in induction and vehicle groups as opposed to normal controls ($p<0.05$). Besides, ezetimibe and tacrolimus groups show considerably diminished counts of total WBC, neutrophils, lymphocytes, monocytes, and eosinophils than induction and vehicle groups as indicated in Table 1.

Effect of tested agents on skin histological abnormalities

The healthy control group has regular skin microstructure; the keratin epidermal layers, dermal uppermost layer, sebaceous glands, and hair proliferating follicles all appear normal, as illustrated in Table 2 and Figure 4. However, the application of DNCB resulted in substantial histopathological changes in the induction group opposed to the healthy untreated controls ($p<0.05$). Those skin changes were recognized by the breakdown of the skin barriers, the presence of clefts, marked hyperkeratosis, marked parakeratosis, increased acanthosis, profound erosion, remarkable edema, profound sloughing, and prominent inflammatory cell infiltration as represented in Table 2 and Figure 4. Except for erosion and extracellular edema, the vehicle group exhibited non-significant histopathological changes as matched to the induction group ($p>0.05$), including increased epidermal thickenings/acanthosis, marked hyperkeratosis, marked parakeratosis, significant erosion, significant edema, and increased inflammatory cell penetration. The erosion and extracellular edema in the vehicle group were markedly alleviated compared to those seen in the induction. In opposition to the induction and vehicle groups, the tacrolimus 0.1% treated group showed substantially smaller histological aberrations, such as reduced cutaneous thickness, minor hyperkeratosis, minor parakeratosis, slight erosion, slight edema, and reduced inflammatory cell invasion as depicted in Table 2 and Figure 4. Additionally, the histopathological changes were markedly diminished in the ezetimibe group versus the induction group ($p<0.05$), as demonstrated by decreased epidermal thickness, mild hyperkeratosis, mild parakeratosis, mild erosion and reduced inflammatory cell infiltration, as revealed in Table 2 and Figure 4.

Table 2. Effect of tested agents on histopathological scores[a]

Variables	Mean±SD					
	Control	Induction	Vehicle	Tacrolimus	Ezetimibe	p
Acanthosis	0	3±0.4*	3±0.04*	1±0.05#	1±0.04#	<0.05
Hyperkeratosis	0	3±0.06*	3±0.08*	1±0.2#	2±0.04#	<0.05
Parakeratosis	0	3±0.04*	3±0.18*	0±0.03#	0±0.04#	<0.05
Erosion	0	3±0.05*	1±0.25*	0±0.05#	0±0.04#	<0.05
Inflammatory cell infiltration	0	3±0.1*	3±0.06*	1±0.1#	1±0.04#	<0.05

[a] data are illustrated as mean±SD, * – denote considerable variation ($p<0.05$) vs. control group, # – denote considerable variation ($p<0.05$) vs. induction and vehicle

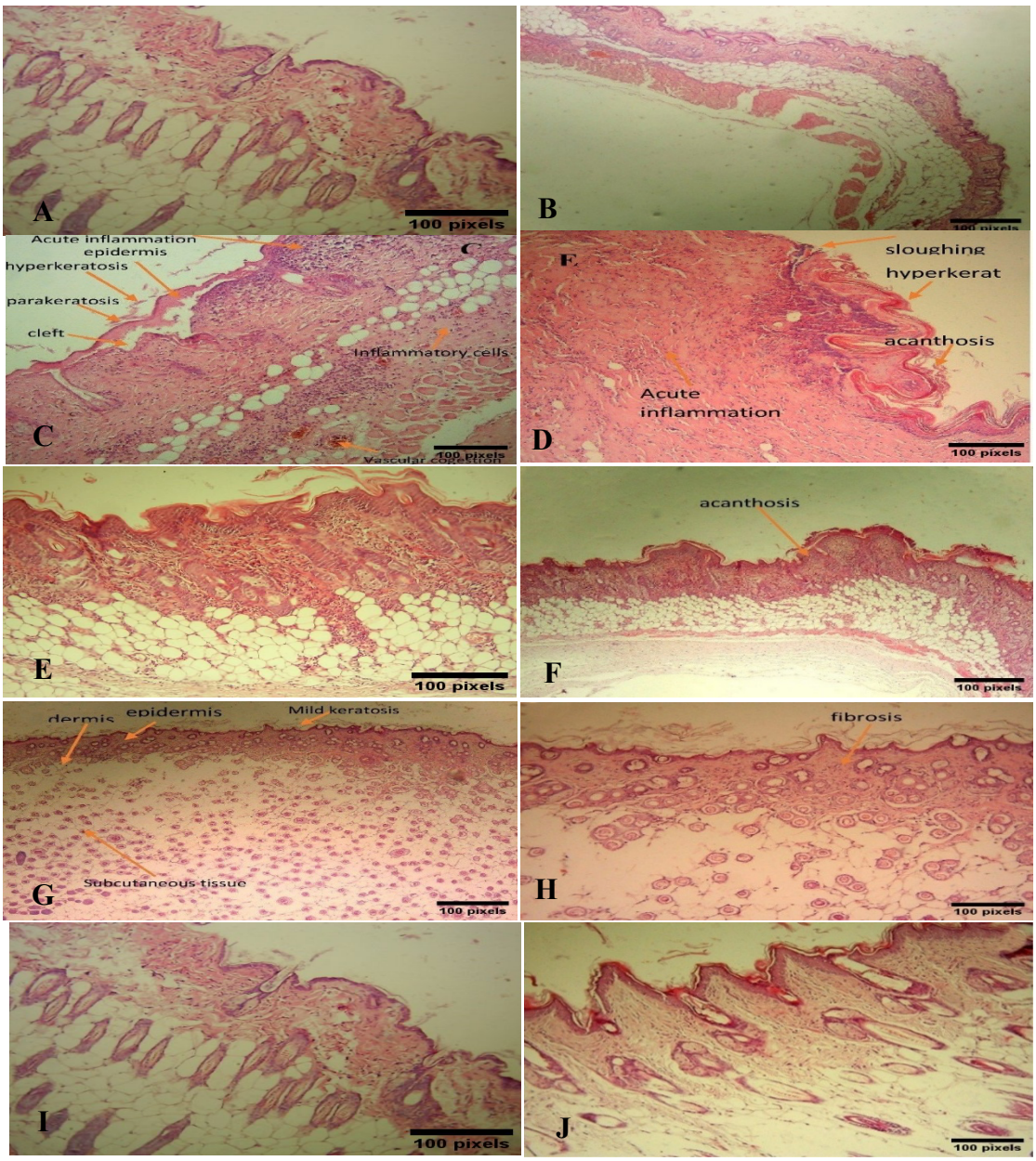

Fig. 4. Effect of studied agents on mouse skin histological alterations: A and B: light microscopy cross-section of mouse skin histopathology for healthy controls (H&E stain=10× and 4×), C and D: light microscopy cross-section of mouse skin histopathology for induction/ model group (H&E stain=10×), E and F: light microscopy cross-section of mouse skin histopathology for vehicle group (H&E stain=10× and 4×), G and H: light microscopy cross-section of mouse skin histopathology for tacrolimus group (H&E stain=10×), I and J: light microscopy cross-section of mouse skin histopathology for ezetimibe group (H&E stain=10×)

groups

Impact of tested agents on skin immunohistochemical levels of IL-4 and IL-13

The immunohistochemistry investigation revealed considerable elevation of IL-4 and IL-13 scores among the model/induction and vehicle groups as opposed to the normal controls ($p<0.05$), but there are no appreciable variations among the induction and vehicle groups ($p>0.05$). The tacrolimus and ezetimibe-treated groups exhibited substantial diminution in IL-4 and IL-13 contents when opposed to the induction and vehicle non-treated groups ($p<0.05$). However, no substantial changes were noted across the tacrolimus and ezetimibe groups in terms of IL-4 and IL-13 scores ($p>0.05$), as clarified in Figure 5 and Figure 6.

Effect of tested agents on skin tissue levels of IgE and MDA

The outcomes uncovered that the induction/model group exhibited markedly greater mean skin amounts of IgE and MDA as matched to the healthy untreated control group ($p<0.05$). No notable variations were seen

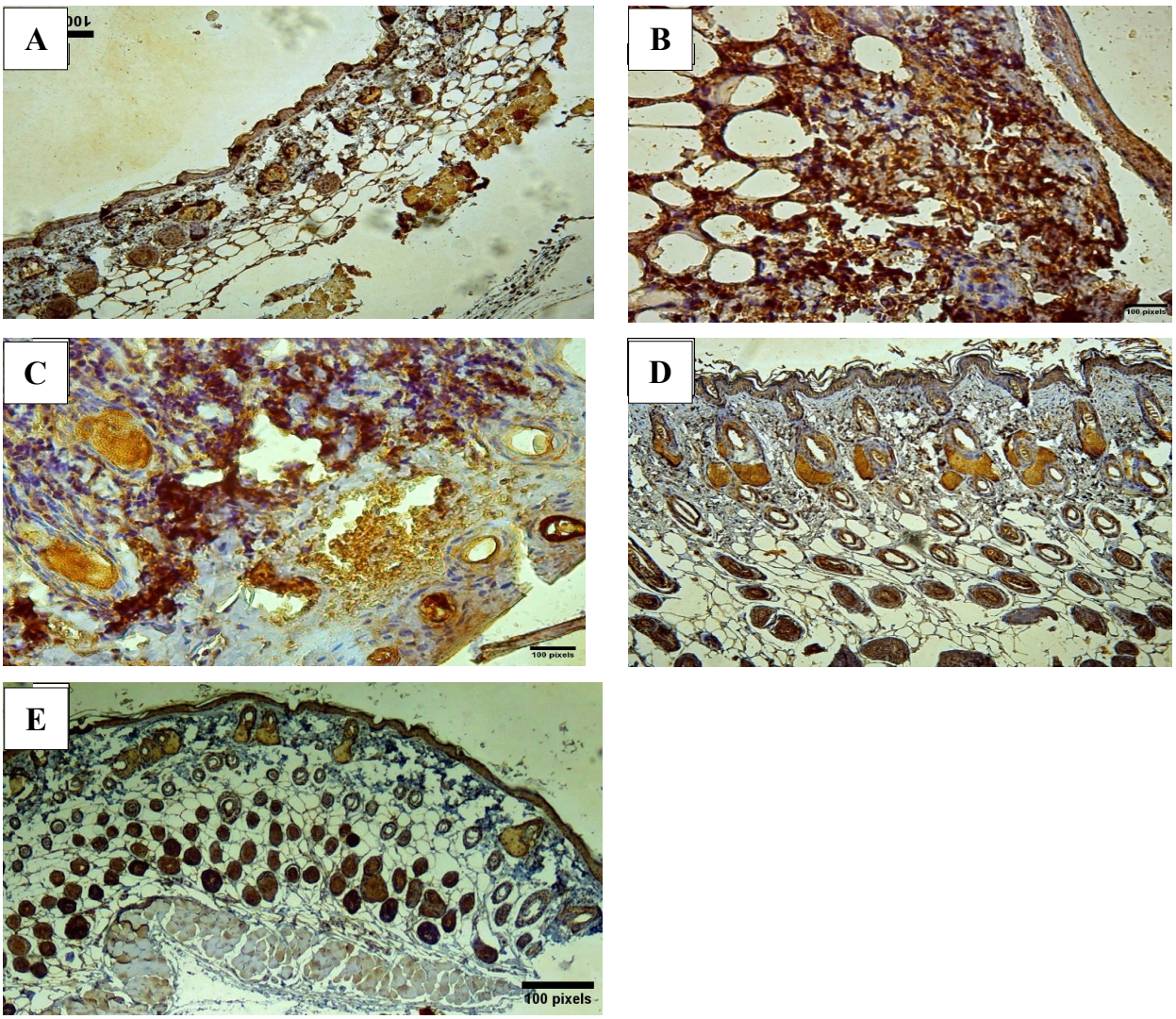

Fig. 5. Impact of studied agents on skin immunohistochemistry scores of IL-4: groups: (scale bar=100 mm), A: microscopic mouse cutaneous slice from the control group illustrating the immunohistochemical levels of IL-4, B: microscopic mouse cutaneous slice from the induction/model group illustrating the immunohistochemical levels of IL-4, C: microscopic mouse skin slice from the vehicle group illustrating the immunohistochemical levels of IL-4, D: microscopic mouse cutaneous slice of the tacrolimus group illustrating the immunohistochemical levels of IL-4, E: microscopic mouse cutaneous slice of the ezetimibe group illustrating the immunohistochemical levels of IL-4

in any of the parameters between the induction and vehicle groups. However, the tacrolimus and ezetimibe groups had dramatically lower levels of IgE and MDA relative to the induction and vehicle groups ($p<0.05$). Both the tacrolimus and ezetimibe groups did not vary considerably from one another on any of the parameters ($p>0.05$), as shown in Figure 7.

Effect of tested agents on skin tissue levels of IL-17, IL-31, TGF-β, and TNF-α

The results demonstrated that the induction group had markedly elevated mean epidermal levels of IL-17, IL-31, TGF-β, and TNF-α in relation to the healthy controls ($p<0.05$). No remarkable variances occurred in all metrics across the model/induction and vehicle groups. Still, the tacrolimus and ezetimibe groups exhibited extensively mitigated amounts of IL-17, IL-31, TGF-β, and TNF-α relative to the induction/model and vehicle groups ($p<0.05$). The tacrolimus and ezetimibe groups exhibited no significant distinctions across all variables ($p>0.05$), as explained in Figure 8.

Discussion

The novelty of this work lies in demonstrating for the first time that ezetimibe, beyond its systemic lipid-lowering action, exerts strong topical anti-inflammatory and antioxidant effects in a murine model of AD. The mouse prototype of DNCB-aggravated atopic dermatitis was extensively utilized to assess innovative therapies and compounds. The research designs and methodologies are markedly varied, revealing distinct patterns of atopic dermatitis that manifest after sensitization and

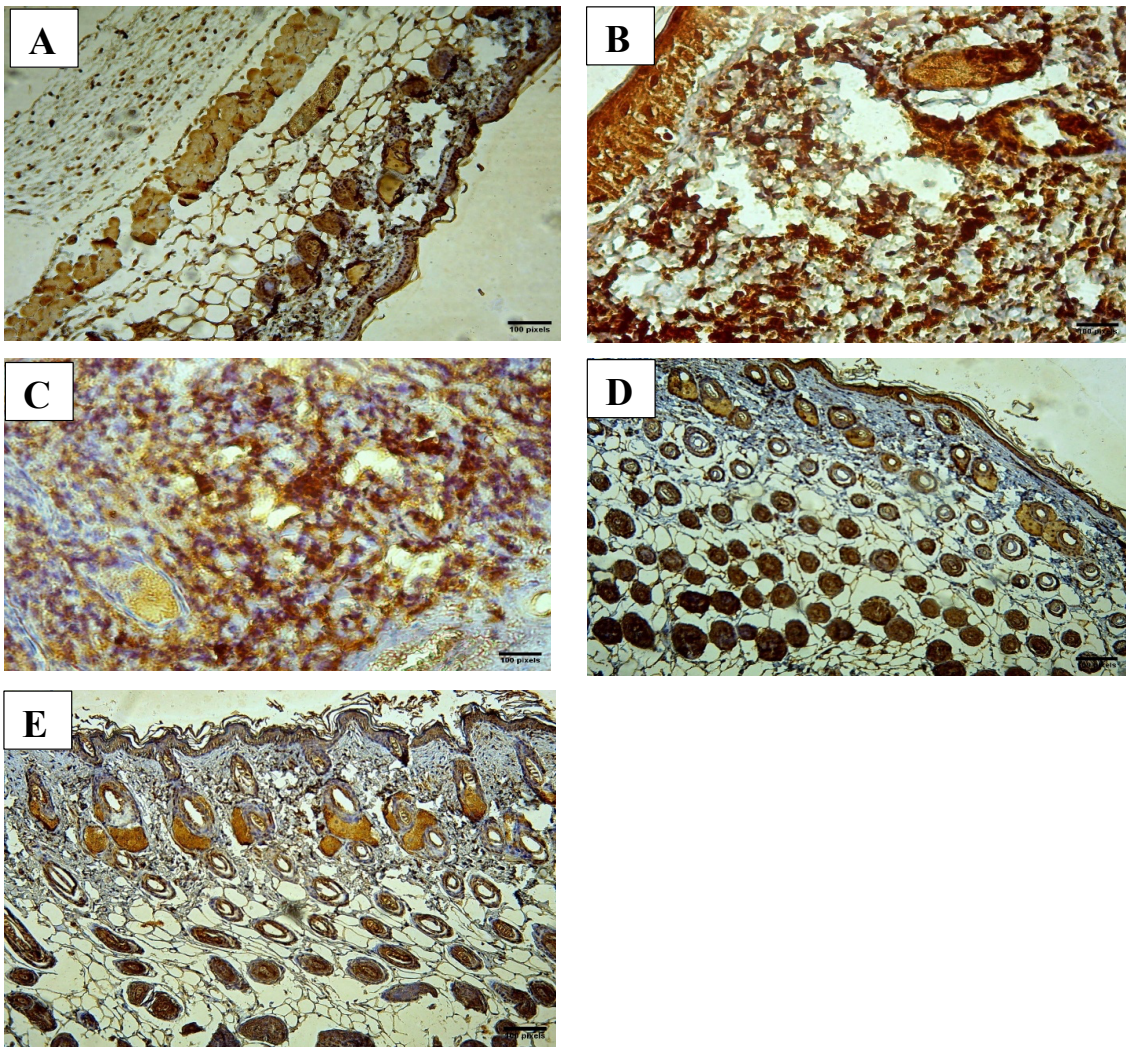

Fig. 6. Impact of studied agents on skin immunohistochemistry scores of IL-13: groups: (scale bar=100 mm), A: microscopic mouse cutaneous slice from the control group illustrating the immunohistochemical levels of IL-13, B: microscopic mouse cutaneous slice of the induction/model group illustrating the immunohistochemical levels of IL-13, C: microscopic mouse cutaneous slice of the vehicle group illustrating the immunohistochemical levels of IL-13, D: microscopic mouse cutaneous slice of the tacrolimus group illustrating the immunohistochemical levels of IL-13, E: microscopic mouse cutaneous slice of the ezetimibe group illustrating the immunohistochemical levels of IL-13

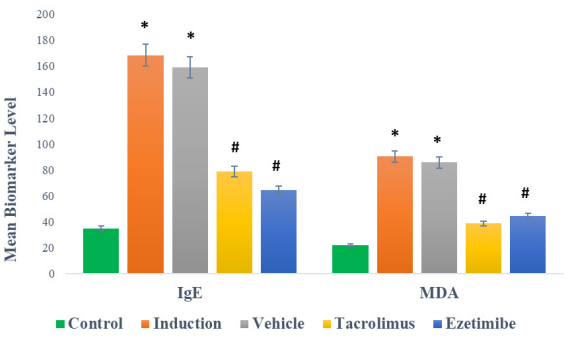

Fig. 7. Impact of tested agents on IgE and MDA (the data expressed as mean±standard deviation; * –implies a statistically marked difference ($p<0.05$) when contrasted to the normal controls, while # – implies a marked variation ($p<0.05$) when contrasted to the model/induction and vehicle groups

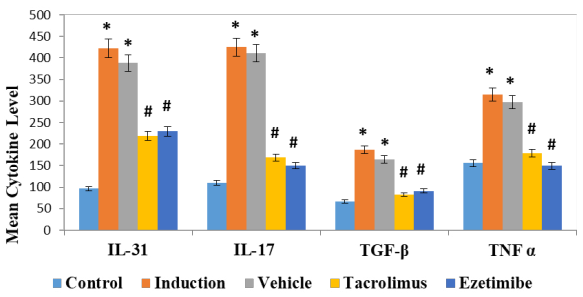

Fig. 8. Effects of tested agents on IL-17, IL-31, TGF-β, and TNF-α, the data expressed in the form of mean ±standard deviation, * – implies a statistically remarkable difference ($p<0.05$) when contrasted to the control group, while # – implies a remarkable variation ($p<0.05$) when contrasted to the model/induction and vehicle groups, IL – interleukin, TGF-β – transforming growth factor-β; TNF-α – tumor necrosis factor-α

frequent exposure to DNCB on the back skin.[112] In this work, the disease was induced with 1% DNCB in the sensitization phase and successive administrations of 0.5% DNCB in the challenge stage, resulting in a mild phenotype of eczematous dermatitis.[83] The DNCB-treated skin exhibited a moderate extrinsic subacute atopic dermatitis lesion on day 12 and a mild extrinsic subacute to prolonged phenotype and endotype on day 22, characterized by a predominant Th2 response. Both timepoints demonstrate hallmark features linked with atopic dermatitis, including dorsal skin thickening, hyperkeratosis, parakeratosis, elevated TH1 and TH2 cytokine levels, and alterations in barrier proteins. Elevated mast cell influx in the epidermis and higher plasma IgE levels signify a type I allergic reaction. [83,113]

In this study, administering DNCB to the murine dorsal regions resulted in evident indications of desquamation, erythema, and blisters, along with a notable rise in eczema severity indices. Furthermore, the aggregate populations of WBCs, neutrophils, lymphocytes, monocytes, and eosinophils were strongly elevated, and immunohistochemical testing indicated a large increment in IL-4 and IL-13 values. DNCB further drastically boosts the amounts of IgE, MDA, IL-17, IL-31, TGF-β, and TNF-α, while histological examination reveals pathological features such as hyperkeratotic changes, dermal thickness, and infiltrating lymphocytes, therefore confirming the AD model. Topical treatment is a popular method of ameliorating clinical manifestations, although it might trigger epidermal thinning and hypersensitivity.[114-116] Tacrolimus is an effective prolonged topical therapy for AD that acts by reducing phosphatase activity in the calcineurin pathway, resulting in decreased T-lymphocyte activation and inflammatory cytokine production. It also improves skin barrier function by stimulating the development and maturation of skin cell. [117-119] Prior research has demonstrated that the anti-atopic benefits of tacrolimus entail the suppression of total and differential leukocytes, IgE activity, oxidative measures notably MDA, and Th1- and Th2-related inflammatory cytokines, notably IL-4, IL-13, IL-17, IL-31, TNF-a, and TGF-β, as well as various molecular signaling cascades.[120-124]

Topical tacrolimus 0.1% yielded an immediate decrement in overall dermatitis scores, inflammation, and pruritus.[117] The most frequent undesirable actions of tacrolimus entail burning irritations at the sites of administration.[125-127] Consequently, developing innovative, efficacious medications for AD is essential.

In the current study, ezetimibe 2% ointment demonstrated a suppressive impact on DNCB-evoked AD-mimicking cutaneous erosions in mice, as evidenced by substantially reduced amounts of inflammatory and oxidative biomarkers, histological alterations, and the dermatitis severity scores. Additionally, a considerable decrement in the numbers of total leukocytes, lymphocytes, monocytes, eosinophils, and neutrophils was observed in comparison to the induction group. These outcomes were similar to those of Suchy et al. who discovered that ezetimibe has direct inhibitory effects on the activation pathways of immune cells, including monocytes and macrophages.[128] Another research indicated that ezetimibe administration yielded in a dose-related decline in both the overall proportion of CD3+CD4+ T cells and the number of $CD3^+/CD4^+$/CD45RO T memory lymphocytes.[129] Aside from the effect on immune cells, ezetimibe additionally offers other benefits, such as lowering the production of C-reactive protein, an influential inflammatory biomarker.[130] Likewise, topical ezetimibe experienced anti-inflammatory properties, reducing ear edema by 64%.[131] supporting current research that suggests it may be applied to treat inflammatory skin conditions. These favorable effects of ezetimibe could be explained by its ability to downregulate inflammatory mediators, suppress macrophage activation, and regulate the NF-kB, a chain accountable for producing various inflammation-related cytokines. In this context, ezetimibe treatment caused IkB decomposition and thereby suppressed NF-kB translation via the mitogen-activated protein kinases (MAPKs) mechanism. This evidence showed that there might be an opportunity of using ezetimibe to treat and prevent inflammatory disorders.[132] Another proof implies that ezetimibe might exhibit anti-inflammatory features alongside its lipid-lowering impacts, as NF-kB stimulation is influenced by chemotactic cytokines and substances involved in intracellular defense.[53] Moreover, ezetimibe alleviated clinical manifestations of ankylosing spondylitis in animals by suppressing Th17 differentiating-associated genes like IL-23R and IL-1R and modifying the Th17/Treg cell harmony resulting in a distinct anti-inflammatory impact irrespective of circulatory lipid lowering. The researchers found that ezetimibe dramatically boosted the overall count of Treg cells while decreasing the proportion of Th17 and Th1 lymphocytes. Ezetimibe also markedly decreased IFN-γ, TNF-α, IL-1β, IL-6, and IL-17 concentrations. As a result, ezetimibe modulates T-cell proliferation and pro-inflammatory cytokine release by immune system cells.[133] Similarly, ezetimibe inhibited lymphocytic production of TNF-α, IFN-γ, and IL-2 in hypercholesterolemic individuals in a lipid-unrelated way.[134] Subsequent studies concluded that ezetimibe successfully managed the rat model of ulcerative colitis by alleviating the output of pro-inflammatory measures like TNF-α, IL-1β, and NF-κB alongside oxidative measures like MDA and MPO in colon homogenate.[135-137] Likewise, literatures showed that ezetimibe is effective in treating of alopecia, an immune-related disease featured by T-lymphocyte stimulation, accumulation mast cells and pro-

duction TNF-a.[138-140] Alopecia sufferers are more likely to acquire other autoimmune disorders, like atopic dermatitis, vitiligo, and skin cancer.[141]

Meanwhile, this work found that ezetimibe remarkably reduced the immunohistochemical expressions of IL-4 and Il-13 in the back cutaneous folds. This outcome concurs with prior research, which indicated that ezetimibe/simvastatin combination contributed to decreased immunohistochemistry expression of chemotactic cytokines among alopecia areata patients.[141] Additional observation revealed that ezetimibe possesses immunomodulatory impacts on antigenic presentation, lymphocytic trafficking, and regulatory T cell function.[142] Ezetimibe was also documented to mitigate inflammatory variables like CRP, monocyte chemoattractant protein-1, IL-1β, IL-6, TNF-α, and pro-oxidant markers.[143-145] In macrophage tests, ezetimibe reduced NF-κB expression and blocked the NLRP3 inflammasome-IL-1β route, resulting in anti-inflammatory activity.[146] Ezetimibe can also attenuate inflammation and oxidative injury induced by caspase-1/IL-1β via the AMP-stimulated protein kinase/NF-E2-relating factor 2 (Nrf2)/thioredoxin-interacting protein (TXNIP) system.[147] In addition, this medication decreases monocyte chemoattractant protein 1-induced recruitment.[148,149] All these outcomes support our hypothesis that ezetimibe could be of an important value in attenuating experimentally-induced model of AD.

Study limitations

Nonetheless, the present investigation encompassed some limitations. Actually, our data exclusively confirmed the inhibitory effect of ezetimibe on a murine male prototype of DNCB-aggravated AD, neglecting the influence of gender-related fluctuations on the experimental results and failing to apply human participants due to the disparities between rodents and humans. Consequently, the effectiveness and security of ezetimibe in the management of AD ought to be assessed in people. Moreover, the further in-depth mechanism by which ezetimibe confers preventive impacts in mice afflicted with AD needs to be elucidated. The issue pertains to implications on other immune-related elements such as the proliferation of systemic immune receptors (sCD25, sCD30), the atopy patch tests, and the filaggrin genes. Further investigation is necessary to assess the efficacy of the combined use of ezetimibe and tacrolimus.

Conclusion

Topical ezetimibe significantly attenuated DNCB-induced eczematous dermatitis in mice by reducing histopathological alterations, suppressing pro-inflammatory cytokines, and lowering total and differential leukocyte counts. These results provide the first evidence supporting ezetimibe as a potential adjunctive therapy for immune-mediated and inflammatory dermatoses. The findings also highlight a novel therapeutic avenue for drug repurposing of ezetimibe in dermatology, particularly in AD.

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Declarations

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Author contributions

Conceptualization, A.M.S. and A.R.A.; Methodology, H.R-S.; Software, W.H.A.; Validation, A.R.A., H.R-S. and A.J.H.A.; Formal Analysis, A.M.S.; Investigation, W.H.A.; Resources, A.M.S.; Writing – Original Draft Preparation, H.R-S., A.M.S. and A.R.A.; Writing – Review & Editing, H.R-S.; Visualization, A.R.A., W.H.A. and A.J.H.A; Supervision, A.R.A. and H.R-S.; Project Administration, A.J.H.A.; Funding Acquisition, A.M.S.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

The corresponding author may obtain data validating the findings of this research on an adequate request.

Ethics approval

The research proposal has been authorized by the Institutional Review Board (IRB) of AL Nahrain University's College of Medicine (authorization number: 2 on October 14, 2021).

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ORIGINAL PAPER

A retrospective study on sickle cell disease-associated cardiovascular anomalies

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ABSTRACT

Introduction and aim. Sickle cell disease (SCD), a chronic inherited hemolytic disorder prevalent in certain regions of India and globally, is associated with various cardiovascular complications that significantly impact morbidity and mortality. The objective is to assess the relationship among clinical, electrocardiographic, and echocardiographic findings in individuals diagnosed with sickle cell disease, and, as a secondary aim, to contrast these findings with those observed in patients with sickle cell trait (SCT) taking into account different age groups across both sexes.

Material and methods. This study included thirty-four individuals previously diagnosed with sickle cell disease or SCT based on hemoglobin electrophoresis. Clinical records were reviewed to obtain data from comprehensive examinations, including electrocardiographic and echocardiographic assessments. Cardiac function was evaluated, interpreted, and compared between the two groups using documented ECG and echocardiography results.

Results. A total of 34 participants were enrolled, including 23 individuals with SCD and 11 with SCT. The mean age was 28.6±9.3 years in the SCD group and 30.2±8.7 years in the SCT group (p=0.57). Males predominated in both groups (SCD: 65.2%; SCT: 63.6%). Clinical examination revealed a statistically significant higher frequency of displaced apex beat in the SCD group (60.9%) compared to SCT (18.2%) (p=0.03). Electrocardiographic abnormalities such as left ventricular hypertrophy were more common in SCD (47.8%) than SCT (18.2%) (p=0.04). ST-T changes were seen in 56.5% of SCD vs. 9.1% of SCT (p=0.01). Echocardiographic findings demonstrated significantly elevated pulmonary artery systolic pressure (PASP) in the SCD group (38.6±6.2 mmHg) compared to SCT (28.4±5.7 mmHg) ($p < 0.001$). Mean pulmonary artery pressure (mPAP) was also higher in SCD (25.3±4.8 mmHg) vs. SCT (19.2±3.9 mmHg) (p=0.002). Tricuspid regurgitation velocity (TRV) was elevated in SCD (2.9±0.3 m/s) compared to SCT (2.4±0.2 m/s) (p=0.001). Right ventricular dilatation and diastolic dysfunction were noted in 39.1% and 34.8% of SCD cases, respectively, while absent in SCT ($p<0.05$ for both). In univariate logistic regression, apex beat displacement (OR=4.6; p=0.04), elevated PASP (OR=5.2; p=0.01), increased mPAP (OR=4.3; p=0.02), and TRV (OR=6.1; p=0.009) were significant predictors of SCD. Multivariate logistic regression identified PASP as an independent predictor of SCD (OR=4.8; 95% CI: 1.3–17.9; p=0.02).

Conclusion. The findings highlight that patients with SCD exhibit a significantly higher burden of cardiac abnormalities than those with SCT. Among the evaluated parameters, pulmonary artery systolic pressure emerged as an independent predictor of sickle cell disease.

Keywords. acute chest syndrome, echocardiogram, pulmonary artery systolic pressure, pulmonary hypertension, sickle cell disease, sickle cell trait.

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Introduction

Sickle cell disease (SCD) is a chronic hemolytic disorder characterized by autosomal recessive inheritance, resulting in the involvement of multiple organs and an increased risk of early mortality. The condition was initially recognized by Herrick in 1910, while the hereditary nature of the disease was recorded by Pauling in 1949. Since then, it has emerged as a major public health issue on a global scale. According to the Centers for Disease Control and Prevention, approximately one in every 365 Black or African American infants born in the United States is diagnosed with sickle cell disease. A birth prevalence study conducted in the United States between 2016 and 2020 indicated that the prevalence of SCD among non-Hispanic Black newborns was 28.54 per 10,000, translating to roughly one in every 350 births.[1] Furthermore, the American Society of Hematology reports that approximately 70,000 to 100,000 individuals in the United States are affected by sickle cell anemia, while around 2 million carry the sickle cell trait (SCT).[2]

In India, it was first described in the Nilgiri Hills of northern Tamil Nadu, and it is found in the central and eastern states like Gujarat, Maharashtra, Chhattisgarh, and Odisha.[3,4] According to a survey conducted by the Indian Council of Medical Research, approximately 20% of children diagnosed with SCD do not survive past the age of two. In comparison, 30% of those in tribal communities with SCD do not live to reach adulthood.[5]

Individuals diagnosed with sickle cell disease possess crescent-shaped red blood cells. This irregular morphology hinders the cells' ability to navigate through blood vessels effectively and obstructs blood flow to various organs, resulting in painful episodes called sickle cell crises. Furthermore, sickle cells have a shorter lifespan than healthy red blood cells, leading to a persistent deficiency of red blood cells and consequently, anemia. When a person has sickle cell trait, it indicates that they carry one copy of the gene responsible for sickle cell disease, resulting in a mixture of normal and sickle-shaped red blood cells. This condition is not classified as a disease. Generally, individuals with SCT lead normal lifespans. However, extreme dehydration and intense physical exertion can pose serious risks, including the potential for sudden death, for those with the trait. Carriers of the sickle cell gene have a 50% likelihood of transmitting the gene to their offspring, which implies that individuals with SCT may face the risk of having a child with either SCT or sickle cell disease. At present, there is no definitive cure for sickle cell disease; however, ongoing research is focused on pain management strategies and gene therapy. Thus, individuals with SCD possess two abnormal sickle cell genes inherited from both parents. In contrast, those SCT carry one normal hemoglobin gene and one sickle hemoglobin gene, with the latter inherited from one parent.

Sickle cell disease arises from a single nucleotide mutation in the beta-globin gene, leading to the replacement of a glutamic acid residue with valine at the sixth position. This alteration results in the formation of mutant hemoglobin known as hemoglobin S. When deoxygenated, this abnormal hemoglobin reveals a hydrophobic region surrounding the valine, which facilitates interactions between the beta chains of adjacent hemoglobin tetramers. Consequently, this interaction leads to the formation of long polymer bundles. The polymerization process diminishes the flexibility of the hemoglobin and distorts its shape, thereby impairing membrane fluidity and modifying its rheological characteristics in circulating blood. The combination of physical entrapment and adhesive interactions between red blood cells, leukocytes, and endothelial cells, exacerbated by secondary inflammation, contributes to the obstruction of the microvasculature.[6] This obstruction results in ischemia-reperfusion injury to essential organs, intensifying inflammatory and oxidative stress, activating the innate immune response, and precipitating infarction in critical organs such as the spleen, kidneys, liver, muscles, brain, lungs, and bones.[7] Cardiac complications frequently occur in SCD and are considered significant contributors to morbidity and mortality.

Sickle cell disease-associated anemia leads to several physiological abnormalities. The presence of anemia induces an elevation in heart rate and stroke volume, subsequently resulting in left ventricular dilation.[8] The extent of left ventricular dilation correlates directly with the severity of the anemia.[9] As the dilated left ventricle responds to the heightened wall stress, left ventricular hypertrophy develops.[10]

SCD is also associated with the development of pulmonary hypertension (PHT) which is of two types, precapillary and postcapillary. Precapillary pulmonary hypertension can be attributed to various factors, such as deficiency in nitric oxide, microvascular obstruction, chronic pulmonary thromboembolism, and vasoconstriction associated with anemia and hypoxemia. In contrast, postcapillary pulmonary hypertension is mainly linked to left ventricular dysfunction. Right heart catheterization reveals that approximately half of the patients with pulmonary hypertension exhibit signs of diastolic dysfunction.[11-13] Pulmonary pressure also rises during episodes of acute chest syndrome, leading to cor pulmonale.[14] Furthermore, direct myocardial injury resulting from microvascular disease and iron deposition has been suggested as contributing factors to cardiac abnormalities. It is essential to recognize that patients with SCD who develop pulmonary hypertension are at a markedly higher risk of mortality compared to those who do not have this condition.[15] Case reports of patients with acute chest syndrome (ACS) show electrocardiogram (ECG) abnormalities and their reversal is noticed following exchange transfusion and aggressive supportive care for the ischemia.[16-18] These findings have been attributed to acute and chronic microvascular occlusion in the setting of chronic endothelial damage.

ECG abnormalities commonly observed in SCD include bi-ventricular hypertrophy, prolonged PR intervals, QT prolongation, and nonspecific ST-T changes. Given that ECG is a non-invasive and readily accessible tool, it is crucial for identifying cardiac abnormalities such as myocardial infarction, left ventricular hypertrophy (LVH), and heart blocks.[19] However echo is the investigation of choice for detecting morphological abnormalities, cardiac function, and motion wall abnormality and it can pick up early most of the cardiac abnormalities associated with SCD.

Hormonal variations between males and females have been identified as a potential factor contributing to the higher morbidity observed in males.[20] Estrogen has been demonstrated to reduce the degradation of nitric oxide while simultaneously promoting its production, and it also plays a role in the transcriptional regulation of fetal hemoglobin. Females typically exhibit higher levels of HbF compared to males, and it is recognized that the severity of SCD is greater in patients with lower HbF levels. Additionally, diminished nitric oxide levels are linked to increased morbidity in individuals with SCD.[21]

The increased occurrence of complications related to SCD in males can be linked to their propensity for insufficient health-seeking behaviors and a hesitance to pursue hospital care during the initial phases of the condition. Additionally, greater exposure to stress and trauma resulting from occupational and physical activities may also contribute to this issue.[22]

Aim

Sickle cell crises encompass sequestration, vaso-occlusive, and aplastic crises. During the intervals between these acute episodes, individuals experience a condition of relative well-being known as the "steady state." This study highlights the diverse cardiac abnormalities linked to CSD and SCT.

Material and methods

This retrospective study included patients who presented to the outpatient and inpatient Department of General Medicine at SUM Hospital, Bhubaneswar, between June 2022 and June 2023. Medical records were reviewed to identify individuals with confirmed diagnoses of sickle cell disease or sickle cell trait, as determined by hemoglobin electrophoresis. The study population consisted of both newly diagnosed and follow-up cases. Patients were excluded if their records indicated a sickle cell crisis within the preceding three months, electrolyte imbalances, or known structural cardiac abnormalities.

Study procedure

Patients who met the inclusion criteria including both newly diagnosed and follow-up cases were identified through medical records. Their clinical notes, along with electrocardiography and echocardiography data, were subsequently retrieved. A standardized resting 12-lead ECG was recorded using the Cardioline AR-600 machine, with a calibration of 0.1 mV/mm and a paper speed of 25 mm/s. All ECGs were interpreted by a single observer based on established American Heart Association (AHA) criteria.

In this study, ECG records were analyzed using standard diagnostic criteria to identify various cardiac abnormalities. LVH was defined by: (1) The sum of SV1 and R wave in lead V5 or V6 must exceed 35 mm for individuals over 26 years of age, and exceed 53 mm for those between 18 and 25 years. (2) The voltage in lead AVL should be greater than 13 mm. The criteria for abnormal ST-T wave patterns include: (1) A depression or elevation (convex) of the ST segment from the baseline greater than 1 mm, and (2) T waves that are notched, excessively peaked, biphasic, flat, or inverted. The criteria for left anterior hemiblock consist of: (1) A left axis deviation with a QRS axis ranging from -30° to -90°, (2) The presence of a small Q wave in leads I and AVL, and (3) A QRS duration of less than 0.12 seconds in the case of an isolated defect.

Archived echocardiographic data were also reviewed. Echocardiograms had been performed using a Hewlett Packard Sonos 2500 ultrasound machine with a 3.7 MHz transducer. The evaluated parameters included cardiac chamber dimensions, regional wall motion, global ventricular function, valvular morphology and function, and abnormal intracardiac flow patterns. Pulmonary artery systolic pressure (PASP) was non-invasively estimated by measuring the tricuspid regurgitant jet velocity using continuous-wave Doppler imaging. Pulmonary hypertension was defined as an estimated mean pulmonary artery pressure (mPAP) exceeding 25 mmHg. Right ventricular systolic pressure (RVSP), equivalent to PASP, was determined using tricuspid regurgitant velocity (TRV). The mPAP was calculated using the formula: mPAP = RVSP×0.6+2.1. None of the patients exhibited pulmonary stenosis.

Ethical consideration

Ethical approval for the study was obtained from the Institutional Ethics Committee – Clinical Research & Studies, SUM Ultimate Medicare, Bhubaneswar (Registration No. ECR/1604/Inst/OD/2021). The study did not interfere with standard clinical care or therapeutic management. The requirement for written informed consent was waived by the ethics committee.

Data analysis

Statistical analyses were conducted using Statistical Packages for Social Sciences (SPSS) software version 23. Data were presented as means ± standard deviation/median as appropriate for continuous variables and as

proportions for categorical variables. Univariate and multivariate logistic regression analyses were used to identify the association of the clinical parameters with sickle cell disease. A $p<0.05$ was considered statistically significant.

Results

The study population consisted of 34 cases, which included 23 individuals with SCD and 11 with SCT. The analysis of gender distribution reveals a higher prevalence of males in most age groups, with a male-to-female ratio of 17:6 for SCD and 7:4 for SCT. A significant number of cases were identified within the 15–25 age range, followed by the 26–35 age group, indicating an earlier age of onset, as presented in Table 1.

Table 1. Distribution of individuals in the study population

Age in years	15–25		26–35		36–45		46–55	
Sex	Male	Female	Male	Female	Male	Female	Male	Female
HBSS	7	3	4	2	6	1	0	0
HBAS	3	1	2	1	1	1	1	1
Total	10	4	6	3	7	2	1	1

Among patients with Sickle Cell Disease, 47% (n=11) exhibited hypertension, 60% (n=14) demonstrated an apex beat shift, and 21% (n=7) presented with a clinically detectable S3. In contrast, individuals with SCT showed lower prevalence rates: 27% (n=3) had hypertension, 9% (n=1) displayed an apex shift, and 18% (n=2) had a detectable S3. These findings suggest a higher incidence of cardiovascular abnormalities in sickle cell disease compared to sickle cell trait, as presented in Table 2.

Table 2. Distribution of clinical features in the study population

Clinical features	SCD (n=23)	SCT(n=11)	Total (n=34)
Hypertension	11 (47%)	3 (27%)	14 (41%)
Apex shift	14 (60%)	1 (09%)	15 (44%)
S3	7 (21%)	2 (18%)	9 (20%)

ECG analysis of patients with sickle cell disease revealed a range of cardiac abnormalities. LVH was the most prevalent finding, observed in 52% (n=12) of SCD patients. ST-T wave changes were noted in 34% (n=8), while QT interval prolongation was present in 20% (n=5) of cases. Less frequent findings included pre-ventricular contractions in 8% (n=2) and first-degree heart block in 4% (n=1) of SCD patients. In contrast, individuals with SCT exhibited a significantly lower incidence of ECG abnormalities, with ST-T wave changes being the only abnormality detected in 11% (n=2) of cases, as detailed in Table 3. These findings emphasize the substantial cardiac involvement in SCD compared to the sickle cell trait, underscoring the necessity for comprehensive cardiac evaluation and monitoring in SCD patients.

Table 3. Distribution of ECG features in the study population

ECG features	SCD (n=23)	SCT(n=11)	Total (n=34)
Left ventricular hypertrophy	12 (52%)		12 (35%)
ST – T changes	8 (34%)	2 (11%)	10 (29%)
QT prolongation	5 (20%)		5 (14%)
Pre ventricular contraction	2 (08%)		2 (06%)
1st degree heart block	1 (04%)		1 (03%)

Echocardiographic evaluations of patients with sickle cell disease revealed a spectrum of cardiac abnormalities. Left ventricular hypertrophy was observed in 43% (n=10) of SCD patients, while dilated left ventricle and motion abnormalities were each identified in 9% (n=2) of cases. Additionally, heart failure with preserved ejection fraction (HFpEF) was detected in 4% (n=1) of SCD patients, and Pulmonary Hypertension was present in 13% (n=3). In contrast, left ventricular hypertrophy was the sole cardiac abnormality observed among patients with sickle cell trait, affecting 27% (n=3) of individuals. These findings are summarized in Table 4.

Table 4. Distribution of echocardiographic features in the study population

ECHO features	SCD (n=23)	SCT(n=11)	Total (n=34)
Left ventricular hypertrophy	10 (43%)	3 (27%)	13 (38%)
Dilated LV	2 (09%)		2 (06%)
Motion abnormality	2 (09%)		2 (06%)
HFpEF	1 (04%)		1 (03%)
PHT	3 (13%)		3 (08%)

The univariate logistic regression analysis presented apex shift, mean PAP (mean pulmonary arterial pressure), PASP (pulmonary artery systolic pressure), and TRV (tricuspid regurgitation velocity) as independent predictors of sickle disease while HTN, S3, LVH, ST changes, and others were not found significant. Table 5 indicates that apex shift has a strong positive association with the outcome, with an odds ratio (OR) of 15.556 (95% CI: 1.7–143.17, p=0.015), suggesting that individuals with apex shift are significantly more likely to experience sickle cell disease compared to those who experience sickle cell trait. Conversely, mean PAP, PASP, and TRV showed predictive associations with the outcome, with odd ratios of 0.595 (95% CI: 0.412–0.861, p=0.006), 0.73 (95% CI: 0.587–0.909, p=0.005), and 0.015 (95% CI: 0.001–0.256, p=0.004), respectively (Table 5).

Table 5. Univariate binary logistic regression analysis presents the association between the mentioned variable and the outcome

Variable	Odds ratio	Lower	Upper	p
Apex shift	15.556	1.7	143.17	0.015
Mean PAP	0.595	0.412	0.861	0.006
PASP	0.73	0.587	0.909	0.005
TRV	0.015	0.001	0.256	0.004
HTN	2.44	0.514	11.61	0.261
S3	1.96	0.335	11.57	0.453
LVH	2.909	0.612	13.828	0.179
ST changes	0.329	0.415	13.895	0.329

The multivariable logistic regression analysis, conducted after considering variables significant in the univariable analysis, shows that PASP is an independent predictor of sickle cell disease with an adjusted odd ratio of 0.73 (95% CI: 0.587–0.909) as shown in Table 6. These findings highlight the clinical significance of PASP in sickle cell disease. The adjusted odd ratio suggests that increases in PASP are associated with a decreased likelihood of sickle cell disease, indicating a potential protective effect.

Table 6. The multivariable logistic regression analysis of significant univariable analysis

Variable	Odds ratio	Lower	Upper	p
TRV	665.90	0	4.09	0.529
PASP	0.73	0.587	0.909	0.005
Apex shift	5.019	0.387	65.172	0.217

Discussion

Sickle cell anemia is associated with significant electrocardiographic abnormalities. Previous research has examined the clinical manifestations, ECG findings, and echocardiographic changes separately, however, this study integrates all these aspects into a single comprehensive analysis. Cardiac complications associated with sickle cell disease are assessed through clinical evaluations, as well as utilizing electrocardiograms and echocardiograms.

The blood pressure (BP) of children and adolescents diagnosed with SCD has been observed to be lower than that of the general pediatric population.[23,24] To establish a definition for hypertension in these patients, prior studies have introduced the concept of relative systemic hypertension (RSH). This condition is defined by systolic blood pressure measurements ranging from 120 to 139 mmHg and diastolic readings between 70 and 89 mmHg. Within this blood pressure range, patients with SCD have shown a decline in renal function and an increased risk of developing pulmonary hypertension. However, in our study hypertension was present in 47% of the cases based on 2017 American College of Cardiology (ACC) guidelines for defining hypertension.[25] The result of our study aligns with recent research that has shown that patients with SCD frequently have increased blood pressure, as evidenced by 24-hour ambulatory blood pressure monitoring (ABPM). Furthermore, children diagnosed with sickle cell disease have been noted to exhibit abnormal blood pressure patterns, which encompass ambulatory hypertension, diminished nocturnal blood pressure dipping, and masked hypertension.[26,27]

The shifting of cardiac apex and the presence of a third heart sound indicate a hyperdynamic circulatory state resulting from chronic anemia, rather than from myocardial failure. This conclusion is supported by the fact that the majority of cases examined in this study were in a steady state. In this study, apex shift is present among 60%, similar to a study done in Nigeria.[28] Heart sound S3 was identified in 21% of the patients. These findings are lower than those from previous research conducted within a pediatric population, which reported S3 in 70% of cases involving SCD.[29] This difference may be attributed to the higher likelihood of S3 occurring in normal pediatric hearts.[30]

Electrocardiographic abnormalities are frequently observed in individuals with SCD.[31] Various studies have documented differing percentages and types of ECG changes, with left ventricular hypertrophy (LVH), QT prolongation, and ST-T changes being the most prevalent. LVH is recognized as a compensatory response to chronic anemia. In our research, LVH was identified as the most common ECG alteration, occurring in 52% of cases, consistent with findings by Oguanobi et al. in a Nigerian study.[28] A similar prevalence was also reported by Aluko et al. in 1985.[32] ST-T changes and QT prolongation are repolarization abnormalities.[33] ST-T changes and QT prolongation are classified as attributed to both acute and chronic microvascular occlusion, which is associated with chronic endothelial damage.[34] In the present study, ST-T changes were observed in 34% of participants, which aligns closely with the aforementioned Nigerian study.[28] In this study, QT prolongation was noted in 20% of cases, which falls within the range reported by Anah MU et al.[35] QT prolongation indicates abnormal repolarization due to various factors and is also recognized as a characteristic of myocardial ischemia.[36] Ventricular premature complexes and first-degree heart block, are less frequently observed in our study likely attributable to ischemic foci arising from perfusion and reperfusion injuries. This aligns with other studies that have indicated a lower prevalence.[37,38]

Chronic anemia associated with sickle cell disease is typically well-compensated through mechanisms such as increased cardiac output, while minimally increasing the heart rate. The left ventricle, which becomes dilated, responds to the heightened wall stress by undergo-

ing eccentric hypertrophy. This adaptation enables the left ventricle to manage chronic volume overload. However, as time progresses, the continued dilation results in heightened wall stress and an increase in left ventricular mass.[39] Studies suggest that LVH could represent an initial phase in the onset of pulmonary hypertension among individuals with SCD.[40] Nevertheless, the timeline and the underlying mechanisms through which LVH advances to pulmonary hypertension remain unclear. Cardiopulmonary abnormalities frequently observed in SCD include pulmonary hypertension, enlargement of both the right ventricular and left ventricular chambers, an increase in LV mass, and diastolic dysfunction of the LV, which serves as an independent predictor of mortality.[41,42] Consistent with other research left ventricular hypertrophy was identified as the most common abnormality, occurring in 38% of the patients. This finding also aligns with the work of Aluko et al. and de Almeida Faro et al.[32,43]

Recent extensive echocardiographic screening studies suggest that contrary to the traditional view that heart failure is prevalent among adult patients with SCD, left ventricular systolic function remains intact in most SCD patients when assessed at rest, and the occurrence of segmental wall motion abnormalities is infrequent.[44,45] Our findings corroborate this, revealing that only 3% of participants exhibited HF, while the majority maintained a preserved ejection fraction. Additionally, left ventricular dilation (LVD) was observed in 7% of our study cohort, contrasting with a 2002 study conducted in New York, which reported a prevalence of 13%.[46] This discrepancy may be attributed to the association of LVD with increased afterload and renal dysfunction in these patients.[36]

Segmental wall motion abnormalities are infrequently observed in sickle cell disease, consistent with our study findings, where merely 6% of participants exhibited such motion abnormalities.[47] PHT is emerging as a significant complication of vasculopathy in SCD. In the current study, pulmonary hypertension PHT was identified in 13% of the cases, which is significantly lower than the 58% prevalence reported in the previously mentioned New York study.[46] The higher incidence of pulmonary hypertension in the New York study may be attributed to factors such as advanced age and a prior history of acute chest syndrome. Recent studies indicate that pulmonary hypertension, as identified through right heart catheterization, is prevalent among adult patients with sickle cell disease, with reported rates ranging from 6% to 11%, which aligns with our study.[48] Pulmonary hypertension resulting from intravascular hemolysis is associated with a heightened risk of mortality in patients with sickle cell disease.[49]

SCT has long been regarded as a harmless condition; however, recent research indicates that individuals with this trait may have cardiovascular risk, kidney damage, and an increased risk of sudden death.[50,51]

We acknowledge the constraints of our study, which include the lack of a control group and the unavailability of hemodynamic data obtained through right heart catheterization. Additional limitations include the small sample size and the baseline disparities observed between the two groups. Furthermore, variations arise from differences in the study setting, participant characteristics, and interviewer criteria, among other factors.

Conclusion

Cardiovascular manifestations are fewer among SCT but found significant among SCD. When compared with western studies, our study exhibits less prevalence of cardiovascular changes. Thus a comprehensive cardiac evaluation is essential for patients diagnosed with sickle cell disease rather than those with sickle cell trait, as the former group exhibits a greater prevalence of cardiac manifestations and findings. Timely identification of the disease can prevent the advancement of complications.

Declarations

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Author contributions

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

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

Data availability

Due to privacy and confidentiality concerns, the data are not publicly available. However, they can be made available upon reasonable request to the corresponding author, subject to the completion of a signed data access agreement.

Ethics approval

This study was approved by the Institutional Ethics Committee - Clinical Research & Studies, SUM Ultimate Medicare, Bhubaneswar (Registration No. ECR/1604/Inst/OD/2021).

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ORIGINAL PAPER

Zn and Se protect toxic metal mixture-mediated memory deficit by activation of Nrf2-hmox-1 signaling in the hippocampus of female rats

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ABSTRACT

Introduction and aim. Heavy metals mediate neurotoxicity by altering some signaling pathways. This work investigated the effect of two nutritional elements Zn and Se on neurotoxicity caused by toxic metals.

Material and methods. Female Sprague Dawley rats (35) were divided in to 5 groups (7 rats/group), treated as follows: orally received deionized water (group I), heavy metals mixture HMM: 20 mg·kg $^{-1}$; 0.40 mg·kg $^{-1}$ of Hg; 0.56 mg·kg $^{-1}$ of Mn; and 35 mg·kg $^{-1}$ of Pb body weight; of Al (group II), HMM+Zn (zinc chloride; 0.80 mg·kg $^{-1}$) (group III), HMM+Se (sodium selenite: 1.50 mg·kg $^{-1}$) (group IV) and HMM+Zn+Se (group V). Before euthanasia of the rats, the Cincinnati Dry Maze, Barnes Maze, and Rotarod tests were performed. After euthanasia, the hippocampus was examined biochemically and histopathologically.

Results. Treatment with HMM induced inflammation with increased concentration of interleukin 6 and tumor necrosis factor α (30.70±6.65, p<0.02 pg/mg protein and 14.20±4.81 pg/mg protein, p<0.0001) respectively, compromised antioxidants levels, potentiated lipid peroxidation (higher malondialdehyde at 0.94±0.04 nmol/g tissue, p<0.0001 and nitric oxide at 3.89±0.16 nmol/g tissue, p<0.0001 levels), disrupted Nrf2 signaling pathway, potentiated acetylcholinesterase activity and induced mild pathohistological alterations in the rat hippocampus.

Conclusion. Supplementation with Zn and Se attenuated HMM mediated neurotoxicity.

Keywords. essential trace elements, heavy metal mixture, neurobehavioral testing, neurotoxicity, oxido-inflammation

Introduction

The hippocampus is one of the key regions of the limbic system. The hippocampus is broadly connected with other gray masses in the brain; through the Papez circuit, the hippocampus is connected with other relevant limbic regions in the medial limbic lobe and the hypothalamus. In addition, through the mesolimbic pathway, the hippocampus is linked to the ventral tegmental area,

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which is the main source of dopamine for the entire limbic system. Limbic system in general, is deeply involved in emotions, moreover, the hippocampus "decides" which new information will enter a long-term memory and which newly acquired information will get forgotten. Therefore, any potential damage to the hippocampus could lead to memory impairment and humans are continuously exposed throughout life to various metals such aluminum (Al^{3+}), lead (Pb^{2+}), mercury (Hg^{2+}) and manganese (Mn^{2+}) which have been shown to be neurotoxic.[1-5] In most developing nations such as Nigeria and other sub-Saharan African countries, various anthropogenic activities including e-waste have negatively impacted the environment with studies showing contamination of potable water and food, etc. with heavy metals (Pb, Hg, Al, and Mn).[6-11]

Aluminum lurks in infant milk formulas,[12] vaccines[13] and various foodstuffs.[14] Jin et al. in their study showed that grains and vegetables are contaminated with lead, whereas tea was measured with highest lead levels (1.937 mg/kg).[15] Fish and seafood represent major source of mercury; moreover, Feingold et al, showed that regions such as Madre de Dios showed that 37.7% of the people living in mentioned regions of Peru exceed the World Health Organization (WHO) recommended mercury permissible level (>2.2 μg/g).[16] Such high levels of mercury were linked with the consumption of fish. It is reasonable to speculate that the majority of mentioned foodstuffs are used daily, i.e. all meals contain them and consequently humans are exposed to heavy metals via gastrointestinal route throughout their entire life.

All studies conducted so far showed that heavy metals-induced neurotoxicity is provoked by oxidative stress, increased lipid peroxidation and diminution of antioxidants.[2] Bittencourt et al. showed that treatment with Al *per os* in rats mediated changes in protein expression and decreased cellular density in some regions of the hippocampal regions such as the cornu ammonis 1 (CA1), CA3 and dentate gyrus;[4] nevertheless, majority of studies that examined adverse effects of heavy metals were focused on a single metals exposure.[2,4] Limitations of such studies are that individuals are exposed to cocktail of metal mixtures on the daily basis, and to a single metal; next, particular combinations of metal mixtures have synergistic hazardous effects such as Pb and Hg.[17] On the other side what remains unknown is which intracellular signaling pathways is dysregulated by heavy metals and as result of ROS production and antioxidant depletion appear.[18,19]

Deficiencies in Se and Zn are linked with decreased activities of some antioxidants; also metal transporter 1 (DMT1), a protein largely expressed in the proximal duodenum, could uptake Zn and Pb and since both metals compete for the same binding place, as a consequence Zn could decrease the bioavailability of heavy metals and as consequently provide some protective effects.[20]

Aim

In view of these, this study has evaluated (a) modulation of hippocampal apoptosis, intracellular glutathione stores, and Nrf2-hmox-1 signaling in rats by the quaternary toxic metal mixture (Pb, Hg, Mn; and Al) and (b) potential neuroprotective effects of Zn and Se against HMM-induced toxicity, after oral exposure.

Materials and methods

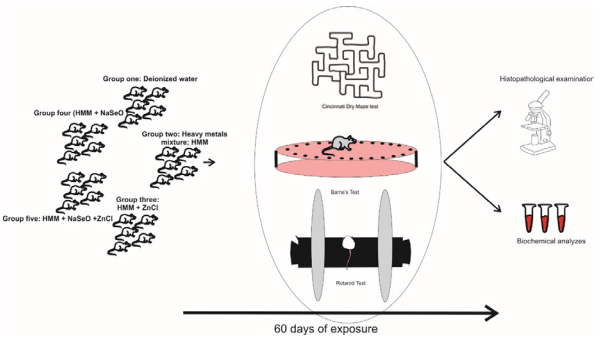

Fig. 1. Schematic representation of methodological approach

Animals' husbandry and treatments

The animal husbandry and treatment protocol adapted our previous protocol reported elsewhere.[21] Briefly, we purchased 35 female Sprague Dawley rats (6-8 weeks of age) from the Faculty of Pharmacy, Animal House, University of Port Harcourt (Animal House), Rivers State, Nigeria. Sprague-Dawley rats were kept at an ambient temperature of 25±2°C with 12-h light/dark cycles. Before the beginning of this study, the Sprague Daley rats were adapted for fourteen days. The rats were placed in five groups (7 per group). The experiment lasted 60 days and treatments were performed by an oral route. Rats in group 1 or controls received deionized water. Group 2 received Pb, Hg, Mn and Al (20 $mg \cdot kg^{-1}$ [22] Pb; 0.40 $mg \cdot kg^{-1}$ of Hg [22]; 0.56 $mg \cdot kg^{-1}$ of Mn; and 35 $mg \cdot kg^{-1}$; of Al [23-25] body weight as a mixture (Sigma Aldrich WGK Germany). Groups 3,4 and 5 received HMM and in addition to 0.80 mg/kg $ZnCl_2$[26], sodium selenite 1.50 mg/kg Na_2SeO_3;[27] and both $ZnCl_2$ and Na_2SeO_3, respectively. Randomization and blinding were used to reduce bias. Doses that mimicked environmental availability, as previously reported in our lab, were used.[28] The metal mixture was rated low,[22] and even lower doses were used elsewhere.[30-31]

Feed and water intakes were recorded every day. The weight of the rats was recorded every week. Pentobarbital (50 mg/kg, ip) was used as an anesthesia agent in sacrificing the animals. The hippocampus of each rat was dissected, separated as previously described in our

laboratory.[26] This was used for both biomarkers and determination of Pb, Hg, Mn and Al.

The protocol was approved by the Research Ethics Committee of the University of Port Harcourt (UPH / PUTOR / REC/12). The study was in line with the "Guide for the Care of Laboratory Animals" ratified by the National Academy of Science (NAS). The rats received standard food and deionized water without restriction.

Cincinnati Dry Maze Test

Cincinnati Maze was employed in the evaluation of egocentric navigation, learning, and memory, as described previously.[32]

The rat was kept at the door of the box and the entrance closed, and allowed it to navigate through the box to the exit, the time it took the rat to navigate its way from the entrance point to the exit point is recorded. The rat was timed for 300 seconds and was expected to find its way to the exit point within that time, the shorter the time it completed its task, the more intact the memory. If the rat cannot find its way to the exit within 5 minutes, the task was assumed to be incomplete.

Barnes maze

Furthermore, Barnes maze is a dry land maze used as a test for visual-spatial learning and memory (hippocampal damage).[33]

The rat was put on the Barnes apparatus and observed for 300 seconds until it located the safe spot. The time to locate the safe spot by the rat was recorded. If the rat was unable to identify the safe area, 0 or 1 is reported.

Rotarod test

This used an accelerating rotarod as described by Jacquez and co-workers.[34] This was done on a rotarod to assess the riding time or endurance, grip strength, motor balance, and coordination of the animals and check for cerebellar damage. This includes a task performed on an accelerating rotarod to determine how long an animal can sustain walking on a rolling rod. On the rotarod, rats were put on a rotating drum (30 mm diameter, 85-mm width). It assesses the rat's capacity to feel equilibrium by measuring how long it lasts on a rotating drum. Over the course of 5 minutes, the rod's velocity increased from 4–20 revolution per minute, and the time to fall was measured. The rats were given at least some time to rest between trials to reduce stress and exhaustion.

Enzyme-linked immunosorbent assay (ELISA) analysis

The assay that determined the levels of cytokines, transcription factors, acetylcholinesterase (AChE), caspase an apoptotic marker in the hippocampus, was previously described in another study.[35]

Oxidative stress markers

The glutathione peroxidase (GPx) was carried out using Paglia and Valentine method,[36] while reduced glutathione (GSH) was Marklund and Marklund method.[37] The method of Habig et al. was to determine glutathione-S-transferase (GST).[37] The activity of superoxide dismutase (SOD) activity was assayed using the method of Jollow et al.[38] Catalase (CAT) activity was estimated by monitoring the rate of breakdown of H_2O_2 at 240 nm according to Aebi's method.[39]

Lipid peroxidation was determined as reactive substances with thiobarbituric acid by the adaptation of Ester Bauer and Cheeseman method at 532 nm. The Griess reaction technique was adapted in the analysis of nitric oxide (NO).[41] In all UV-vis spectroscopy (Agilent Technologies, Cary 8454, UV–visible spectrophotometer, Santa Clara, California, USA) was used.

Estimation of Al, Mn, Pb and Hg levels in the hippocampus

The hippocampal levels of Al, Mn, Pb, and Hg were determined using the atomic absorption spectrometer.[42]

Histopathological examination

Rats were sacrificed one week after the behavioral test. Histopathological slides were prepared and examined as previously described in our lab.[26]

Statistical analysis

Data were expressed as mean±SD. Microsoft Xlstat 2014 was used in performing ANOVA and Tukey multiple comparison pairwise tests all significant differences were at a $p<0.05$.

Results

Absolute and relative weight of the hippocampus; feed and fluid intakes of female Sprague-Dawley rats after HMM (Pb, Mn, Hg, and Al)

After the end of the experiment, the control group 1 had higher body weight compared to the HMM, HMM+Zn, HMM+Se and HMM+Zn+Se groups ($p<0.05$); moreover, HMM and HMM+Zn animals had a higher body weight compared to HMM+Se and HMM+Zn+Se ($p<0.05$). On the other hand, feed and water intakes were similar between groups ($p<0.05$). Also, the weight of the hippocampus did not vary between the examined groups ($p<0.05$). The HMM-exposure rats had significantly higher hippocampus weight compared to either controls ($p<0.05$) or the HMM+Zn, HMM+Se and HMM +Zn+Se groups ($p<0.05$) (Table 1).

Accumulation of heavy metals in the hippocampus

Effect of essential elements on heavy metal accumulation in the hippocampus

The effect of the combination of Zn alone, Se alone, and Zn+Se on heavy metals accumulation in the hip-

pocampus is shown in Figure 2. We found the following percentage of reduction 44.53% in HMM+Zn, 42.34 in HMM+Se, and 51.82 in HMM+Zn+Se. The HMM+Zn animals had a mean Hg value of 0.092±0.002 mg/kg, HMM+Se had a mean value of 0.089±0.002 mg/kg and HMM+Zn+Se had a mean value of 0.080±0.001 mg/kg (Fig. 2A).

Table 1. Effect of Zn and Se on body weight, absolute and relative weight of hippocampus feed and fluid intakes of female Sprague Dawley rats after exposure to heavy metal mixtures (Pb, Mn, Hg, and Al)

Treatment	Absolute (g)	Relative (%)	Body weight (g)	Feed intake (g)	Fluid intake (mL)
Control	0.12±0.08^a	7.04	200.00 ±14.14	154.10 ±23.42^a	242.98 ±35.36^a
HMM only	0.10±0.01^a	5.28	180.50 ±0.71^b	148.27 ±16.42^a	210.07 ±33.46^a
HMM+Zn (0.8 mg/kg)	0.10±0.01^a	6.06	162.50 ±12.02^b	147.94 ±22.13^a	195.62 ±43.20^a
HMM+Se (1.5 mg/kg)	0.13±0.08^a	8.02	155.50 ±0.71^c	127.63 ±35.47^a	209.74 ±51.27^a
HMM+Zn +Se	0.10±0.03^a	6.25	155.00 ±7.07^c	127.82 ±29.85^a	203.85 ±53.26^a

* mean±SD, dissimilar characters a, b, c, d denote a significant difference between the means at p<0.05 but when they are the same there is no significant difference (n=5)

The following concentrations of Pb, 0.448±0.006 mg/kg, 0.511±0.009 mg/kg and 0.410±0.003 mg/kg for HMM+Zn, HMM+Se and HMM+Zn+Se rats, respectively, were observed. The essential elements show a percentage reduction of 38.38 in HMM+Zn rats, while HMM+Se rats showed a percentage reduction of 29.78 and HMM+Zn+Se rats showed a percentage reduction of 43.60 (Fig. 2B).

A mean concentration of Mn in HMM+Zn rats was 0.038±0.001 mg/kg, 0.040±0.003 mg/kg in HMM+Se rats, and 0.033±0 mg/kg in HMM+Zn+Se rats. The percentage reduction in HMM +Zn was 48.02, HMM+Se showed a percentage reduction of 49.72 and HMM+Zn+Se showed a percentage reduction of 59.80 (Fig. 2C).

The effect of the combination of Zn only, Se only, and Zn+Se caused significant decrease (p<0.05) in the level of aluminum in the hippocampus (Fig. 2D). HMM+Zn had a mean aluminum concentration of 0.407±0.002 mg/kg, HMM+Se had a mean value of 0.539±0.007 mg/kg and HMM+Zn +Se had a mean value of 0.388±0.007 mg/kg. HMM+Zn had a percentage reduction of 57.52, HMM+Se had a percentage reduction of 43.67 and HMM+Zn+Se had a percentage reduction of 59.46 due to treatment with essential trace elements (Fig. 2D).

Effects of HMM (Pb, Mn, Hg and exposure to Al) and Zn and Se on antioxidants (SOD, GPx, CAT, GSH) and MDA and NO levels in the hippocampus of female Sprague Dawley rats

SOD activity decreased significantly in rats exposed to HMM in comparison to controls (p<0.05). The SOD activity was the same as in the HMM and HM-

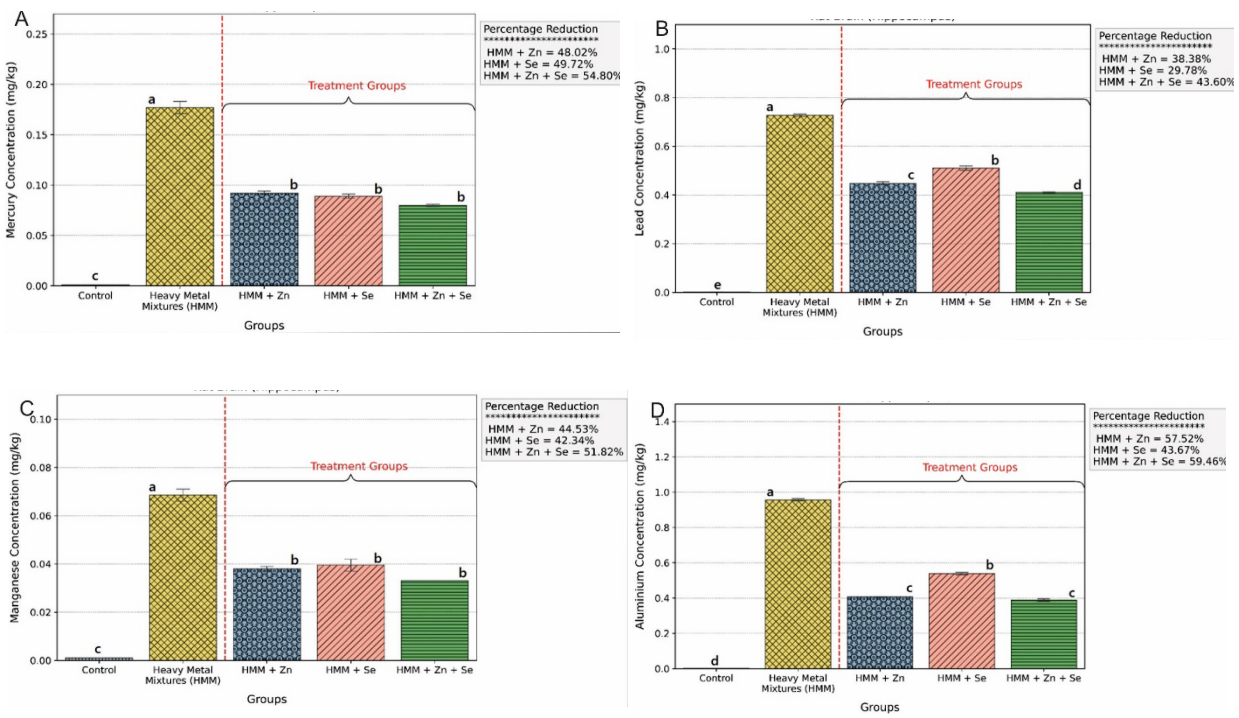

Fig. 2. Graphical representation of heavy metal accumulation in the hippocampus – A: mercury, B: lead, C: manganese and D: aluminum (mean±SD, dissimilar characters a, b, c, d denote significant difference between the means at p<0.05 but when they are the same there is no significant difference (n=5)

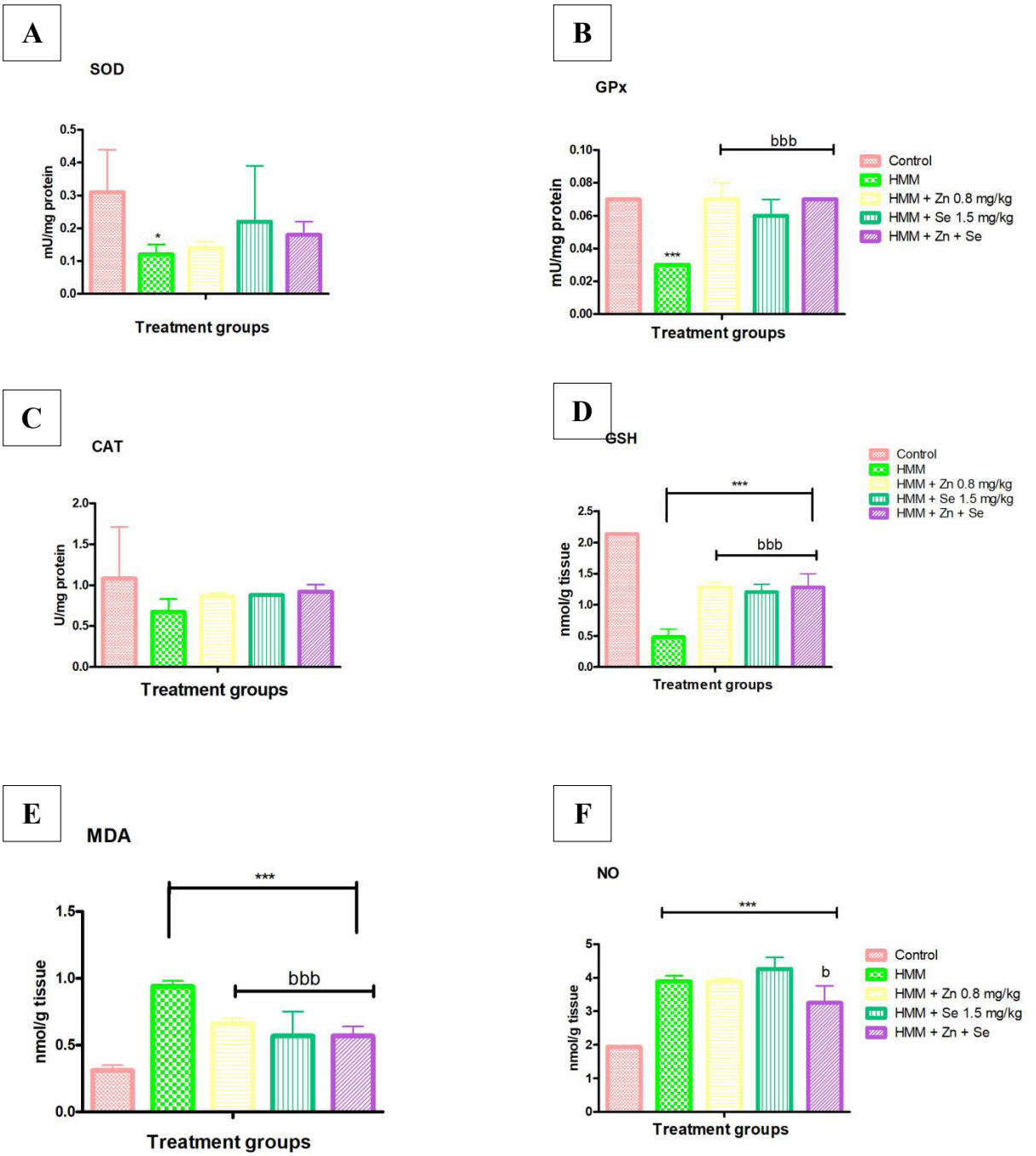

Fig 3. Effect of essential elements on antioxidants (SOD, GPx, CAT, GSH) and MDA and NO levels in the hippocampus of female Sprague-Dawley rats after HMM (Pb, Mn, Hg, and Al) (*** – significantly different compared to control at p<0.001; ^{bbb} – significantly different compared to HMM at p<0.001)

M+Zn groups of animals; however, HMM+Se and HMM+Zn+Se animals had the SOD activity like unexposed controls rats and significantly higher than HMM exposed rats (Fig. 3A). GPx levels did not vary between controls, HMM+Zn, HMM+Se and HMM+Zn+Se groups (p> 0.05), while the controls, HMM+Zn, HMM+Se and HMM+Zn+Se groups (p>0.05), while all four mentioned groups (controls, HMM+Zn, HMM+Se, and HMM+Zn+Se) had higher (p<0.05) levels compared to the HMM treated rats (Fig. 3B). CAT activity was similar (p>0.05) between the five examined groups of animals (Fig. 3C). We observed a significant decrease in GSH levels in the HMM treated animals compared to controls (p<0.05), while Se and Zn supplementation significantly but incompletely recovered GSH levels; that is, all three groups co-treated with essential trace metals (HMM+Zn, HMM+Se and HMM+Zn+Se) had higher GSH levels higher than HMM exposed group (p<0.05)

and significantly lower than unexposed controls rats ($p<0.05$) (Fig. 3D).

HMM-treated rats had higher levels of MDA compared to the unexposed controls rats ($p<0.05$), moreover, all three groups co-treated with essential trace metals (HMM+Zn, HMM+Se and HMM+Zn+Se) had MDA levels higher than controls ($p<0.05$) and significantly lower ($p<0.05$) than HMM-exposed animals (Fig. 3E). In Figure 3F, HMM, HMM+Zn, HMM+Se and HMM+Zn+Se had similar NO levels while ($p>0.05$). Unexposed controls rats had lower ($p<0.05$) NO levels ($p <0.05$) compared to groups treated with heavy metals treated groups (HMM, HMM+Zn, HMM+Se and HMM+Zn+Se).

Effect of Zn and Se on levels of inflammatory cytokines (IL-6 and TNF-α), transcription factors (Nrf2 and NF-kB) and caspase 3 in the hippocampus of female Sprague Dawley rats after exposure to HMM (Pb, Mn, Hg, and Al)

There were significantly higher levels of IL-6 and TNF-α in rats exposed to HMM compared to controls not exposed (Fig. 4A and 4B). Regarding IL-6, we obtained that all three groups co-treated with HMM and Zn, Se and Zn+Se had similar levels as controls did ($p>0.05$), while on the other side, all three groups co-treated with HMM and Zn, Se had significantly ($p<0.05$) lower levels of TNF-α compared to the group treated only treated group.

The Nrf2 level decreased significantly in animals exposed to HMM only when compared with the control, HMM+Zn, HMM+Se and HMM+Zn+Se (Fig. 4C). There was a significant increase in Caspase-3 (Casp-3) levels in animals exposed to HMM compared to the unexposed control (Fig. 4D). HMM+Zn, HMM+Se and HMM+Zn+Se had significantly lower Casp-3 levels than exposed rats (Fig. 4E).

Effect of Essential Elements Zn and Se on Cincinnati Dry Maze Test, Barnes and Rotarod Performance Tests

Cincinnati Dry Maze Test

Figure 5A represents the Cincinnati Dry Maze performance test of rats exposed to HMM treated with Zn and Se. The unexposed control group showed significantly lower escape latency in comparison to HMM exposed rats. The HMM exposed rats showed significantly ($p<0.05$) higher escape latency than the unexposed control. The escape latency of the groups co-treated with essential elements decreased significantly compared with the HMM only group.

Barnes test

The Barnes performance test is shown in Figure 5B. The unexposed group had a lower escape latency time for locating the hole when compared with the HMM only exposed group. The escape latency of the groups treated with Zn alone,, Se only and the combination of Zn plus Se was significantly ($p<0.05$) lower than the group with HMM only group.

Rotarod test

The performance test is shown in Figure 5C. Animals treated with essential elements in the Rotarod test are shown in Figure 4C. Rats that received deionized water (control group) had significantly ($p<0.05$) longer fall

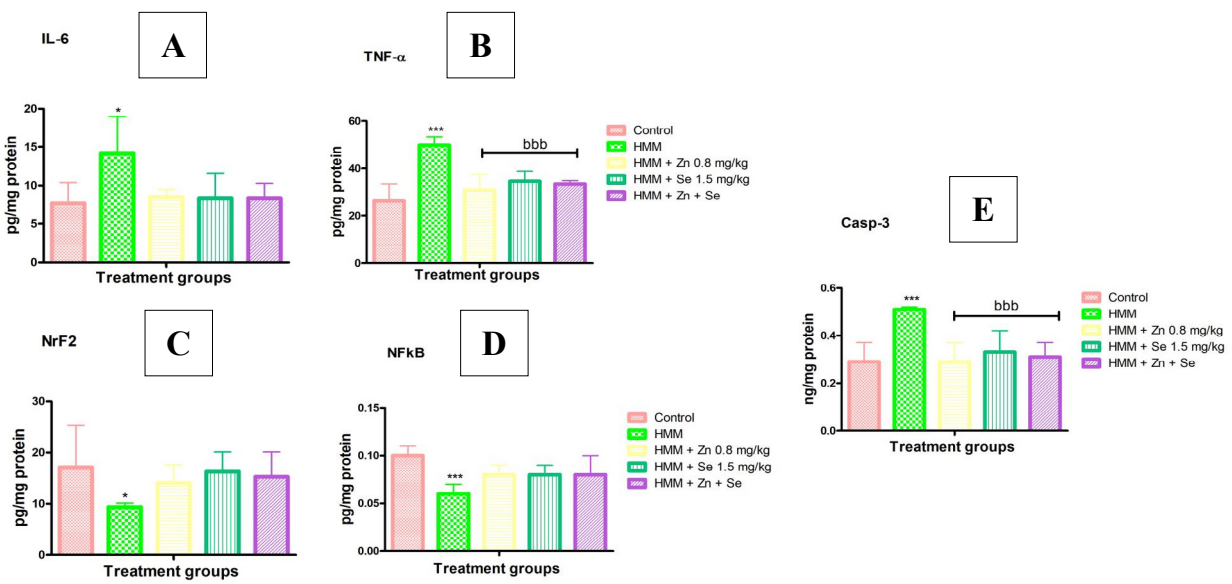

Fig. 4. Effect of Zn and Se on the pro-inflammatory cytokines IL-6, TNF-α, transcription factors (Nrf2; NF-kB) and caspase 3 in the Hippocampus of female Sprague Dawley rats after exposure to HMM (Pb, Mn, Hg, and Al) (* – significantly different compared to control at $p<0.05$; *** – significantly different compared to control at $p<0.001$; ^{bbb} – significantly different compared to HMM at $p<0.001$)

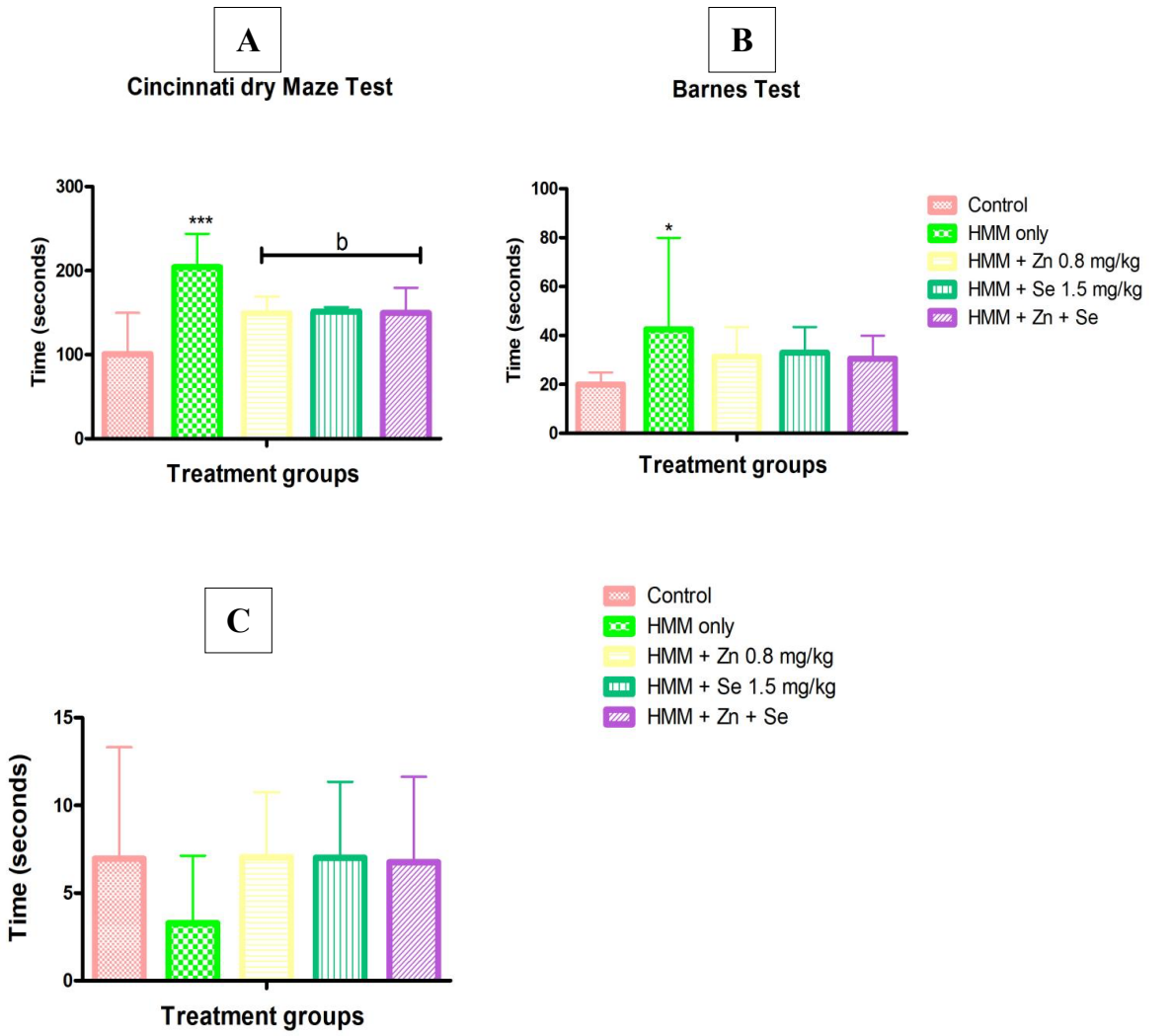

Fig. 5. Effect of Zn, Se on Cincinnati dry Maze, Barnes and Rotarod tests (time sec, * significantly different compared to control at $p<0.05$, *** – significantly different compared to control at $p<0.001$, * – significantly different compared to HMM at $p<0.05$)

time from the rotating wheels/rod of the rotarod than all groups when compared with the HMM-only group. The HMM only group had significantly ($p<0.05$) shorter latency of fall. The latency of fall in the HMM plus Zn, HMM plus Se, and HMM plus Zn plus Se were longer than that of the HMM only group.

Effect of essential trace element on AChE activity female Sprague Dawley rats after HMM (Pb, Mn, Hg and Al) exposure

We found the following levels of AChE, 95±7.07 µmol/mL, 285±7.07 µmol/mL, 110±0.00, 135±49.50 µmol/mL, 170.5±14.85 µmol/mL for control, HMM, HMM+Zn, HMM+Se and HMM+Zn+Se, respectively. There was a significant increase in animals exposed to AChE activity in HMM compared with other groups.

Histopathological examination

The hippocampus of rats exposed to only deionized water showed a regular architecture of the CA1 and CA3 regions, the dentate gyrus (DG), and the subicular cortex (subiculum); while HMM treated rats had severe neuronal cell loss of neurons with associated diffuse chromatolysis. The number and morphology were similar in HMM+Zn as in controls, while features of HMM+Se neurons showed minimal darkening. The hippocampus of animals exposed to HMM+Zn+Se showed normal neuronal density with dysplasia in the CA2 region (Fig. 6).

Discussion

In this study, low environmental levels of heavy metals have been shown to induce inflammation, deplete antioxidants, disruption of the Nrf2 signaling pathway, potentiated acetylcholinesterase (AChE) activity in the hippocampus with associated memory impairment, and mild histological alterations. The wide range of parameters examined (IL-6, Nrf2, Casp-3, GPx, CAT, and NO) which were reversed after supplementation with essential trace elements tend to reflect the significance of Zn and Se in neuroprotection. There was an increase in brain

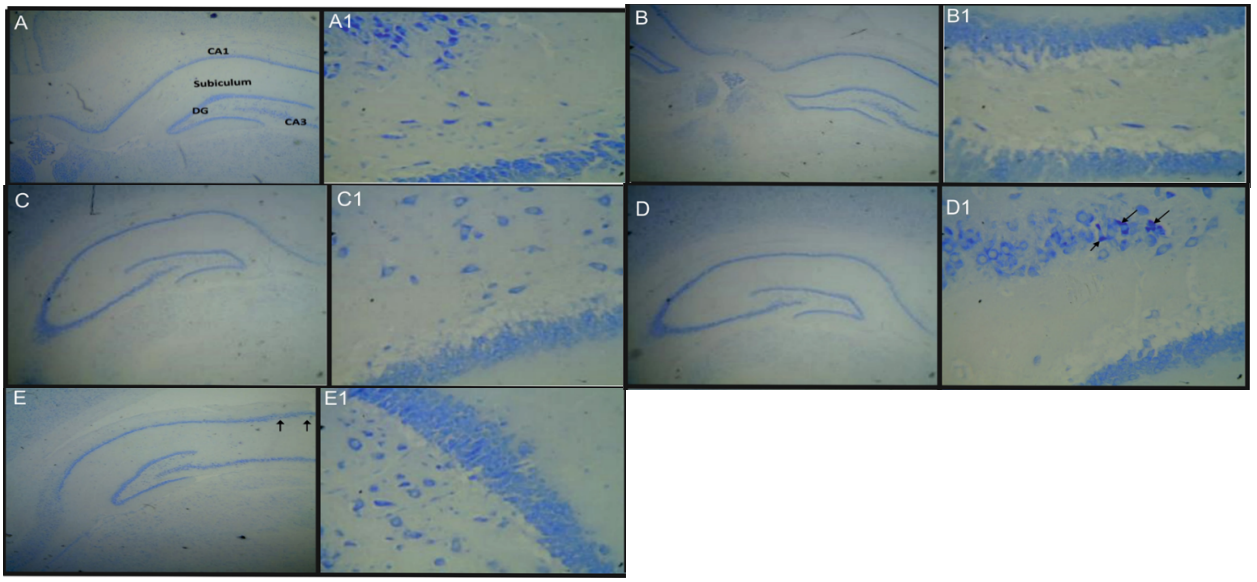

Fig. 6. A: Photomicrograph (toluidine X40) and 3A1: normal hippocampus (toluidine X400) exposed to only deionized water showing the CA1 and CA3 regions, the dentate gyrus (DG) and the subicular cortex (subiculum), B: hippocampus (toluidine X40) exposed to only HMM showing mild loss of neuronal cells, B1: hippocampus (toluidine X400) exposed to only HMM showing severe loss of neuronal cells with an associated diffuse chromatolysis; C: hippocampus, normal (toluidine blue X40) exposed to HMM+Zn, C1: hippocampus, normal (toluidine blue X40) exposed to HMM+Zn, D: hippocampus (toluidine blue X40) exposed to HMM+Se showing minimal neuronal darkening, D1: hippocampus (toluidine blue X400) exposed to HMM+Se showing multiple foci of necrosis (arrows), E: hippocampus (toluidine blue X40) exposed to HMM+Zn+Se showing normal neuronal density with dysplasia at CA2 region (arrow), E1: hippocampus (toluidine blue X400) exposed to HMM+Zn+Se showing normal cellularity

weight in HMM exposed rats, highly likely due to edema induced by inflammation; similar findings regarding increased brain weight are observed in similar studies. Some studies have shown that HMM heavy metal mixture decreased body weight gain, increased relative organ weight, with brain edema, and induction of apoptosis.[43-44]

As expected, all the heavy metals accumulated in abundance in the hippocampus of treated rats; Interestingly, there were studies that showed that some of the metals used in this study, eg aluminum, are found to a AD patients in greater extent.[45-46] Histology s of the hippocampus in Bittencourt et al., study, where authors treated male Wistar rats with aluminum 8.3 mg/kg for two months, revealed a decrease in the thickness of hippocampal cell bodies and the dentate gyrus; also, by using water maze test, they found that used low levels of Al may induce memory deficit of the rats.[4] Other *in vivo* studies showed that single treatment with other metals used in our study, e.g. lead also may lead to disturbances in neurobehavioral testing (radial arm maze).[47]

It is well known that iron is an integral part of CAT, in other words, its antioxidant activity is iron dependent.[48] and the observed presence of heavy metals (Al, Pb, Mn and Hg) led to decreased levels of CAT; There are no ways to eliminate excess of iron from the centrals nervous system (CNS), so iron that originated from damaged CAT would likely accumulate in the CNS, or actually in the hippocampus in this case. When cellular iron levels overcome the cellular ability to bind it, iron starts to exert neurotoxicity in a reaction known as ferroptosis.[49]

Karri et al. conducted an in vitro study in which authors exposed HT-22 cell line (originate from immortalized mouse hippocampal neuronal precursor cells) to single heavy metals (Cd, Pb, Hg and As) and to various combination of them[50] for eight days. They found that all metals examined, as a single exposure, decreased cell vitality and provoked apoptosis in a dose-dependent manner and also noted that among the four heavy metals, not all of them have similar potential to induce neurotoxicity (e.g., mercury appeared to induce apoptosis and decrease neuronal viability at lower doses than Pb and Cd). Interestingly, Karri et al. also noted that some combinations of metals have synergistic adverse effects (eg Pb and Hg) posing a significant hazard to hippocampal precursors, while on the other side some combinations such as Cd and As had antagonistic effects,[50] i.e., induce cell damage to a lesser extent compared to single exposure (either Cd or As). This study raises the significance of our study since in our mixture, Pb and Hg were used. Jia et al. divided pregnant 8 week old Sprague-Dawley rats to four groups and treated them with metal mixture (MM; metal mixture; Cd, Pb and Hg) *per os,* following pattern 1 x metal mixtures (MM), 5 x MM, 10 x MM and controls;[51]

animals were exposed to MM until the end of weaning (post-natal day 23). They found that 1 x MM, 5 x MM pups did not experience any significant neurotoxicity while 10 x MM showed suboptimal search strategies which underlies the early cognitive deficits; Transmission electron microscope revealed severe neuronal loss in CA1 and CA3 areas coupled with reduced organelles and mitochondrial swelling.[51] In the similar experiment, which had the same groups and exposure pattern, but lasted until 83 postnatal day (PND), ultrastructure analyzes revealed that 10 x MM induced reduction in synapse density in the CA1 region.[31] Both studies examined effects of metals mixtures in the hippocampus, involved rats' puppies and examined morphological and cognitive aspects of metal toxicity while pathophysiological mechanisms on which metal induced neurotoxicity in the hippocampus is based were not evaluated.

Behavioral effects

The ability to advantageously maneuver through the environment in search of either feed and avert danger deemed central to the survival of most animals is mostly dependent on learning and recalling different locations and is encoded in the brain by two systems:[52] allocentric navigation and egocentric navigation.[53,52]

Exposure to heavy metals caused learning and memory deficits in animals with longer escape latency,[31,54] It is believed that this must be due to heavy metal-mediated hippocampal impairments. Heavy metals are known to alter hippocampal N-methyl-D-aspartate NMDA and muscarinic cholinergic receptors involved in spatial learning.[31,55,56]

With respect to the Barnes test, the HMM caused significantly longer time to locate the hole than the control group that received only deionized; this is indicative of spatial memory and learning ability loss mediated by the heavy metal mixture. Supplementation with Zn alone, Se only and Zn plus Se combination significantly improved the latency in locating the hole than the HMM only exposed group suggestive of improved spatial memory and learning ability. Although unlike the cerebellar areas of the brain, the hippocampus is often not associated with balance or motor functions, there are more recent facts that tend to implicate the hippocampus in maintaining balance.[57-58]

There were distortions in motor coordination, as shown in this study. In the Rotarod test, the latency is measured when the rats fall from the rotating cylinder. Diverse animal models of neuropathologies such as cerebellar ataxia, Parkinsonism, stroke show poor performance on the Rotarod test.[59-60] This work has shown that rats treated with HMM only showed loss of balance in the Rotarod test.[59-60] Rats treated with HMM only expended less time than the control group on the rotating cylinder, whereas rats that received zinc, selenium, and zinc and selenium combination expended more time on the rotating cylinder compared to the rats treated with HMM only group. The Rotarod test is characterized by exercise with the production of free radicals that overwhelm the rat's antioxidant machinery of the rat, culminating in the destruction of the cell membrane and skeletal muscle damage.[61-62] This cascade is even exacerbated in the HMM-only exposed rats and may have accounted for the reduced latency required for rats to fall from the rotating cylinder. It has been opined that ROS mediated cellular injury may be annulled by skeletal muscle antioxidant enzymes.[61] Exaggerated levels of ROS following HMM can alter intracellular signaling pathways by altering cellular functions.[63] Since selenium is a known antioxidant enhancer, this may explain the increased latency taken for rats to fall from the rotating cylinder seen in groups treated with essential trace elements.[61]

One of the most striking findings in our study was that HMM-induced disruption and decrease in Nrf2. Nrf2 attenuates oxidative and xenobiotic stress.[64] Increased oxidative stress mediated by accelerated ROS generation has been implicated in neurogenerative diseases.[65-68] Nrf2 also activates genes that encode antioxidant apparatus in a cell, including SOD, CAT, and GPx[69] and depletion of Nrf2, observed in HMM exposure rats, led to downregulation of antioxidants and afterwards accumulation of ROS became inevitable. When ROS accumulates in abundance, cells damage may appear and injured cells can stimulate innate immunity.[70] NF-kB is a key regulator of innate immunity and could possibly affect TNF-α levels, nevertheless, we did not detect disturbances in the NF-kB levels between the group exposed to HMM and controls. So, following axis, exposure to HMM - Nrf2 pathway disruption - ROS antioxidants depletion – generation - neuroinflammation could partly explain HMM-induced neurotoxicity. Today, the Nrf2 signaling pathway and its link with neurodegenerative disorders, as well as a potential therapeutic approach, attract the attention of the scientific community more than ever.[71-76] Nrf2 decreases the levels of α-synuclein aggregates that have been implicated in (PD).[77,72] Sarlette et al. examined the expression of Nrf2 levels in the primary motor cortex and spinal cord of 5 individuals with diagnosed ALS) and five controls and found lower levels of Nrf2 in subjects with ALS for both examined locations.[78] Down-regulation of the Nrf2 is hallmark of aging and neurodegenerative disease.[79] Von Otter et al. reported association between one NFE2L2 haplotype (GAAAA) with faster AD disease progression.[80] All mentioned neurodegenerative disorders are highly complex and still insufficiently evaluated and although we did not aim to examine contributing effects of heavy metals in onset of various neurodegenerative disorders some similarities are doubtless. Interestingly we found in our study that cholinergic disruption appeared due to increased AChE activity and low levels of acetylcholine in the basal fore-

brain correlates with decreased walking speed among individuals with PD[81] and AD.[82]

To counteract oxidative stress, a plausible modulator of cellular defense Nrf2 and Keap1 serves as a usual intrinsic defense system.[83] Exposure to heavy metals and metalloids and metals through diet, drinking water, air and diverse anthropogenic activities is linked to production of ROS in many organs, including the brain by both direct and indirect means.[84] Nrf2 signaling sustains antioxidant balance and can have two roles depending on the biological setting. Nrf2 is protective against metal-induced toxicity. The absence of female reproductive hormones, which would have offered more information to support the earlier assertion that even when female rats are used in neuroscience experiments without regard to the estrous cycle stage, their data are not more variable than those of male rats, may be considered a likely weakness of this study.[85]

Taken together, HMM-induced neurotoxicity mimics the cellular basis of neurodegenerative diseases in many ways: i) increased brain accumulation of heavy metals (observed in both), ii) ROS-mediated cellular damage, iii) disruption of the Nrf2-signaling pathway, and iv) low levels of acetylcholine. These pathological findings were attenuated by Zn and Se supplementations.

Metals (Al, Pb, Hg, and Mn)-induced neuronal toxicity in the hippocampus may be mediated by ROS accumulation, antioxidant depletion, Nrf2 pathway disruption, and increased activity of AChE. Importantly, most of altered parameters were reversed when animals were co-treated with Zn and Se, reflecting neuroprotective effects.

Declarations

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Author contributions

Conceptualization, C.N.O. and O.E.O..; Methodology, C.S.D., C.N.O, A.N.E.; Validation, C.N.O. and O.E.O.; Formal Analysis, C.S.D., C.N.O. and A.N.E.; Investigation, C.S.D., C.N.O. and A.N.E.; Data Curation, C.S.D., C.N.O. and A.N.E.; Writing – Original Draft Preparation, C.N.O., Ai.C., An.C., T.C.U. and O.E.O.; Writing – Review & Editing, Ai.C., An.C., T.C.U. and O.E.O.; Visualization, O.E.O.; Supervision, C.N.O., A.N.E. and O.E.O.

Conflicts of interest

Authors confirm that there was no conflict of interest.

Data availability

All data have been provided.

Ethics approval

Ethical approval was given by the University of Port Harcourt Research Ethics Committee (UPH/PUTOR/REC/12).

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ORIGINAL PAPER

Elevated estrogen receptor beta and oxidative stress in postmenopausal women with ovarian cancer

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ABSTRACT

Introduction and aim. Ovarian cancer in postmenopausal women is associated with hormonal dysregulation and oxidative stress. This study investigates the relationship between estrogen receptor beta (ERβ), selected reproductive hormones, and oxidative stress markers in women with ovarian cancer compared to healthy controls. To our knowledge, this is one of the first studies to evaluate these parameters in an integrated way, offering new insights into the pathophysiological mechanisms underlying postmenopausal ovarian cancer.

Material and methods. Blood samples were collected from 45 postmenopausal women with ovarian cancer immediately after diagnosis to be compared with 45 healthy women. ERβ and some hormones were evaluated using an enzyme-linked immunosorbent assay. Chemiluminescence imunoassays and miniVIDAS, while spectrophotometric methods were used to evaluate variables associated with oxidative stress.

Results. The results show a significant increase in beta estrogen receptor values for women with ovarian cancer 12.69±1.79 ng/mL, p<0.001 compared to healthy women 0.47±0.06 ng/mL. Furthermore, a significant increase was observed in the values of each estrogen (E2) 18.4±2.19 pg/mL vs. 16.20±3.45 pg/mL, p=0.001, anti-Müllerian hormone (AMH) 15.56±2.88 pmol/L vs. 1.22±0.29 pmol/L, p<0.001, and total oxidant status 2.93±0.63 µmol/L vs. 0.65±0.09 µmol/L, p<0.001. On the contrary, a significant decrease in total antioxidant capacity 3.22±0.72 mmol/L vs. 10.04±1.50 mmol/L, p<0.001. The results also show a positive correlation between the values of total oxidants and the hormones studied, compared to the negative correlation with total antioxidants.

Conclusion. The significant increase in the values of ERβ as well as the estrogen hormone that may be derived from adipose tissue in women with ovarian cancer in the postmenopausal stage, has multiple effects, for example, by altering some hormones such as progesterone, dehydroepiandrosterone sulfate, testosterone, and AMH. These hormonal disturbances resulting from granulosa cell tumors play a role in increasing the metabolic rate and therefore increasing the oxidative stress of cells.

Keywords. β-estrogen receptors, hormonal disorders, ovarian cancer, oxidative stress, postmenopausal

Introduction

Ovarian cancer affects postmenopausal women and is the second most deadly gynecologic cancer worldwide. About 90% of all ovarian cancers are epithelial ovarian carcinomas, according to histological subgroups.[1] High-grade serous ovarian cancer (HGSOC), the most prevalent and deadly variety, is responsible for 70 to 80% of ovarian cancer fatalities.[2] This is because the pathology is asymptomatic, which causes the diagnosis to be made after the disease has already advanced and has a poor

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prognosis. Surgery is typically the first step in treatment and then platinum-based chemotherapy. However, resistance to treatment frequently arises despite early favorable responses, leading to a poor 5-year survival rate of 20% in stage IV and 40% in stage III.[3]

Estrogen receptor β and ovarian cancer

When estrogen attaches to the ER, receptor1dimerization occurs. In the cytoplasm, "ligand-bound ER dimer molecules translocate" to the cell's nucleus. Estrogen receptor elements (ERE) are specific DNA sequences present in the coding regions of target genes to which ER dimers attach in the nucleus.[4,5] ER-mediated transcriptional-1regulation causes changes in the expression of specific genes involved in several cellular activities, such as division, growth, survival, and metabolism (Fig. 1). G protein-coupled estrogen receptor 1 (GPER1) is a binding to the membrane protein receptor that binds to estrogen and activates a number of subsequent signaling cascades, including the pathway involving the PI3K, AKT and m and the mitogen-activated protein kinase (MAPK) circuit. This further enhances classical genomic signaling. Rapid cell proliferation, motility, and survival alterations can be caused by this so-called non-genomic signal.[6,7]

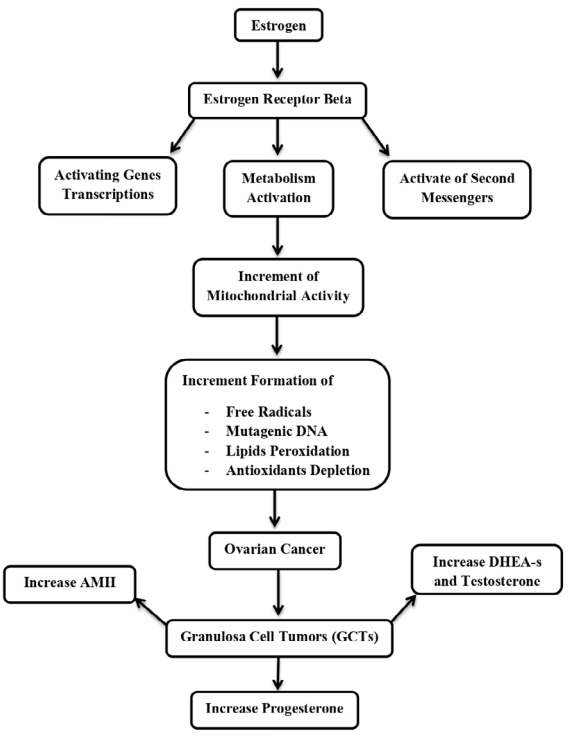

Fig. 1. Impact of estrogen and estrogen receptor on ovarian cancer

Anti-Mullerian hormone and ovarian cancer

The production by the tumor directly causes endocrine symptoms in the majority of cases of granulosa cell tumors (GCTs) cases (two-thirds). Although there are occasional reports of androgenic effects, most of the effects are estrogenic. Menstrual irregularities are observed in premenopausal women, whereas prepubertal women can experience early isosexual development as a result of these effects. Postmenopausal bleeding is the most common symptom among older women. This hormone release has led to a well-documented correlation between GCT and endometrial hyperplasia, as well as adenocarcinoma.[8] AMH is a highly helpful indicator of cancers originating from granulosa cells of ovarian follicles because it is released exclusively by these cells in women. According to reports, 76-93% of women with "granulosa cell tumors (folliculomas)" have elevated AMH levels (Fig. 2). AMH levels could be noticeably higher. AMH levels may rise to 16 months before a tumor becomes clinically noticeable. Compared to estradiol and inhibin, AMH appears to be a more specific biomarker for these tumors and its levels are correlated with tumor size.[9,10] Additionally, AMH is a highly sensitive and specific marker that helps patients with folliculoma who have had the ovaries removed detect a recurrence early. Even 10 to twenty years after the main tumor has been removed, there is a significant chance of recurrence.[11]

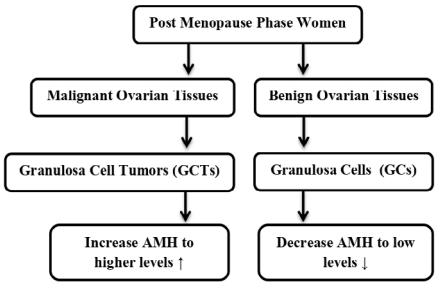

Fig. 2. AMH levels in benign and malignant ovarian cancer in women in the postmenopause phase

Oxidative stress and ovarian cancer

An imbalance between the body's antioxidant defense mechanisms and the generation of reactive metabolites, or free radicals, including oxidants such as reactive nitrogen species (RNS) and reactive oxygen species (ROS), is what leads to oxidative stress (OS). This imbalance may have a cumulative effect and damage biological molecules and cells. Increased oxidant production can lead to an OS state, which can irreversibly damage oxidative biomolecules such as proteins, lipids, and nucleic acids. This OS state is caused by the stimulation of the antioxidant defense mechanism. The development and progression of cancer are closely associated with these lesions. Prior research has indicated that OS has a major impact on the development of cancer.[12] The RNS or ROS can encourage molecular genetic alterations that can accelerate the development and spread of cancers and increase their resistance to treatment (Fig. 1). Although previous studies have explored hormonal disturbances or oxidative stress in ovarian cancer, few

have comprehensively investigated the concurrent alterations in estrogen receptor beta (ERβ), reproductive hormones, and markers of oxidative stress in postmenopausal women. To our knowledge, this is one of the first case-control studies to examine the integrated relationship between ERβ expression, hormonal profiles, and systemic redox status in this patient population.[12]

Aim

The study aims to explain the effect of the presence of estrogen receptors and estrogen hormone in the postmenopausal stage and its effects on disturbances in other hormone levels such as progesterone, testosterone, DHEA, and AMH, as well as high levels of total oxidant status and low levels of antioxidant capacity in women with ovarian cancers compared to healthy women of similar age.

Material and methods

Sample collection and processing

This retrospective study included postmenopausal women diagnosed with ovarian cancer, confirmed by histopathological examination and ultrasound. The patients were age-matched with a control group of healthy women. Blood samples were collected after ethical approval from both the patients and the Iraqi Ministry of Health. Under the supervision of specialist physicians, samples were obtained immediately after diagnosis at the Maternity and Children's Teaching Hospital in Babylon Governorate, Iraq, between February 1 and November 30, 2024. For comparison, blood samples were also taken from healthy controls. Serum was separated from all samples for analysis of hormonal, oxidative stress, and antioxidant parameters.

Study design

The experimental design of this research is a case-control study. Samples were taken from (45) women diagnosed with ovarian cancer after clinical, laboratory, and ultrasound diagnosis, and before starting chemotherapy or radiation therapy to compare with (45) women apparently healthy as control. Patients with chronic diseases or hormonal or endocrine problems were also excluded. The sample size was calculated according to the following equation:

$$n = Z2P\ (1 - P) / d2$$

Ninety samples were gathered; the 'Z score (1.96), the population (0.14)", the number 1 of samples (n), and the absolute marginal error (d), which is equal to 5%, are all indicated.

Inclusion criteria: All women who are suspected of ovarian cancer. Women in the postmenopausal phase.

Exclusion criteria: All women suffering from autoimmune diseases, diabetes mellitus, chronic kidney diseases, hypertension, viral infections such as HPV and any chronic disease. All women with estrogen- or estrogen-like effect therapy.

Procedures

ERβ levels were measured using a competitive quantitative ELISA technique (Catalog No: ab285292). The optical density (OD) was measured at 450 nm. The standard curve included the following concentrations: 20, 10, 5, 2.5, 1.25, 0.625, 0.313, and 0 ng/mL.

Serum estradiol concentrations were determined using a competitive ELISA method (Catalog No. E-FS-E117). The OD was measured at 450 nm. The assay had a detection limit of 5 ppb.

Progesterone levels were quantified using a competitive ELISA (Catalog No: EIAP4C21). The OD was read at 450 nm. The assay demonstrated a sensitivity of 47.9 pg/mL, calculated by adding two standard deviations to the mean OD obtained after 20 replicates of the zero standard.

Dehydroepiandrosterone sulfate (DHEA-S) levels were assessed using a competitive immunoluminometric assay on a fully automated chemiluminescence immunoassay (CLIA) analyzer (MAGLUMI system). The assay used an anti-DHEA-S monoclonal antibody labeled with FITC and a purified DHEA-S antigen labeled with ABEI. Each reagent cartridge was equipped with an RFID tag to ensure proper identification and performance. The procedure strictly followed the manufacturer's operating instructions.

Testosterone and AMH: These hormones were measured using an enzyme-linked fluorescent assay based on competitive immunoassay principles. The solid-phase receptacle functioned both as the solid phase and as a pipetting system. All reagents were predispensed in sealed strips and ready to use. The assay was fully automated on the MiniVIDAS® system, with all steps performed by the instrument. The reaction mixture was repeatedly cycled through the SPR to obtain optimal binding and detection.

Total antioxidant capacity (TAC) was measured using the CUPRAC method (Cupric Reducing Antioxidant Capacity), which is based on the redox reaction involving copper (II) ions. The absorbance was recorded at 450 nm using a spectrophotometer. A 1 mM uric acid solution was used as the standard.[14]

$$\text{Antioxidant} + Cu^{2+} \longrightarrow Cu^{+}$$

$$Cu^{+} + \text{2.9-dimethyl-1,10-phenanthroline} \longrightarrow \text{complex (at 450 nm)}$$

The total oxidant status (TOS) was determined spectrophotometrically using a method developed by Erel. This technique quantifies total oxidant molecules in serum on their ability to oxidize ferrous ion–o-dianisidine complexes to ferric ions. Glycerol, present in the reaction medium, enhances the oxidation process. In an

acidic environment, the resulting ferric ions form a colored complex with xylenol orange. The intensity of the color, measured at 530 nm using a spectrophotometer, is directly proportional to the oxidant concentration in the sample. The assay is calibrated with hydrogen peroxide, and results are expressed in micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L).

Statistical analysis

Statistical analysis was done using SPSS version 20 (IBM, Armonk, NY, USA), independent T test and the p-value less than 0.05, and the correlation analysis is performed in Person and (r) is the correlation coefficient. Normal distribution assessed by the Shapiro-Wilk test.

Ethical considerations

The study was approved by the Ethics Committee of the Women's and Maternity Teaching Hospital and Babylon University (IRB No. 2-17, dated February 12, 2024). Informed consent was obtained from all participants.

Results

Table 1 presents the results of female patients with ovarian cancer in terms of age and weight, as well as the results of some of the hormones studied, in addition to the values of total oxidation and total antioxidants.

Table 1. Age, BMI, TOS, TAC, and ERβ results in combination with some associated hormones

Parameters	Groups	Means±SD	95% confidence interval		p
			Lower	Upper	
Age (years)	Patients	56.66±3.11	55.77	57.74	0.440
	Control	57.17±3.12	56.27	58.22	
BMI (kg/m^2)	Patients	25.92±3.71	24.90	27.12	0.001
	Control	22.43±2.56	21.74	23.24	
ERβ (ng/mL)	Patients	12.69±1.79	12.15	13.42	<0.001
	Control	0.47±0.06	0.45	0.49	
Estrogen (pg/mL)	Patients	18.4±2.19	17.58	19.01	0.001
	Control	16.20±3.45	15.12	17.03	
Progesterone (ng/mL)	Patients	0.82±0.15	0.76	0.87	<0.001
	Control	0.32±0.1	0.28	0.35	
Testosterone (ng/dL)	Patients	7.31±1.21	6.97	7.69	<0.001
	Control	4.07±0.7	3.82	4.30	
DHEA-S (ng/mL)	Patients	1.13±0.22	1.07	1.20	<0.001
	Control	0.66±0.1	0.63	0.70	
AMH (pmol/L)	Patients	15.56±2.88	14.62	16.57	<0.001
	Control	1.22±0.29	1.15	1.29	
TOS (μmol/L)	Patients	2.93±0.63	2.78	3.14	<0.001
	Control	0.65±0.09	0.61	0.68	
TAC (mmol/L)	Patients	3.22±0.72	2.99	3.51	<0.001
	Control	10.04±1.50	10.20	11.80	

The correlation between oxidation antioxidants with the studied hormones, which include estrogen receptors, estrogen, progesterone, testosterone, DHEAs, and AMH, as well as with BMI as shown in (Table 2). There are positive correlations between TOS and BMI, also with the hormones studied. On the contrary, negative correlation for TAC with these parameters.

There is a positive correlation between estrogen and the beta estrogen receptor with progesterone, DHEA-s, testosterone, and AMH and the results appear in (Table 3).

There is also a positive association between AMH and the hormones studied in women with ovarian cancer (Table 4).

Obesity is considered a risk factor for ovarian cancer due to the adipose tissues from estrogen sources. Therefore, a positive correlation between BMI and estrogen, progesterone, DHEA, testosterone, and AMH for women with ovarian cancer in the postmenopausal period (Table 5).

Table 2. TOS and TAC correlations with ERβ and some related hormones[a]

Hormones		Eβ	Estrogen	Progesterone	DHEA-s	Testosterone	AMH	BMI
TOS	r	0.930*	0.904**	0.398**	0.781**	0.836**	0.873**	0.486**
	p	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001
TAC	r	-0.946**	-0.930**	-0.333**	-0.785**	-0.781**	-0.902**	-0.438**
	p	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001

[a] * – the correlation significant at p less than 0.05 (2-tailed), ** – the correlation significant at p less than 0.01 (2-tailed)

Table 3. Correlations of estrogen and ERβ with some related hormones[a]

Hormones		Progesterone	DHEA-s	Testosterone	AMH
Estrogen	r	0.390**	0.267*	0.289**	0.342**
	p	<0.001	0.011	0.001	0.001
ERβ	r	0.877**	0.828**	0.841**	0.944**
	p	<0.001	<0.001	<0.001	<0.001

[a] * – the correlation significant at p less than 0.05 (2-tailed), ** – the correlation significant at p less than 0.01 (2-tailed)

Table 4. Correlations of AMH and related hormones[a]

Hormones		ERβ	Estrogen	Progesterone	DHEA-s	Testosterone
AMH	r	0.944**	0.342**	0.816**	0.754**	0.703**
	p	<0.001	0.001	<0.001	<0.001	<0.001

[a] * – the correlation significant at p less than 0.05 (2-tailed), ** – the correlation significant at p less than 0.01 (2-tailed)

Table 5. Correlations of BMI and related hormones[a]

Hormones		ERβ	Estrogen	Progesterone	DHEA-s	Testosterone	AMH
BMI	r	0.113	0.463**	0.415**	0.340**	0.386**	0.440**
	p	0.289	<0.001	<0.001	0.001	<0.001	<0.001

[a] * – the correlation significant at p less than 0.05 (2-tailed), ** – the correlation significant at p less than 0.01 (2-tailed)

Discussion

The notion that postmenopausal obesity is associated with increased mortality from ovarian cancer is supported by the findings of this large prospective investigation. However, among women who were slim and of normal weight, the death rate from ovarian cancer

did not increase steadily. Only women who were overweight or obese had a higher risk. As predicted, there was no indication of increased risk with rising BMI between overweight and obese women who had previously taken postmenopausal estrogen.[15]

The possible roles of estrogen and androgenic hormones in the development of ovarian cancer provide biological reasons for the relationship between BMI and the death rate from ovarian cancer. After menopause, the main origin of endogenous estrogen is adipose tissue, and postmenopausal women have higher circulating estrogen levels. In the culture of cells from malignant and normal sources, estradiol and estrone promote cell proliferation.[16] Therefore, estrogens of extra glandular origins may encourage ovarian epithelial cells to proliferate and undergo malignant transformation. The documented elevated risk associated with long-term postmenopausal estrogen use lends credence to an estrogen-related role in the development of ovarian cancer. Elevated blood testosterone concentrations have been linked to high BMI in postmenopausal women. Additionally, in vitro proliferation of both malignant and normal cells is stimulated by testosterone and 5-dihydrotestosterone. In a prospective analysis of blood hormone levels and the appearance of ovarian cancer, prediagnostic levels of androstenedione and dehydroepiandrosterone were linked to an increased risk of ovarian cancer.[17]

Granulosa cells in the ovaries are among the human cells that exhibit the highest levels of ERβ mRNA and protein expression.[18] This expression is sustained or elevated in granulosa cell tumors of the sex-cord stromal tumor type. However, considering the uncommon granulosa cell malignancies and few cell lines that adequately express this subtype or preserving ERβ expression.[19] Therefore, nothing is known about ERβ's functional role of ER in granulosa cell malignancies. Only two studies conducted on this particular subtype in the last five years. The mitochondria of Granulosa tumor cells include a cytoplasmic ERβ2 that binds to the proteins of the 'Bcl2 family' to prevent apoptosis.[20] According to a different study, when ERα is present, ERβ promotes tumor growth. Both results suggest that ERβ plays a tumor-promoting function in these malignancies, albeit through different methods. These results highlight the need for a more thorough comprehension of ERβ and its isoforms in granulosa cells.[21]

Like certain tumor suppressor transcripts, ERβ's mRNA may be quickly destroyed if it functions as a tumor suppressor.[22] However, to ascertain the expression of a protein, validation using a non-antibody-dependent technique is necessary. According to several findings, ERβ in particular, normal tissues, may prevent the occurrence of cancer, as it is linked to enhanced apoptosis, decreased cell proliferation, and anti-inflammatory qualities when present and activated.[22]

An analysis of ERβ and its function in malignant neoplasms requires navigating a difficult and demanding field characterized by disagreements, variant-specific roles, and structural complexity. A vital component of researching ERβ is the use of antibodies in organs with minimal to non-existent protein expression. Since each isoform may have a different impact on its activity, the complex interaction between ERβ subtypes and their cellular location adds even another dimension to their functional variety. It is worth mentioning that antibodies directed against neglected tropical diseases (NTD) would identify every variation of ERβ rather than discriminate between distinct isoforms.[23] High levels of estrogen and progesterone receptor activation are frequently associated with LGSOC. Furthermore, they typically exhibit a wild-type expression pattern, in contrast to individuals with HGSOC, and active mitogen-activated protein kinase (MAPK) pathways with KRAS and BRAF mutations.[24]

Rare ovarian cancers known as Sertoli-Leydig cell tumors (SLCT) are triggered by stromal cells and rudimentary sex cords in the ovary. These tumors usually appear during the second and fourth decades of life and account for fewer than 0.5%1of ovarian malignancies. The self-secretion of testosterone or its precursor androstenedione, by several of these tumors induces a significant androgen excess in females. The remaining tumors either do not function at all or produce estrogen. Because SLCT is so rare, knowledge of its prognostic variables is rather poor.[25] Numerous investigations have noted that the most important prognostic factors are tumor stage at assessment and level of differentiation.[26] Serum testosterone often indicates the amount of androgen produced by the ovaries in women. Adrenal androgens, such as androstenedione, are converted to a minor amount of testosterone. Adrenocortical carcinoma is known to cause markedly high serum testosterone levels, which are correlated with circulating precursor substances of adrenal androgen precursor substances.[27]

About 10% of all ovarian tumors are stromal neoplasms of the sex cord, which are formed from mesenchymal stem cells found in the ovarian cortex. These consist of Sertoli–Leydig cell tumors, granulosa–theca cell tumors, and granulosa cell tumors (GCT). Three percent of ovarian tumors are GCTs. Based on histological characteristics and clinical presentation, two different forms of GCT have been identified, the pediatric and adult form have been identified.[28]

The elevation of serum AMH for patients with GCT because of gene expression of neoplasm specimens. AMH expression was found in each of the GC tumors in a limited number of ovarian tumors, but was absent in the seven epithelial carcinomas. Although AMH positivity was consistently detectable in the GC tumors, it did not appear as prominent or diffuse in alpha-inhibin employing paraffin-fixed tissues and an antibody di-

rected against AMH. It has recently been confirmed that AMH expression can be found in young and adult-type GCT regardless of the ovarian or metastatic location.[29–31]

Recently, it has been discovered that human ovarian epithelial cancer expresses AMH type II receptors (AMH-RII). AMH-RII mRNA was detected by RT-PCR in ovarian cancer cells and peritoneal cells in those suffering from ovarian papillary squamous cystoadenocancer. Ten out of the 15 patients had cell lines with AMH-RII transcripts. The existence of AMH-RII mRNA predicted binding to AMH in 89% of the cases. AMH-RII immunostaining was also shown in the solid tumors of four individuals.[32–34]

According to studies, the Fenton reaction is how ferrous and its metabolites increase the creation of ROS. Old blood from the ovary, which has an exceptionally high iron concentration during the development of the chocolate cyst, might increase ROS creation of ROS and cause DNA damage, hence raising the likelihood of the endometriosis turning malignantly into mast cell ovarian cancer.[35–38] The transferrin receptor 1 (TfR1) axis is boosted by the hydroxyl radicals created via H_2O_2 in the "Fenton reaction". This can result in many double-strand breaks in the DNA of the oviduct's epithelial cells, which will encourage OV. Furthermore, OS affects different immune cells, including regulatory T cells, myeloid-derived suppressor cells, neutrophils, and tumor-associated macrophages. Our findings expand current knowledge by demonstrating strong correlations between ERβ and multiple hormonal and oxidative stress parameters, which may reflect a mechanistic link between estrogen signaling and redox imbalance in the tumor microenvironment. Unlike previous studies that focused on isolated markers, this investigation combines endocrine and oxidative markers, offering a more integrative perspective on tumor biology in postmenopausal ovarian cancer.[39,40]

Study limitations

Despite the study's thoroughness, numerous aspects remain unexplored and could be investigated in future research. These include taking into account the duration of the illness and reviewing patient follow-ups both before and after the prescription of antioxidants and vitamins supplements. Furthermore, the patient's lifestyle was neglected because it is integrally related to behavior, decisions about life, and food, all of which have a strong connection with elevated oxidative stress levels.

Conclusion

This study provides novel insights into the interplay between ERβ, hormonal dysregulation, and oxidative stress in postmenopausal women with ovarian cancer, highlighting the potential of ERβ and AMH as candidate biomarkers for future diagnostic and prognostic applications. Hormonal disturbances in postmenopausal women can contribute to the occurrence of ovarian cancer in women. A large percentage of these women who have ovarian cancer have positive estrogen receptors. Estrogen at this age plays a role in increasing the growth rate of granulosa cell tumors. These tumor cells play a role in increasing the levels of estrogen, DHEA, testosterone, and AMH. These hormonal disturbances in granulosa cell tumors increase metabolic processes and thus increase oxidation levels due to mitochondrial activity and the electron transport chain, which in turn leads to a decrease in the total concentrations of antioxidants in patients. Weight gain in postmenopausal women can also be considered a risk factor for ovarian cancer. Because adipose tissue is a source of estrogen production, which is a risk factor after binding to the estrogen receptors alpha and beta, which in turn disrupts the body's hormones and balance of oxidant-antioxidants.

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Declarations

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Author contributions

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

Conflicts of interest

There are no conflicts of interest.

Data availability

The corresponding author can be contacted for the raw data upon request.

Ethics approval

The study was approved by the Ethics Committee of the Women's and Maternity Teaching Hospital and Babylon University (IRB No. 2-17, dated February 12, 2024).

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ORIGINAL PAPER

Impact of multicomponent training on functional fitness among older adults in rural India

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ABSTRACT

Introduction and aim. In rural India, preserving functional fitness is crucial for older adults to maintain independence and perform daily activities without assistance. This study examines the effectiveness of multicomponent training (MCT) in improving functional fitness among older adults in rural India.

Material and methods. Thirty participants (mean age 64.50±3.67 years) engaged in a structured low-to-moderate intensity exercise program three times per week for six weeks. Functional fitness was assessed at baseline, three weeks, and six weeks using the Senior Fitness Test (SFT).

Results. In a short period of six weeks, significant improvements were observed in lower-body strength ($p<0.001$), endurance ($p<0.001$), flexibility ($p<0.01$), and overall functional fitness ($p<0.001$), with lower-body strength and endurance surpassing minimal detectable change thresholds. Females had lower baseline performance but showed greater improvement.

Conclusion. MCT effectively enhances functional fitness in older adults. However, additional upper-body strength and balance exercises may be beneficial for comprehensive improvements.

Keywords. aged, aging, endurance training, exercise, muscle strength, physical fitness, resistance training

Introduction

Aging is a natural and inevitable process that affects all individuals, regardless of socioeconomic status or background. It is characterized by progressive anatomical and functional changes across various physiological systems, leading to a decline in mobility, strength, and overall functional capacity.[1,2] With increasing life expectancy, the global population of older adults is growing rapidly, particularly in developing nations.[3] According to the World Health Organization, individuals aged 60–74 are classified as elderly, while those aged 75 years and older are considered to be in the late-old age group.[4]

Aging-related physiological changes can impair an individual's ability to maintain physical independence and perform activities of daily living (ADLs).[5] However, regular physical activity can mitigate these effects by enhancing mobility, flexibility, and functional capacity while preserving muscle strength and joint range of motion.[6,7] Exercise also contributes to reduced body fat, improves body composition, aids in weight control, and lowers the risk of chronic illnesses.[8] Despite these benefits, older adults often exhibit low participation in structured exercise programs, which can accelerate functional decline and dependence.[9,10]

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Functional fitness is defined as the ability to perform everyday tasks safely and independently without undue fatigue.[11] Although traditionally associated with younger populations, functional fitness is especially critical for older adults, as it enables them to maintain their independence and quality of life. Regular exercise is defined as structured, repetitive movement intended to improve physical fitness,[12] plays a vital role in delaying physical decline and promoting healthy aging.

Various forms of physical activity programs have demonstrated benefits in improving the health and functional ability of older adults. These include aerobic exercises for cardiovascular endurance, resistance training to build muscle strength, balance and flexibility routines to reduce fall risk, and mobility-focused activities that support daily functioning.[13–16] Multicomponent approaches, which incorporate a combination of these elements, are considered effective in promoting overall functional fitness.

Aim

Although many previous studies have employed longer intervention durations, emerging evidence suggests that even six-week programs can produce meaningful improvement in physical function.[17,18] Motivated by these findings, the present study aims to examine whether a shorter-duration training program can effectively enhance senior fitness, particularly within the practical and logistical constraints of rural settings in India.

Material and methods

Setting and research design

The study was a six-week longitudinal study of an exercise program among older adults. All the assessments were conducted by the research investigator, and the collected data were subsequently analyzed. The study utilized an interrupted time series design without a control group, conducted in 2019. All participants provided written informed consent prior to participation. The study obtained Institutional Ethics Committee permission (Ref no:G2-312/2015/CONTSR(7)).

The study was conducted in Ward XIII of Mundur village, involving a purposive sample of 30 older adults aged 60 years and above. No dropouts occurred during the intervention. Exclusion criteria included individuals who were unavailable during data collection, already engaged in regular exercise, or had sensory or cognitive impairments, acute or chronic illnesses, complete physical dependency, or cardiac and musculoskeletal conditions.

Socio-demographic data were collected through structured interviews, and functional fitness was assessed using an observational checklist. Data collection took place at the Peramangalam sub-center in Thrissur district.

Sample size calculation

The required sample size was determined using G*Power 3.1 software based on a repeated-measures ANOVA within-subjects design. The power analysis was conducted with the following parameters: medium effect size (f=0.25), significance level (α=0.05), power (1-β)=0.80, one group, three measurement points, assumed correlation among repeated measures=0.5, and nonsphericity correction=1.

The estimated minimum sample size was 28 participants. To account for potential attrition, 2 additional participants were included, resulting in a final sample of 30 older adults.

Data collection instruments and procedures

Socio personal variables

The validated questionnaire was used to gather sociodemographic information through a face-to-face interview. Variables include age, gender, marital status, educational status, previous employment, type of family, and previous knowledge regarding exercise programs. Experts review was conducted to assess the tool's clarity, relevance, simplicity, yielding a content validity index (CVI) of 0.97.

Functional fitness

Functional fitness was assessed using the Senior Fitness Test (SFT) also known as Fullerton Functional Fitness Test (FFFT). This standardized tool evaluates key components of functional fitness in older adults and measures an individual's capacity to safely and independently perform daily activities without excessive fatigue. It typically takes around 30–40 minutes to complete.

The test included six components:

1. Lower-body strength: (30-second chair stand test) number of complete chair stands a person can perform within 30 seconds while keeping their arms folded across the chest.

2. Upper-body strength: (arm curl test), number of biceps curls completed in 30 seconds using 5 lb (2.27 kg) for women and 8 lb (3.63 kg) for men.

3. Endurance: (2-minute step test) number of steps with knees raised to a predefined height in 2 minutes.

4. Lower-body flexibility: (chair sit-and-reach test), the distance between the fingertips and toes while sitting at the edge of a chair and extending their hands toward their toes.

5. Upper-body flexibility: (back scratch), distance between fingers reaching from over the shoulder and behind the back.

6. Agility and balance: (8-foot up-and-go test), time taken to stand up from a seated position, walk 8 feet (2.44 m), turn around, and return to the seated position.

Six testing stations were established, one for each component. Standardized procedures were followed as

per the SFT Manual.[19] Participants were briefed on each test, procedures, potential risks, and safety measures. Participants' performance was scored using age-specific normative values.

The reliability of the SFT employed in this investigation was determined using a test-retest methodology developed following a pilot study yielding a reliability coefficient of r=0.9352, indicating high dependability.[20]

Multicomponent training (MCT)

Thirty older adults participated in a 6-week MCT program, developed by the researcher in consultation with trained physical therapists. After receiving appropriate training, the researcher personally supervised and conducted all sessions to ensure the exercises were performed correctly, focusing n improving flexibility, strength, and posture through chair-based movements. The program included 12 sessions, each lasting 45 to 60 minutes, conducted three times a week (Tuesday, Thursday, and Saturday) with the aid of an LCD projector for guidance. Participants were divided into small groups of five for personalized instruction. The exercise routine included deep breathing (3 to 10 times) to enhance lung capacity and endurance. Chest stretches (hold for 5–10 seconds, repeat 5 times) improved posture and increased upper arm flexibility. Hip marching (5 lifts per leg) targeted lower arm strength, while ankle stretches (2 sets of 5 per foot) enhanced flexibility and reduced the risk of blood clots. Arm raises (5 repetitions) helped build upper arm strength.

Additionally, neck rotation (3 times per side) and neck stretching (holding for 5 seconds, repeating 3 times per side) improved upper arm flexibility and mobility. Sideway bend (3 times per side, hold for 2 seconds) promoted core flexibility and balance. Finally, sit-to-stand (5 slow repetitions) strengthened the legs, enhancing mobility and balance.

Participants followed six key exercise rules to ensure safety and effectiveness, including using a stable chair**,** maintaining steady movements, and progressing comfortably. Regular practice of these exercises can significantly enhance mobility, balance, and overall well-being in older adults. Additionally, an information booklet was provided for future reference, and a post-test assessment was conducted on days 21 and 42 to evaluate progress.

Plan for data analysis

Statistical analyses were conducted using SPSS Version 21 (IBM, Armonk, NY, USA). Descriptive statistics were computed to summarize the demographic and baseline characteristics of the participants, including frequencies, percentages, means, and standard deviations. The normality test (Kolmogorov-Smirnov indicated that the data were not normally distributed ($p<0.05$). For the overall fitness scores, z-scores were calculated to normalize the data, allowing for comparison across the different components of the SFT. The Friedman test was used to assess differences in functional fitness across multiple test attempts, while the Wilcoxon signed-rank test was employed as a post hoc analysis to compare baseline with 3 weeks and 6 weeks of observation. To assess the practical relevance of observed changes, results were compared with established minimum detectable change (MDC) and minimal clinically important difference (MCID) values for each component of the SFT. Generalized estimating equations (GEE) were also employed to examine gender-related effects over time, considering the main effects of gender and time, and the interaction effects (gender × time). Wald chi-square statistics were used to determine significance, and unstandardized coefficients (B) with 95% confidence intervals (CI) were reported.

Table 1. Frequency and percentage distribution of demographic variables

Sl no	Variable (n=30)	Frequency	Percentage
1.	Age in years (Mean±SD)	64.50±3.67	
	60–64	15	50
	65–69	15	50
2.	Gender		
	Male	7	23.3
	Female	23	76.7
3	Marital status		
	Married	24	80
	Widow/widower	6	20
3	Education		
	No formal education	7	23.3
	Primary	20	66.7
	Secondary	3	10
4	Previous job		
	Coolie	7	23.3
	Homemaker	17	56.3
	Self-employed	2	6.7
	Private	3	10
	Government	1	3.3
5	Present job		
	Coolie	2	6.7
	Homemaker	21	70
	Self-employed	1	3.3
	Unemployed	6	20
6	Family		
	Joined	6	20
	Nuclear	24	80
7	Previous knowledge		
	Yes	7	23.3
	No	23	76.7

Statistical significance was set at $p<0.05$ for all analyses. Both statistical and clinical interpretations were used to evaluate the impact of the 6-week MCT intervention on strength, endurance, flexibility, agility, and overall functional fitness in older adults.

Results

The study included 30 participants with a mean age of 64.50±3.67 years, evenly distributed between the 60–64 (50%) and 65–69 (50%) age groups. Most were female (76.7%) and married (80%). Most participants had completed primary education (66.7%), while 23.3% had no formal education, and 10% had secondary education. Regarding employment history, 56.3% were homemakers and 23.3% had worked as coolie workers. At the time of the study, 70% remained homemakers, and 20% are unemployed. Additionally, 80% lived in nuclear families, and 76.7% reported no prior knowledge of the exercise program (Table 1).

There were no dropouts, falls, fall-related injuries, emergencies or hospitalizations reported during the intervention.

Functional fitness improvements

Table 2. Effectiveness of MCT on functional fitness at baseline, 3 weeks, and 6 weeks[a]

Sl no	Activity/observation	Mean±SD	Median (IQR)	Mean Rank	Z value[#]
1	Lower-body strength (chair sitting)				
	Baseline	9.33±1.66	9.50 (9 to 10)	1.03	
	3 weeks	12.43±2.11	13 (12 to 14)	2.08	55.83**
	6 weeks	14.06±0.95	14 (14 to 14)	2.88	
2	Upper-body strength (arm curl)				
	Baseline	7.96±2.11	9 (5 to 9)	1.88	
	3 weeks	8.20±2.08	9 (6 to 9)	2.08	5.20
	6 weeks	8.16±2.09	9 (6 to 9)	2.03	
3	Endurance (2-min step test)				
	Baseline	72.23±7.65	74 (64 to 79)	1.23	
	3 weeks	75.93±5.67	77 (72 to 82)	2.30	35.42**
	6 weeks	76.33±6.10	76 (72 to 82)	2.47	
4	Lower-body flexibility (chair sit and reach)				
	Baseline	-4.12±1.58	-4.8 (-5.99 to -2.3)	1.32	
	3 weeks	-3.26±1.08	-3.2 (-4.07 to -2.3)	2.20	31.00**
	6 weeks	-3.08±0.91	-3.05 (-4.0 to -2.27)	2.48	
5	Upper-body flexibility (back scratch)				
	Baseline	-5.58±1.84	-6 (-7 to -3)	1.57	
	3 weeks	-5.02±1.59	-5 (-6.02 to -3.75)	2.02	16.48**
	6 weeks	4.19±2.73	-4 (-5.08 to -3.57)	2.42	
6	Agility and balance (8-foot up and go)				
	Baseline	58.4±9.93	60 (52 to 60)	2.55	
	3 weeks	54±7.10	55 (50 to 59.5)	1.87	19.32**
	6 weeks	52.66±6.42	52 (50 to 58)	1.58	
7	FFT (overall)[##]				
	Baseline	2.69±1.30	2.57 (1.59 to 3.57)	1.27	
	3 weeks	3.31±1.06	3.17 (2.59 to 3.97)	2.67	29.60**
	6 weeks	3.15±3.74	2.95 (2.63 to 3.74)	2.07	

[a #] – Friedman test, ** – p<0.01

The Friedman test results indicate significant improvements across several fitness activities over time. For Chair Sitting, there was a notable increase in performance from pretest to post-tests, with median scores rising from 9.50 to 14 (p<0.01). The 2-min step test, chair sit and reach, back scratch, and 8 ft up and go all showed significant enhancements, with p-values less than 0.01, reflecting improved strength, endurance, flexibility, and agility. In contrast, the arm curl test did not show significant changes, suggesting no substantial improvement in upper-body strength. The FFT also significantly improved, with scores increasing from pretest to post-test (p<0.01). These results highlight that most fitness measures were substantially enhanced, except for upper-body strength (Table 2). The mean and standard deviation for each parameter of the SFT are shown in Fig. 1, which reflects the improvement of all SFT parameters over the study period.

Table 3. Post-Hoc test to assess the effectiveness of Multicomponent training between baseline, 3rd week, and 6th week[a]

Sl no	Activity/observation	Enhancement (reps/steps/sec/cms)	Test Statistics	p	MDC	MDC interpretation
1	Lower-body strength (chair sitting in reps)					
	Baseline vs 3 weeks	3.1	-4.06	<0.001		CS
	Baseline vs 6 weeks	4.7	-7.16	<0.001	1.15	CS
	3 weeks vs 6 weeks	1.63	-3.09	0.006		CS
2	Upper-body strength (arm curl in reps)					
	Baseline vs 3 weeks	0.23	NS	NS		CNS
	Baseline vs 6 weeks	0.20	NS	NS	2.53	CNS
	3 weeks vs 6 weeks	0.03	NS	NS		CNS
2	Endurance (2-min step test in steps)					
	Baseline vs 3 weeks	3.70	-4.13	<0.001		CS
	Baseline vs 6 weeks	4.10	-4.77	<0.001	1.40	CS
	3 weeks vs 6 weeks	0.40	-0.64	>0.99		CNS
3	Lower-body flexibility (chair sit and reach in cms)					
	Baseline vs 3 weeks	0.85	-3.42	0.002		CNS
	Baseline vs 6 weeks	1.04	-4.51	<0.001	1.55	CNS
	3 weeks vs 6 weeks	0.18	-1.09	0.817		CNS
4	Upper-body flexibility (back scratch in cms)					
	Baseline vs 3 weeks	0.56	-0.43	0.28		CNS
	Baseline vs 6 weeks	0.82	-0.76	0.009	1.41	CNS
	3 weeks vs 6 weeks	0.26	-0.33	0.59		CNS
5	Agility and balance (8-foot up and go in sec)					
	Baseline vs 3 weeks	0.44	2.64	0.024		CNS
	Baseline vs 6 weeks	0.57	3.74	<0.001	1.47	CNS
	3 weeks vs 6 weeks	0.13	1.097	0.81		CNS
6	Functional Fitness test- FFT (overall)					
	Baseline vs 3 weeks	NA	-1.40	<0.001	NA	NA
	Baseline vs 6 weeks	NA	-0.80	<0.006	NA	NA
	3 weeks vs 6 weeks	NA	0.60	0.06	NA	NA

[a] Test statistics – Wilcoxon Sign test, Reps – repetition, NS – no post hoc performed due to non-significance, NA – not applicable, MDC – minimum detectable change, CS – clinically significant, CNS – clinically non-significant

Post-hoc analysis using the Wilcoxon signed-rank test showed improving trends in most of the components of SFT. Lower-body strength, assessed via the Chair Sitting Test, improved significantly from base-

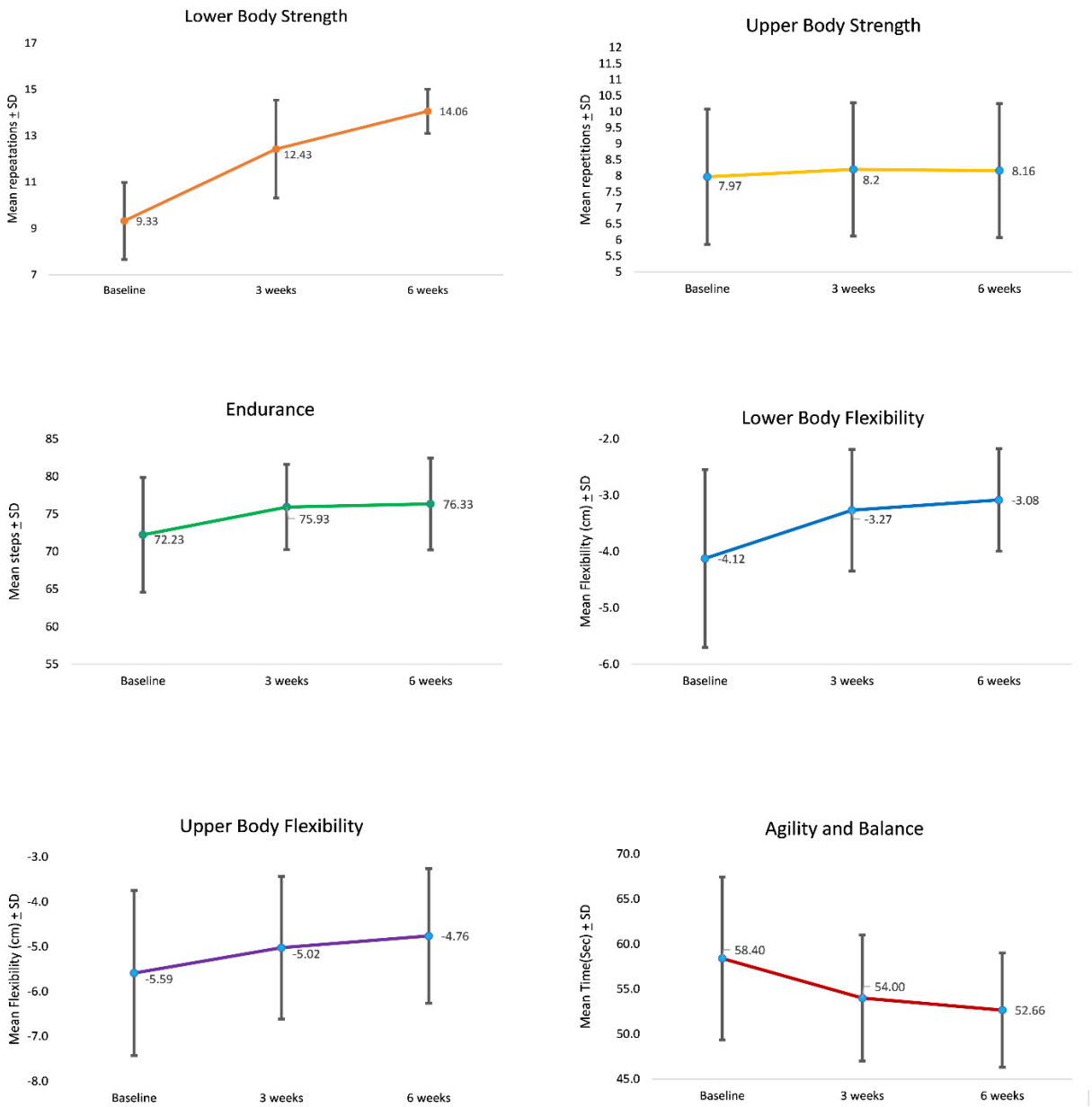

Fig 1. Mean±SD of each parameter of the SFT among the older adults

line to 3 weeks ($p<0.001$) and further enhanced at 6 weeks ($p<0.001$). A significant difference was also observed between 3 and 6 weeks ($p=0.006$), indicating progressive gains in strength. Endurance, measured through the 2-Minute Step Test, showed significant improvements from baseline to both 3 weeks ($p<0.001$) and 6 weeks ($p<0.001$), but no significant change between 3 and 6 weeks ($p>0.99$), suggesting early adaptation with no further substantial gains. For lower-body flexibility (chair sit and reach), significant improvements were observed from baseline to 3 weeks ($p=0.002$) and 6 weeks ($p<0.001$), though no significant difference was noted between 3 and 6 weeks ($p=0.817$). Similarly, upper-body flexibility (back scratch) significantly improved from the baseline to 6 weeks ($p=0.009$). In contrast, changes between baseline and 3 weeks ($p=0.28$) and 3 to 6 weeks ($p=0.59$) were not statistically significant. Complex coordination, agility, and balance (8-ft up and go) demonstrated a significant improvement between baseline and 3 weeks ($p=0.024$) and an even more significant improvement at 6 weeks ($p<0.001$). However, no significant change was observed between 3 and 6 weeks ($p=0.81$), indicating that most improvements occurred within the first 3 weeks.

The overall FFT scores showed significant improvements from baseline to both 3 weeks ($p<0.001$) and 6 weeks ($p<0.006$), while the difference between 3 and 6 weeks was not significant ($p=0.06$). Upper-body strength (arm curl test) could not be analyzed post hoc due to a lack of significant differences between time points (Table 3).

These findings suggest that substantial gains in strength, endurance, flexibility, and coordination occur within the first 3 weeks of training, with further significant improvements seen at 6 weeks in most areas, particularly in lower-body strength and functional fitness.

Table 4. Comparison of senior fitness parameters between male and female over time[a]

Sl no	Activity/ observation	Frequency (Percentage)						Source	B	W
		Total (30)		Male (7)		Female (23)				
		BA	A/AA	BA	A/AA	BA	A/AA			
1	Lower-body strength (chair sitting)									
	Pretest	26 (86.7)	4 (13.4)	6 (85.7)	1 (14.3)	20 (87)	3 (13)			
	Post-test 1	5 (16.7)	25 (83.3)	1 (14.3)	6 (85.7)	4 (17.4)	19 (82.6)	G(F)	-0.53	0.22
	Post-test 2	1 (3.3)	29 (96.7)	-	7 (100)	1 (4.3)	22 (95.9)	GxT(F)	-0.08	0.120
2	Arm curl									
	Pretest	30 (100)		7 (100)	-	23 (100)	-			
	Post-test 1	30 (100)		7 (100)	-	23 (100)	-	G(F)	-1.11	1.09
	Post-test 2	30 (100)		7 (100)	-	23 (100)	-	GxT(F)	-0.56	0.137
3	Endurance (2-min step test)									
	Pretest	16 (53.3)	14 (46.7)	3 (42.9)	4 (57.1)	13 (56.5)	10 (43.5)			
	Post-test 1	14 (46.7)	16 (53.3)	3 (42.9)	4 (57.1)	11 (47.8)	12 (52.2)	G(F)	0.70	0.02
	Post-test 2	15 (50)	15 (50)	4 (57.1)	3 (42.9)	11 (47.8)	12 (52.2)	GxT(F))	-1.79	4.33*
4	Lower-body flexibility (chair sit and reach)									
	Pretest	26 (86.7)	4 (13.3)	7 (100)	-	19 (82.6)	4 (17.4)			
	Post-test 1	25 (83.3)	5 (16.7)	7 (100)	-	18 (78.3)	5 (21.7)	G(F)	-1.003	1.60
	Post-test 2	25 (76.7)	5 (16.7)	7 (100)	-	18 (78.3)	5 (21.7)	GxT(F)	0.101	0.23
5	Upper-body flexibility (back scratch)									
	Pretest	23 (76.7)	7 (23.3)	6 (85.7)	1 (14.3)	17 (73.9)	6 (26.1)			
	Post-test 1	23 (76.7)	7 (23.3)	7 (100)	-	16 (69.6)	7 (30.4)	G(F)	-1.64	2.15
	Post-test 2	21 (70)	9 (30)	7 (100)	-	14 (60.9)	9 (39.1)	GxT(F)	0.56	2.05
6	Complex coordination: agility and balance (8-foot up and go)									
	Pretest	6 (20)	24 (80)	-	7 (100)	6 (26.1)	17 (73.9)			
	Post-test 1	3 (10)	27 (90)	-	7 (100)	3 (13)	20 (87)	G(F)	0.359	0.25
	Post-test 2	2 (6.7)	28 (93.3)	-	7 (100)	2 (8.7)	21 (91.3)	GxT(F)	0.017	0.007
7	FFT (Mean±SD)									
	Pretest	9.06±1.52		9.71±1.38		8.87±1.54				
	Post-test 1	10.63±1.58		10.85±1.57		10.56±1.61		G(F)	-1.348	3.86*
	Post-test 2	11.00±1.48		10.85±1.06		11.04±1.60		GxT(F)	0.516	5.44*

[a] BA – below average, A – average, AA – above average, G(F) – gender (female), GxT – gender x time, GEE model test: W – Wald Chi-square, * – significant p<0.05, B – unstandardized coefficient

The GEE model analysis revealed significant effects in the 2-minute step and FFT, with the female gender as the reference. In the 2-minute step test, a significant interaction between gender and time was observed (W=4.33, B=-1.79; 95% CI=-3.49, -0.10*), indicating that females showed a lower rate of improvement over time compared to males. Similarly, in the Functional Fitness Test, a significant gender difference was found (W=3.86, B=-1.348; 95% CI=-2.69, -0.004*), suggesting that females had lower baseline performance than males. However, a significant gender-by-time interaction (W=5.44, B=0.516; 95% CI=0.08, 0.94*) indicated that females improved more over time than males (Table 4).

Discussion

This study demonstrates that a 6-week, low-to-moderate intensity MCT program performed three times weekly significantly improves functional fitness in older adults. These results support the hypothesis that structured exercise positively impacts age-related functional decline.

Several studies have been conducted to enhance senior fitness among older people over varying durations, such as 12 weeks[21], 24 weeks[16], and 32 weeks[15], often incorporating control groups.[22–25] However, only a few studies have reported that even a 6-week training program can lead to improvements in physical attributes among older adults.[17,18]. Some Studies have explored links to psychological well-being[23], Cognition, and Activities of Daily Living,[13] and, in some cases, extended their scope to examine relationships with factors like resilience, happiness, perceived stress, and overall well-being.[26] Notably, this is the first study conducted in a rural South Indian setting, offering a unique perspective and aiming to evaluate whether a shorter, 6-week intervention can effectively enhance senior fitness in a context where longer-duration programs may not be feasible.

Compared with the MDC values established for the Indian older adult population[27] highlights the effectiveness of the Multicomponent training in enhancing various fitness components (Table 3). Notably, the program led to clinically significant gains in lower-body strength (30-second chair stand) and aerobic endurance (2-minute step test), surpassing the MDC thresholds of 1.15 repetitions and 1.40 steps, respectively. Upper-body flexibility (back scratch) showed near-clinical significance, with a gain of 1.39 cm, just below the MDC of 1.41 cm. However, lower-body flexibility (chair sit-and-reach test) and agility and dynamic balance (8-foot up-and-go test) did not reach their MDC thresholds of 1.55 cm and 1.47 sec, respectively, suggesting that the observed changes may not be functionally significant.

Additionally, upper-body strength (30-second arm curl test) showed minimal changes (0.23 reps at 3 weeks and 0.20 reps at 6 weeks), which fall well below the MDC value of 2.53 reps, indicating no clinically mean-

ingful improvement. Currently, minimal clinically significant difference (MCID) values are not established for the Indian context; hence, they are not compared with it. In contrast, in a 10-year study conducted in the USA (2005–2016),[28] reported clinically significant improvements across all SFT parameters**.** Further, the study also revealed that less improvement was associated with being female, being aged more than 75, not being married, having one or more falls, and having a disability.

Upper and lower-body strength (arm curl and chair sitting)

The study intervention resulted in a notable increase in lower-body strength, while no significant improvement was observed in upper-body strength. Maintaining optimal upper and lower-body strength is crucial for older adults to preserve physical function, prevent chronic diseases, and perform daily activities independently.[29] Research has consistently shown that enhancing upper and lower-body strength is vital in safeguarding functional ability in older adults.[30] This study's results are similar to a study conducted in Hungary [26] where a 12-week FFFT, which is otherwise called the SFT, was conducted along with the control group, reported a significant increase in upper-body strength at 8 and 12 weeks, with notable improvements in lower-body strength observed at the 12-week mark. This difference may be attributed to the longer session duration (50 minutes) and the inclusion of three specific exercises targeting both upper and lower limb strength in the Hungarian study.

Upper and lower-body flexibility (chair sit and reach, back scratch)

The flexibility had increased significantly in 3 weeks and 6 weeks after the intervention. A decline in flexibility among older adults is strongly associated with the onset of musculoskeletal impairments and the advancement of disabilities. Reduced flexibility leads to a decline in functional ability, making everyday tasks such as getting up from a bed or chair, using the toilet, and navigating stairs increasingly challenging.[31] Studies have shown that older men are more flexible than older women.[32] The results are similar to the studies conducted in Australia[33], USA[28], 16-week programs, where improved upper and lower flexibility was significant; additionally, studies have found an association between Pilates flexibility exercises and reduced fall risk.[34,35] A study comparing a 16-week program with sessions three times versus twice per week found a significant difference in upper-body flexibility, suggesting that both frequency and duration of exercise play a crucial role in enhancing flexibility.[25]

Agility and balance (8-foot up and go)

Agility and balance are essential for many daily activities, including walking, climbing stairs, navigating obstacles, using the bathroom, boarding and exiting vehicles, crossing the street, and responding to a phone call or doorbell**.**[25] The study revealed a statistically significant time reduction in completing the 8 Ft up and test ($p<0.05$), But when compared to the MDC value of the Indian population[27], it's not clinically significant. Research suggests that performance in the Eight-Foot Up-and-Go Test can effectively distinguish between different functional levels in this population and is responsive to improvements resulting from increased physical activity. Studies have shown that physically active older adults completed the Eight-Foot Up-and-Go Test faster than their inactive counterparts.[9,36] Similarly, a study conducted in S. Africa[25], found that all SFT parameters showed statistically significant improvements from baseline ($p<0.05$), except for agility and balance, which remained unchanged even after 16 weeks of a structured exercise program. The no significant improvement may be related to the large number of older women; the population under study may not be active.

Endurance/aerobic capacity (2-min step test)

Endurance or Aerobic capacity is directly related to oxygen intake capacity.[37] Studies have shown a decline of 15 to 20 % per decade.[38] Endurance exercise can increase cardiorespiratory fitness, decrease insulin resistance, and reduce body fat [39] and lipid levels in diabetic patients, one of the most chronic disorders in Old age.[40] The study revealed that the Multicomponent training increased endurance and was significant statistically and clinically. The 2-minute step test numbers improve from 72 steps to 76 steps at the end of the program. On the contrary, a study was conducted to validate a 2-minute step test among healthy older adults and found a mean step of 81 in men and 92 steps in women, which is higher than the current study.[41]

The Multicomponent training led to clinically meaningful improvements in lower-body strength and aerobic endurance, with borderline effects on upper-body flexibility. However, gains in upper-body strength, lower-body flexibility, agility, and balance did not exceed the MDC, suggesting they may not significantly impact daily functional activities in older adults. Additionally, the sample consisted predominantly of female participants, potentially limiting the findings' generalizability to older men. These results highlight the need for program modifications, including enhanced flexibility and balance training, to maximize functional fitness benefits.

Study limitations

This study has several limitations. The sample was predominantly female, which may limit the generalizability of the results to older men. Additionally, the lack of data on participants' sports background and current physical activity levels makes it difficult to account for individual differences, as improvements are often more pronounced

in those with lower baseline activity. The absence of a control group also limits our ability to draw definitive conclusions about the specific effects of the intervention, as observed improvements could be influenced by other factors. While significant improvements were noted in some areas, certain measures (e.g., upper-body strength, lower-body flexibility, and agility) did not exceed the MDC thresholds, suggesting these changes may not be functionally significant. Lastly, the 6-week duration may have been too short to produce substantial improvements in all fitness domains. Future research should include a more balanced sample, a control group, and longer interventions to better assess the impact on functional fitness.

Conclusion

The findings highlight the effectiveness of structured exercise programs in improving strength, endurance, flexibility, agility, and balance in older adults. While lower-body strength, flexibility, endurance, agility, and balance showed statistically significant improvements, upper-body strength showed limited improvements. Enhancing exercise frequency, duration, and targeted training may optimize functional fitness outcomes.

Declarations

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This study received no funding.

Author contributions

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

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available from the first author, on request.

Ethics approval

The study obtained Institutional Ethics Committee permission (Ref no:G2-312/2015/CONTSR(7)).

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ORIGINAL PAPER

Aprocitentan mitigates ischemia reperfusion induced kidney injury in rats by attenuating inflammation and pyroptosis through suppressing NF-κB/NLRP3/caspase-1 signaling pathway

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ABSTRACT

Introduction and aim. Renal ischemia-reperfusion (I/R) injury is a primary cause of acute kidney injury (AKI). The NF-κB/NLRP3/caspase-1 signaling system is crucial for I/R-induced kidney damage, which leads to inflammation, pyroptosis, and tissue damage. Aprocitentan has anti-inflammatory and vasoprotective properties, suggesting its potential nephroprotective advantages. The aim of this investigation was to evaluate the protective advantages of aprocitentan against renal I/R injury in rat models. This was achieved by evaluating the impact of aprocitentan on inflammation, pyroptosis, and kidney function by altering the NF-κB/NLRP3/Caspase-1 pathway.

Material and methods. Using twenty-four adult male Sprague-Dawley rats, four separate groups were formed: sham group, I/R control group, dimethyl sulfoxide group (DMSO) and aprocitentan group (10 mg/kg). Renal ischemia/reperfusion was induced by a period of forty minutes of bilateral ischemia, which was followed by two hours of reperfusion. To evaluate the renoprotective effect of aprocitentan urea, creatinine, neutrophil gelatinase-associated lipocalin (NGAL), phosphorylated nuclear factor kappa B p65 (NF-κB p65), NOD-like receptor protein 3 (NLRP3), and cysteine-aspartic protease-1 (caspase-1), were measured, along with histopathological examination.

Results. Compared to the sham group, the I/R control group had significantly increased concentrations of urea, creatinine, NGAL, NF-κB p65, NLRP3, as well as caspase-1. These signs decreased in the aprocitentan group, leading to enhanced renal function, reduction of inflammation, and inhibition of pyroptosis. Histology showed a decrease in tubular inflammation and necrosis in the aprocitentan group.

Conclusion. Aprocitentan decreases inflammation and inhibits pyroptosis by suppressing the NF-κB/NLRP3/Caspase-1 pathway. These results illustrate its promising potential to prevent ischemic renal diseases, including acute kidney injury.

Keywords. acute kidney injury, caspase-1, endothelin receptor antagonist, ischemia-reperfusion, NLRP3 inflammasome, pyroptosis

Introduction

Renal I/R injury is a significant clinical concern and one of the main causes of AKI, leading to a high incidence of morbidity and mortality globally.[1] This disorder occurs when the kidney blood supply is temporarily interrupted (ischemia) and then resumed (reperfusion). Despite the fact that reperfusion is necessary to avoid irreversible tissue damage, it conversely worsens kidney injury, resulting in decreased kidney function and, in serious cases, end-stage kidney disease.[2]

Damage to renal I/R is a crucial subject in both therapeutic and experimental research, as it manifests frequently in clinical conditions, including kidney transplants, partial nephrectomy, septic shock, and car-

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diovascular procedures.[3] Renal I/R injury is particularly crucial in the transplantation of the kidney; it is a strong predictor of delayed graft function as well as long-term allograft survival.[4] While techniques for preserving organs have improved, I/R injury is still an important issue that makes it difficult for transplants to succeed.

Growing evidence indicates that pyroptosis – a pro-inflammatory type of programmed cell death regulated by the NLRP3 inflammasome and caspase-1– has a pivotal role in AKI after ischemia reperfusion injury.[5,6] During renal I/R, damage-associated molecular patterns (DAMPs) initiate NF-κB and the NLRP3 inflammasome, leading to caspase-1 activation and the liberation of mature interleukins, this includes interleukin-1 beta (IL-1β) and interleukin-18 (IL-18) that trigger an intense inflammatory cascade.[6] In experimental I/R models, treatments that inhibit NLRP3 or caspase-1 have shown lower kidney damage, suggesting that the pyroptosis pathway is a viable target.[7] Endothelin-1 has been shown to worsen renal damage by resulting in vasoconstriction and inflammation,[8] aprocitentan is a new dual endothelin receptor blocker. Therefore, we proposed that aprocitentan could reduce I/R-induced kidney damage by suppressing NLRP3/caspase-1-mediated pyroptosis and NF-κB-driven inflammation.

Aprocitentan is a dual endothelin receptor antagonist (ERA) approved for the treatment of resistant hypertension in adults. Aprocitentan blocks endothelin receptor type A (ETA) and endothelin receptor type B (ETB) receptors that decrease blood pressure and improve cardiovascular health.[9] Aprocitentan has shown the ability to reduce the levels of pro-inflammatory cytokines, especially IL-1β and tumor necrosis factor-alpha (TNF-α), thus decreasing the inflammatory response in I/R.[10]

NF-κBp65 it is an important subunit of the NF-κB transcription factor and plays an essential role in the inflammatory response associated with renal I/R. Oxidative stress and inflammatory mediators activate NF-κBp65, which moves to the nucleus and starts the transcription of pro-inflammatory genes after I/R. This procedure improves tissue damaging and increases pro-inflammatory cytokines, adhesion molecules, and enzymes, resulting in kidney damage. In the I / R animal model, reducing NF-κBp65 particularly reduces renal damage and inflammation.[11] Moreover, NF-κBp65 controls tubular cell apoptosis and fibrosis, each of which are significant characteristics of I/R-induced kidney failure.[12]

The NLRP3 inflammasome is an important contributor to kidney damage during renal I/R. I/R promote NLRP3 activation due to cellular stress and mitochondrial malfunction as well, leading to the formation of reactive oxygen species (ROS) and Generation of pro-inflammatory cytokines (IL-1β and IL-18). Stimulation of NLRP3 inflammasomes plays a role during kidney tubular injury, inflammation, and fibrosis during I/R.[13] NLRP3 activation may worsen kidney damage by inducing pyroptosis, which causes more tissue destruction.[14] Medications that reduce kidney injury after an ischemic episode incorporate in the inhibition of the NLRP3 inflammasome. Research indicates that targeting NLRP3 may enhance kidney function by decreasing inflammation and oxidative stress, possibly providing a method to address renal I/R.[15]

Caspase-1 is a critical enzyme involved in cell death and inflammation and is an important contributor to renal I/R. In I/R, caspase-1 activation during the inflammatory response, breakdown of pro-inflammatory cytokines (IL-1β and IL-18) to their mature form, results in tissue damage. Inhibition of caspase-1 reduces renal damage and improves kidney function after I / R, suggesting that caspase-1 is a mediator of kidney inflammation and injury. It also appears to trigger renal tubular cell pyroptosis, thus worsening tissue damage following I/R.[16]

NF-κB p65 represents a transcription element that increases the synthesis of pro-inflammatory cytokines and components of the inflammasome, effectively priming cells for pyroptosis. NLRP3 is an intracellular sensor that forms the NLRP3 inflammasome complex when detecting cellular stress or damage signals; this then activates pro-caspase-1. Caspase-1 mediates the processing of pro–IL–1β and pro–IL-18 into active cytokines and cleaves gasdermin D, the executor protein that produces membrane pores that promote cell lysis and inflammatory cell death.[17] These three factors were measured as key markers of NF-κB inflammasome and pyroptosis in the kidney tissue.

Limited studies have directly examined therapeutic agents targeting the NF-κB/NLRP3/caspase-1 axis as a coordinated pathway, despite emerging evidence highlighting the impact of inflammation and pyroptosis on renal I/R injury. Although the protective potential of NLRP3 or caspase-1 inhibitors has been independently shown, few interventions have concurrently suppressed upstream inflammatory signaling and downstream pyroptotic mechanisms. Aprocitentan presents a unique opportunity to modulate vascular and inflammatory responses by dual blocking of endothelin A and B receptors. However, its mechanistic efficacy in the context of renal I/R injury and its impact on NF-κB/NLRP3/caspase-1 cascade of NF-B/NLRP3/caspase-1 remain unexplored. Since NLRP3 and caspase-1 are essential for renal ischemia-reperfusion injury, this study aims to investigate the less studied potential of aprocitentan, which can affect these inflammatory pathways by antagonizing endothelin receptors. Aprocitentan could provide upstream modulation in addition to direct inflammasome inhibition, improving the possibilities for treatment beyond traditional inflammasome-targeted therapies.

Aim

The objective of this investigation is to estimate whether the dual endothelin receptor antagonist aprocitentan exhibits any possible nephroprotective advantages in a rat

model of I/R. The research project relies on the mechanisms of pyroptosis and inflammation to offer a detailed understanding of their beneficial effects.

Material and methods

Animals' preparation

A total of 24 mature male Sprague-Dawley rats, weighting between 200 and 250 grams, were sourced from the Faculty of Science University of Kufa. They were kept in the University of Kufa's Faculty of Science's animal department, under standard settings. The average daily temperatures were preserved at 24 ± 2°C, and 60-65% humidity, with the rats having unrestricted access to food and tap water. The animals were kept in cages, with a 12 hour light/dark cycle.

Experimental design

Twenty-four male adult rats (Sprague-Dawley) randomly assigned to four groups using a simple randomization method (n=6 each group). The sample size of n=6 per group was chosen based on previous studies of I/R injury that detected significant differences with similar group sizes[18,19]: Sham, I/R control, I/R+DMSO, and I/R+aprocitentan. The Sham group underwent a surgical operation without renal ischemia, while the I/R control group underwent renal ischemia-reperfusion injury without any drugs or vehicle interference. The I/R+DMSO group suffered an I/R injury and received an injection of DMSO as medication solvent, while the I/R+aprocitentan group suffered I/R injury and received 10 mg/kg of aprocitentan (TargetMol Chemicals, China). Aprocitentan (or an equivalent DMSO vehicle) was injected intraperitoneally twice, 24 hours and 60 minutes before the onset of ischemia to guarantee systemic drug exposure throughout the I/R insult. The dose of 10 mg/kg was chosen after previous studies indicating effective endothelin receptor blockade at this dose in rats.[20] After the duration of the experimental period, blood samples and kidneys were collected for further assessment, and then all animals were sacrificed. The experiments carried out in this study were reviewed and approved by the Ethics Committee of the Faculty of Pharmacy of the University of Kufa, Najaf, Iraq (Approval No. 3177/2025-2-4).

Renal I/R model

The animals received ketamine (100 mg/kg) (Interchem, Holland) and xylazine hydrochloride (10 mg/kg) (Alfasan, Holland) intraperitoneally to induce anesthesia.[21] After induction of anesthesia, the limbs were secured, the area around the incision was shaved and it was then disinfected with iodine spray. Subsequently, a retroperitoneal flank incision. The renal pedicles were cautiously recognized by blunt dissection to gently separate the connective tissues and adipose tissue surrounding the left and right renal arteries and veins, anteriorly and posteriorly. The abdominal cavity was accessed via a retroperitoneal flank incision, exposing both kidneys. Bilateral renal occlusions were performed for 40 minutes using non-traumatic microvascular clamps (Minechina/Germany). The color shifted from red to dark maroon in 10 minutes, and the whole surface was an obvious indication of occlusion. Then a sterilized gauze was placed on the rat. When the ischemia phase and withdrawing the clamp was removed, the color of the kidney rapidly change from a dark maroon to dark pink, signifying successful reperfusion. After verifying reperfusion, the wound was bound and wrapped in clean gauze, both saturated with 0.9% saline to avoid dehydration. After 2 hours of reperfusion, blood was taken directly from the heart for parameter measurements, and the kidneys were harvested for evaluation of the experimental parameter assessment; afterwards, the rats were sacrificed by cardiac puncture. The kidney was sagittally divided into two parts; one was kept in 10% formaldehyde for histological purposes, and the other was placed in the freezer at -80°C in phosphate buffered saline (PBS) for the enzyme-linked immunosorbent assay (ELISA) analysis.[22] All outcomes assessments, including histopathological scoring and biomarker measurements, were performed by investigators blinded to group allocations.

Blood sample collection

At the end of the procedure, when the rats remained under anesthesia, approximately 2-4 milliliters of blood were collected directly from the cardiac chamber. The blood sample was placed in a gel tube free of anticoagulant agents and centrifuged at 3000 rpm for approximately 10 min to extract serum. Serum was next used to evaluate urea and creatinine spectrophotometrically and NGAL levels using a commercial ELISA kit (Shanghai Ideal Medical Technology Co., Ltd./China, Cat No: ADLEL-RT00241).

Tissue sample preparation

Samples of kidney tissue were taken after the blood samples were collected. For histopathological evaluation, one piece of tissue was embedded in 10% formaldehyde, while the other was placed in a freezer at -80°C before homogenizing using a high-frequency ultrasonic liquid processor within a 1:10 W/V solution of buffered phosphate buffered saline with 1% Triton X-100 and a cocktail of protease inhibitors (Roche Germany).[23] The homogenate went through a centrifuge at 5000 rpm for 10 min at 4°C. The obtained supernatants were used to assess NF-κB p65, NLRP3, and Caspase-1 using available ELISA kits (Shanghai Ideal Medical Technology Co., Ltd./China, Cat No: ADL-EL-RT01064, Cat No: ADL-EL-RT01063, Cat No: ADL-EL-RT00752).

Histopathological examination

Kidney tissues have been extracted and preserved in 10% neutral buffered formalin. The samples were placed in paraffin, which is cut to 5 μm pieces. Tissue slices were stained with hematoxylin and eosin (H&E) for histological purposes. The kidney tissues were examined for histopathological changes, including tubular necrosis, inflammation, and severity of damage. Injury scores were given according to the level of damage, and tubular necrosis and inflammation were documented in each section. Histological changes were determined by the percentage of damaged kidney tubules. Tissue damage was determined semi-quantitatively as follows: 0=no damage (intact tubules); 1=<25% damage; 2 = 25 50% damage; 3=50–75% damage; and 4 => 75% damage.[24]

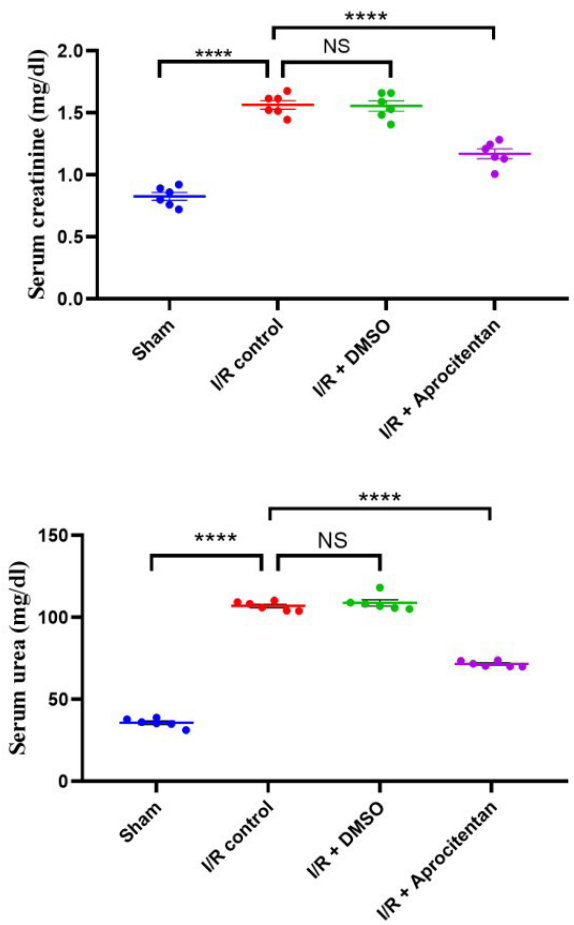

Fig. 1. Means of serum urea and creatinine concentration in the various groups (data resemble mean ±SD; n=6 biological replicates, **** – p<0.0001, and ns – p>0.05 nonsignificant vs. I/R control group, one-way ANOVA, Tukey's test)

Statistical analysis

GraphPad Prism version 8.1 was employed to conduct statistical analysis. Data for normally distributed variables were presented as mean ± SD. The Shapiro-Wilk test was used to determine the normality of the data distribution. A one-way analysis of variance (ANOVA) was used to assess differences between groups, followed by Tukey's multiple comparison test. To compare histopathological changes between groups, nonparametric Kruskal-Wallis and Dunn's post-hoc tests were used. A p-value of <0.05 was considered statistically significant.

Results

Effect of aprocitentan on urea and creatinine

In contrast to the sham group, the serum urea and creatinine in the I/R control group were considerably higher (p<0.0001). The I/R control and vehicle (DMSO) groups did not differ significantly (p>0.05). Compared to rats treated with aprocitentan with the I/R control group, rats blood urea levels were considerably lower (p<0.0001) (Fig. 1).

Effect of aprocitentan on the level of NGAL

Unlike the sham group, the I/R control group's NGAL concentrations of the I/R control group were considerably higher (p<0.0001). There was no apparent difference between the vehicle and the I/R control groups (p>0.05). NGAL levels exhibited a significant decline in rats receiving aprocitentan compared to the I/R control group (p<0.0001) (Fig. 2).

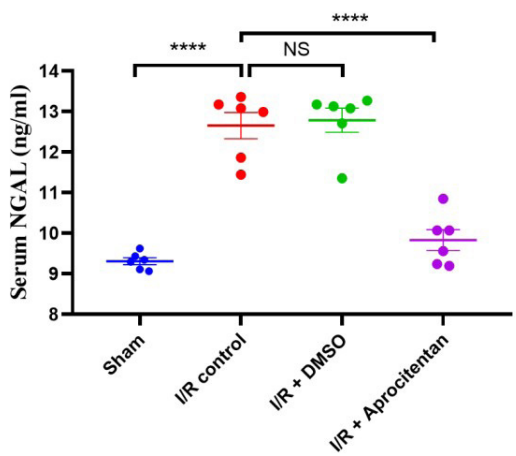

Fig. 2. Means of serum NGAL concentration in the various groups (data resemble mean±SD; n=6 biological replicates, **** – p<0.0001, and ns – p>0.05 nonsignificant vs. I/R control group, one-way ANOVA, Tukey's test)

Effect of aprocitentan on the level of the inflammatory biomarker NF-κBp65 level

The NF-Bp65 concentration of the tissue homogenate NF-κBp65 concentration was significantly higher than that of the sham group (p<0.0001). When comparing the DMSO group with the I/R control group, there was no noticeable difference in the NF-κBp65 level (p>0.05). Rats given aprocitentan had significantly lower NF-κBp65 levels of NF-Bp65 relative to the I/R control group (p<0.0001) (Fig. 3).

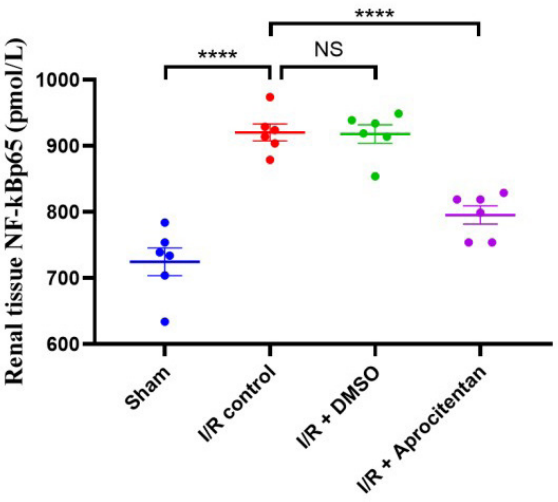

Fig. 3. Means of tissue NF-κBp65 concentration in the various groups (data resemble mean±SD; n=6 biological replicates, **** – p<0.0001, and ns – p>0.05 nonsignificant vs. control group, one way ANOVA, Tukey test)

Effect of aprocitentan on the level of NLRP3

The from the I/R control group of tissue homogenate NLRP3 concentration was significantly greater (p<0.0001) than that of the sham group. When comparing the DMSO group with the I/R control group, there was no noticeable variation in the level of NLRP3 level (p>0.05). Rats who received aprocitentan demonstrated substantial reductions in NLRP3 levels compared to the I/R control group (p<0.0001) (Fig. 4).

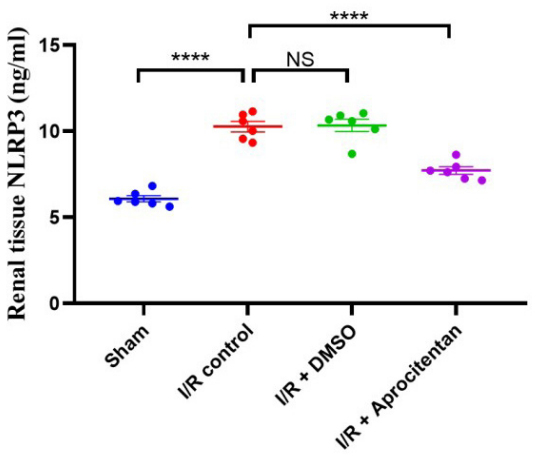

Fig. 4. Means of tissue NLRP3 concentration in the various groups (data resemble mean±SD; n=6 biological replicates, **** – p<0.0001, and ns – p>0.05 nonsignificant vs. I/R control group, one-way ANOVA, Tukey's test)

Effect of aprocitentan on the level of the caspase-1 pyroptosis biomarker

The concentration of tissue homogenate caspase-1 from the I / R control group was significantly higher than that of the sham group (p<0.0001). When comparing the DMSO group with the I/R control group, there was no noticeable difference in the level of caspase-1 level (p>0.05). Rats treated with the aprocitentan group showed a significant decline in the caspase-1 levels compared to the I/R control group (p<0.0001) (Fig. 5).

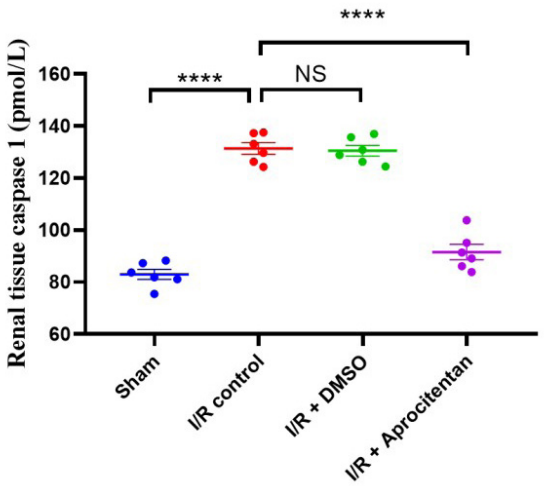

Fig. 5. Means of tissue caspase 1 concentration in the various groups (data resemble mean±SD; n=6 biological replicates, **** – p<0.0001, and ns – p>0.05 nonsignificant vs. I/R control group, one-way ANOVA, Tukey's test)

Histopathological findings

The I/R control and DMSO groups showed a significant histological change (neuronal tubular damage) in comparison with the normal tissue of the sham group (p<0.001). Further comparison that according to the histopathology and scoring system, the score of renal tubular damage was significantly lower in the aprocitentan group in contrast to the I/R control and vehicle groups (*p<0.05) (Fig. 6-10).

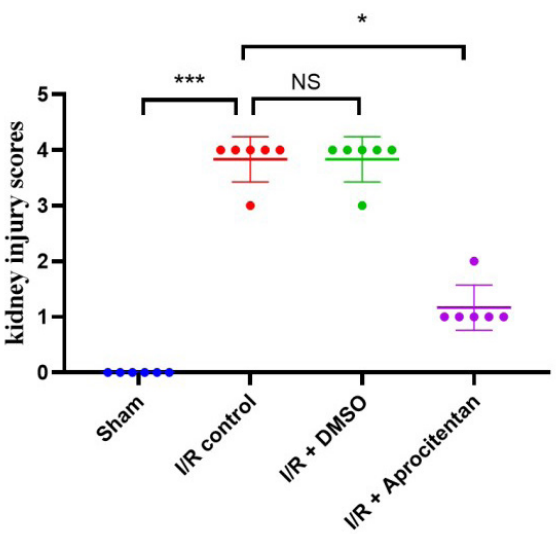

Fig. 6. Scores of kidney injury in the various groups (data resemble mean ± SD; n=6 biological replicates, *** – p<0.001, * – p<0.05, and ns – p>0.05 nonsignificant vs. I/R group, nonparametric Kruskal Wallis and Dunn's post hoc tests)

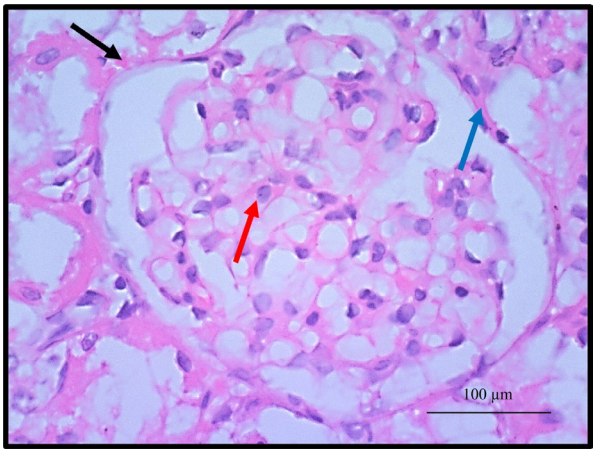

Fig. 7. Histological section in kidney of rat in sham group (the section shows normal renal tissue texture including normal glomeruli (black arrow), normal glomerular tuft (red arrow) and normal glomerular capsule (blue arrow), the proximal renal tubules show normal epithelial cells that line the tubules with normal lumen (green arrows), the tissue is stained by H&E stain and the section was taken with a digital camera attached to a light microscope at 40×magnifier scale)

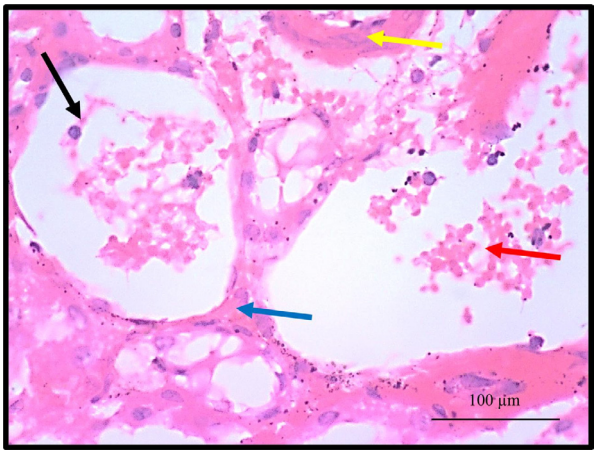

Fig. 9. Kidney histopathological section in rats of DMSO group (intraperitoneal injection with aprocitentan DMSO solvent 24 hours prior to ischemia and then one hour before ischemia, then underwent the surgical procedure, followed by 40 minutes of bilateral renal ischemia then 2 hours of reperfusion), renal tissue shows severe atrophied lesion of renal glomeruli and glomerular tuft (black arrows) with severe hypertrophic changes in the glomerular capsule (blue arrow), blood vessels show clear wall damage congestion (red arrow), the proximal renal tubules show narrowing in the lumen with hypertrophy of lining cells (yellow arrows), tissue is stained by H&E stain and the section was taken with a digital camera attached to a light microscope at 40×magnifier scale

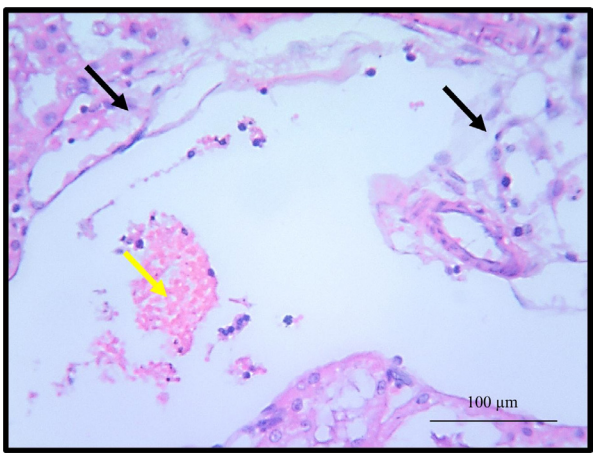

Fig. 8. Kidney histopathological section in rats of I/R control group (rats underwent clamping of the renal artery using the dorsal approach to cause ischemia for 40 minutes and reperfusion for 2 hours), the section shows a clear necrotic lesion in the renal tissue (coagulative necrosis, black arrows) with glomerular atrophy can be seen in the section of renal tissue (red arrows), the glomerular tuft shows severe necrotic change for epithelial cells of the tuft (blue arrow), blood vessels congestion as severe pathological changes can be seen in the section (yellow arrows), the tissue is stained by H&E stain and the section was taken with a digital camera attached to a light microscope at 40×magnifier scale

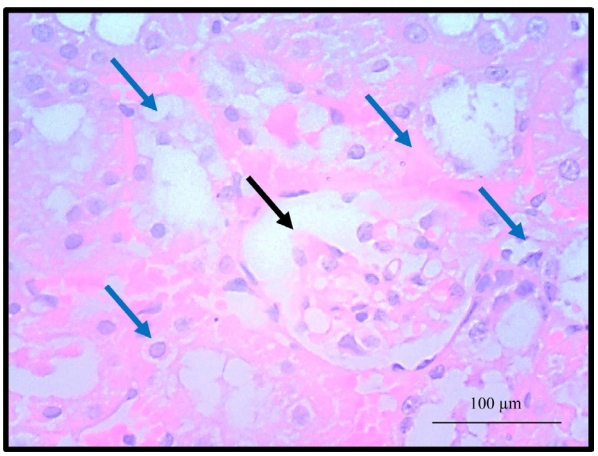

Fig. 10. The histopathological section of the kidney in rat of the treated group with aprocitentan (intraperitoneal injection with aprocitentan (10 mg/kg) both 24 and 1 hour before induction of ischemia), the section shows normal glomerular texture including normal capsule and tuft (black arrow) and some glomeruli show mild atrophied lesion (red arrow), the renal proximal tubules show mild hypertrophy and hydropic degeneration and cytoplasmic vacuolation in tubular epithelial cells (blue arrows), the tissue is stained with H&E stain and the section was taken with a digital camera attached to a light microscope at 40×magnifier scale

Discussion

Urea and creatinine are conventional indicators of renal function that reflect the rate of glomerular filtration. Impaired filtration in AKI, including I/R injury, causes an elevation of these substances in the blood. This is in line with the I/R control rats that had considerably higher blood levels of urea and creatinine compared to the sham group rats.[25–27]

Aprocitentan treatment leads to significantly lower levels of urea and creatinine than untreated I/R control rats, which indicates preserved renal excretory function. This improvement in biochemical indicators of renal function suggests that aprocitentan maintained the glomeruli and tubular filtration systems. Although there are no comparable experiments on aprocitentan in I/R, our findings are consistent with its documented renal effects in other situations. Particularly in hypertensive rat models, dual endothelin-1 (ET-1) receptor blockage with aprocitentan lowers blood pressure without leading to renal damage.[20,28] The ability of aprocitentan to avoid rapid increase in urea and creatinine after I/R corresponds with its history of maintaining renal function, showing that it reduced the acute loss of filtration capacity that often occurs with ischemic damage.

NGAL is an early tubular injury biomarker that increases immediately in blood and urine after renal trauma. It is considered a sensitive early sign of AKI.[29] In our I/R model, serum levels of NGAL were significantly higher in the untreated I/R group in contrast to the sham group, showed significant tubular damage, and confirm the role as an accurate early sign of ischemic AKI.[30] This elevation is consistent with previous research, which suggests that NGAL is significantly increased by ischemic kidney injury and is consistent with the degree of tubular damage.[31]

Aprocitentan treatment led to significantly lower NGAL levels compared with I/R controls. The decreased NGAL shows that aprocitentan decreased acute tubular injury in the ischemic kidneys, providing a degree of protection. The outcome is consistent with previous research on endothelin antagonists. In particular, double ERA bosentan has been found to decrease NGAL levels while improving renal function in the diabetic nephropathy model by lowering oxidative stress and inflammation.[32] Aprocitentan potentially uses comparable defensive mechanisms. Aprocitentan has been shown to relieve the effects of oxidative stress in cellular models.[33] By reducing ROS formation of ROS in the mitochondria,[33] aprocitentan could reduce the oxidative injury to tubules, which would otherwise promote elevation of NGAL. Thus, the impact in I/R suggests reduced acute tubular injury, suggesting effective renal protection, most likely by antioxidant and anti-inflammatory activities.

NF-κB p65 is an essential part of the NF-κB transcription factor complex that controls the expression of inflammatory genes. NF-κB p65 plays a critical role in the inflammatory response during renal I/R.[34] The I/R control group had substantially higher levels of renal NF-κB p65 compared to sham rats, indicating that ischemia-reperfusion stimulated this pathway. Previous investigations have shown that NF-κB p65 levels are significantly elevated in ischemic kidneys, leading to further inflammation and cell death.[12]

As an example, studies indicate that I/R increases NF-κB signaling pathways, leading to renal impairment and tissue damage.[12,35] In a similar way, another study demonstrated that NF-κB p65 levels were significantly higher in kidney damage due to I/R, which was connected to enhanced inflammatory reactions and cell death.[36]

Endothelin-1 itself can enhance inflammatory signaling; ET-1 activates the NF-κB-mediated pathway in macrophages, leading to TNF-α release.[37] Aprocitentan treatment significantly decreased NF-κB p65 in kidney tissue compared with untreated I/R rats, a sign of an anti-inflammatory effect. Aprocitentan inhibits ETA and ETB receptors, so it breaks the loop of ET-1-induced NF-κB activation.[37] This limits the transcription of pro-inflammatory genes. These findings are unique in the context of renal I/R, since previous literature has not specifically studied the influences on NF-κB in acute kidney injury. However, it is comparable to the broad anti-inflammatory effects of endothelin antagonism. Reducing NF-κB activity might limit cytokine release and leukocyte infiltration, preserving renal tissue from inflammation. Therapies that limit NF-κB activity in I/R models may mitigate renal injury.[38] The results we see suggest that aprocitentan produces a similar benefit. Aprocitentan decreases NF-κB p65, indicating that it has the capacity to reduce I/R's early inflammatory pathways. This is essential for reducing tissue damage and preserving renal function.

NLRP3 is an intracellular pattern recognition receptor that polymerizes to form the NLRP3 inflammasome in response to danger signs (oxidative stress, ATP, mitochondrial DNA) after tissue injury.[7] The multiprotein complex activates caspase-1, leading to the production of pro-inflammatory cytokines IL-1β and IL-18.[7] In the kidneys, NLRP3 is found primarily in tubular as well as immune cells, and it is usually inactive.[3] Renal I/R activates NLRP3 by ischemic cell stress, reperfusion-induced ROS production and DAMPs.[7,12]

As a result, we found that I / R significantly increased NLRP3 levels in renal tissue (I/R control versus sham), which agrees with previous studies pointing to NLRP3 as an important modulator of inflammation and injury in I/R models.[1,39]

Rats treated with aprocitentan had considerably lower renal levels of NLRP3 compared to untreated I/R rats, indicating that the drug inhibited inflammasome activation. This is a significant finding, since it reflects that aprocitentan can modify innate inflammatory sen-

sors beyond its hemodynamic effects.[39] To the best of our knowledge, there is no previous investigations have looked into the impact of aprocitentan on NLRP3 on renal I/R. Endothelin-1 signaling has been linked to increased production of cytokines and possibly inflammasome activity in cardiac injury models.[40]

Aprocitentan could reduce upstream triggers (including oxidative stress or NF-κB dependent signals) by blocking ET receptors, which allow NLRP3 activity.[10] Notably, aprocitentan was shown to drastically reduce mitochondrial ROS generation and malfunction in a cellular model, which indicates that its antioxidant properties may be involved in this context.[41] Aprocitentan may help preserve renal tubular cells and decrease dysfunctional inflammation after I/R by inhibiting NLRP3.[42]

Notably, aprocitentan's multifunctional cytoprotective activity appears in different models of organ injury. As an example, aprocitentan has been shown to reduce doxorubicin-induced heart injury by reducing mitochondrial impairment and oxidative stress.[42] These results support the idea that aprocitentan can positively alter stress response pathways (including NLRP3 activation) in many types of tissues, thus promoting organ protection.

Caspase-1 activity led to a cascade of inflammatory damage, which involves the production of IL-1β and IL-18, which intensify inflammation. Furthermore, it cleaves gasdermin D, which results in cell swelling and lytic death.[7] In the present study, I/R caused a substantial increase in caspase-1 in kidney tissue (I/R control versus sham), showing activation of the inflammasome/pyroptosis pathway. Previous studies have shown that caspase-1 activity in AKI can result in an increase in IL-1β/IL18 levels, worsening inflammation, and tissue destruction.[1,12]

Compared to untreated I/R rats, rats treated with aprocitentan exhibited lower levels of caspase-1. This suggests that aprocitentan successfully reduced inflammasome activity and pyroptosis in the kidney.

The direct impact on caspase-1 in I/R has not been investigated in previous studies; however, our results are in line with the protective properties of other treatments that target connected pathways. In fact, it has been suggested that treatments that block ET-1 signaling could reduce ischemic kidney injury by decreasing caspase-1 activation.[40] Dual ETA/ETB blockade of aprocitentan probably interferes with pro-inflammatory signaling sufficient to prevent the NLRP3/caspase-1 axis from full activation. This effect has important consequences: aprocitentan stops the loss of pyroptotic cells and the inflammatory cycle that would cause more damage to the kidneys by inhibiting caspase-1.

Aprocitentan-treated rats showed lower levels of NF-κB p65, NLRP3, and caspase-1, implying a reduction in the inflammasome-driven pyroptosis pathway. Thus, in the current study, inhibition of NLRP3 inflammasome and the caspase-1 action may result in less renal cells undergoing pyroptosis, which is consistent with better renal function and histology found in the aprocitentan group. Research suggests that inhibiting the NF-κB/NLRP3/caspase-1 axis could mitigate renal I/R injury.[20] Lower NF-κB p65 in the treated rats implies reduced transcription of NLRP3 and pro-IL-1β, which contributes to lower damage caused by inflammation. These findings confirm the pathological significance of inflammasome-mediated pyroptosis in AKI and the preventive potential for targeting this pathway with drugs such as aprocitentan.

Renal I/R in rats causes classic acute damage: severe tubular necrosis (loss of epithelial cells and brush boundaries, associated with cast formation), marked vascular congestion, and even glomerular structural injury. These results agree with the observations of Ghada and Tweij et al., whose work reported significant changes in the internal structure of kidneys and significant damage to tubules after I/R.[27,43] in rats treated with aprocitentan, these lesions were markedly attenuated: the glomerular architecture remained intact, milder tubular necrosis, and reduced infiltration of inflammatory cells compared to untreated I/R rats. This protective histopathological profile aligns with previous reports on endothelin blockade in AKI – for example, dual ERA tezosentan almost completely prevented acute cortical tubular necrosis (ATN) and preserved renal architecture in ischemic rat kidneys,[44] and selective ETA blocking (ambrisentan) and dual ETA/ETB blocking (bosentan) similarly decreased tubular injury, apoptosis, and inflammation in renal I/R models.[32]

The decline in NGAL level and histological injury scores in the Aprocitentan-treated group supports the hypothesis that this agent confers meaningful protection against I/R-induced AKI. NGAL reduction implies not only changes in molecular signaling, but also preserved renal function because it is a sensitive marker of tubular injury. These findings are especially relevant considering the existing clinical use as an antihypertensive agent.[45] It could be repurposed to treat perioperative AKI due to its ability to attenuate inflammation and pyroptosis, including situations such as transplantation or cardiac bypass.

This study had various limitations that need to be addressed. To begin with, the sample size was modest (n=6 each group), which limited the range to which the results can be generalized. Second, we only looked at short-term results for two hours after reperfusion; we did not assess the longer-term effects on kidney remediation or the development of chronic injury. Third, it is unclear whether other dose timing or repeated doses might increase the protective effect of aprocitentan because it was only studied at a specific dose and time point. Additionally, we evaluated pyroptosis indirectly

through the measurement of NLRP3 and caspase-1 as substitute indicators instead of directly measuring the downstream executors, such as gasdermin D activation or IL-1β production. As a result, we conclude a decrease in pyroptosis from indirect signs; direct experiments should be used in the future to verify this process. Furthermore, since only male rats were used for this study, any possible sex-based variation in response was not taken into account. Finally, the use of ELISA to assess NF-κB p65 is another limitation, because it does not distinguish between active and inactive forms or indicate nuclear translocation. Future research can use immunohistochemistry (IHC), reverse transcription polymerase chain reaction (RT-PCR), or Western blotting to verify NF-κB activation and obtain more details about the mechanistic insights. Further studies are required to support our findings, and these limitations should be considered when evaluating the data.

Conclusion

This research shows that aprocitentan has significant nephroprotective properties in rat models with renal I/R. Aprocitentan efficiently reduces kidney damage by decreasing inflammation and reducing pyroptosis by inhibition of NFκB/NLRP3/Caspase-1 signaling pathway.

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Declarations

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Author contributions

Conceptualization, T.T.; Methodology, L.A.; Software, L.A.; Validation, L.A. and T.R.; Formal Analysis, T.T.; Investigation, L.A.; Resources, L.A.; Data Curation, L.A.; Writing – Original Draft Preparation, L.A.; Writing – Review & Editing, L.A.; Visualization, L.A.; Supervision, T.R.; Project Administration, T.T.

Conflicts of interest

The authors declare that they have no conflict of interest.

Data availability

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

Ethics approval

Ethical approval for the experiments in this study was obtained from the Ethics Committee of the Faculty of Pharmacy, University of Kufa, Najaf, Iraq (Approval No. 3177/2025-2-4).

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ORIGINAL PAPER

Apelin, C-reactive protein, and serum protein correlation with blood pressure – a biomarker analysis

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ABSTRACT

Introduction and aim. Hypertension is a major global health burden and a leading cause of cardiovascular disease and premature death. This study aimed to evaluate the association between serum apelin, inflammatory markers, and protein metabolism parameters in hypertensive patients compared to normotensive controls. This study uniquely explores the interplay between inflammatory and protein metabolism biomarkers using advanced multivariate models in hypertensive adults, a combination not previously examined in this population.

Material and methods. Two hundred adults aged 35–65 years were divided into hypertensive (n=100) and normotensive (n=100) groups. Serum apelin and C-reactive protein (CRP) levels were measured using enzyme-linked immunosorbent assay, while albumin and total protein were assessed via spectrophotometry. Statistical analyses included t-tests, multiple regression, structural equation modeling (SEM), and Cox regression.

Results. Hypertensive patients had significantly higher blood pressure ($p<0.001$), CRP (7.52±4.21 vs. 1.35±0.51 mg/L; $p<0.001$), globulin (3.4±1.0 vs. 1.8±0.9 g/dL; $p<0.001$), and total protein, but lower apelin (2386.2±401.7 vs. 2873.4±572.8 pg/mL; $p<0.001$) and albumin levels. SEM revealed a direct association between CRP and systolic blood pressure ($\beta=0.45$, $p<0.001$), and a negative association between apelin and systolic pressure ($\beta=-0.20$, $p=0.03$). CRP (HR=1.75, $p=0.005$) and systolic BP (HR=1.52, $p<0.001$) were independent predictors of cardiovascular events.

Conclusion. The findings suggest that systemic inflammation and dysregulation of serum protein metabolism are significantly associated with hypertension and cardiovascular risk. Apelin may play a protective role by mitigating the impact of inflammation on blood pressure.

Keywords. adipokines, albumin, apelin, blood pressure, C-reactive protein

Introduction

Hypertension generally is the leading cause of cardiovascular disease and early death. Over the past forty years, the global mean blood pressure (BP) has either stayed the same or slightly dropped due to the extensive usage of antihypertensive drugs.[1] According to the Global Burden of Disease report, blood pressure-related disorders have killed over 50 million people and injured many more. They have a devastating effect on already fragile economies. Hypertension is sometimes referred to as the "silent killer" because most people with it have no symptoms. However, symptoms like headache, dizziness, dyspnea, angina, palpitations, and epistaxis can occasionally result from hypertension.[2] With 66% of strokes, 50% of cases of coronary heart disease, and 9.4 million deaths each year, hypertension is the leading cause of disease globally. The association between hypertension and the risk of sudden cardiac death has

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been the subject of several cohort studies.[3] Numerous consequences brought on by hypertension include heart disease, stroke, and chronic renal disease, all of which are leading causes of death globally.[4]

Prognostication can still be challenging despite medicines being guided by patient complaints, functional capacity evaluations and pulmonary artery pressures. Age, blood pressure, renal function, and biomarker abnormalities have all been found in studies to be indicators of a bad prognosis. These clinical characteristics might be helpful when discussing prognoses with patients and directing treatment.[5] White adipose tissue secretes adipokines, which are "molecular messengers" that regulate several bodily functions, including energy balance. They can interact with several organs, including the brain, liver, pancreas, muscle, and vasculature, and they can operate in an autocrine, paracrine, or endocrine fashion.[6] The bioactive peptide apelin was first discovered in a bovine stomach tissue extract in some organs. In addition to the central nervous system, adipocytes and the placenta contain apelin and its receptors. An adipocytokine called apelin binds to the G protein-coupled receptor known as the APJ.[7]

Endothelium-dependent vasodilation, smooth muscle vasoconstriction, angiogenesis, cardiac contractility stimulation, water intake, diuretic action, angiotensin II antagonistic effects, positive inotropes, and other cardiovascular activities of the apelin-receptor system are just a few of the biological impacts of apelin. These findings have spurred a study on apelin as an endogenous mediator essential for cardiovascular disorders.[8] Apelin is a vasoactive endogenous peptide produced from the C-terminal of a 77-amino acid pre-proapelin. It is cleaved enzymatically to form different apelin fragments (apelin 13, 16, 17, 19, 36). The most active fragment is apelin-13, and its receptor (APJ) is a member of the G-protein coupled receptors. Apelin and APJ are widely distributed in the cardiovascular system. The apelin-APJ signalling is also crucial for appropriately developing the cardiovascular system and forming blood vessels.[9] The condition can easily impact serum proteins, which are freely accessible. The most significant proteins in human plasma are serum proteins, such as albumin, which are crucial for preserving osmolality and nutrition.[10] Conditions like infection, inflammation, and disorders of the liver and connective tissues can raise serum globulin levels. Still, nephrotic syndrome and starvation can lower them because of decreased protein synthesis and loss through the kidney, respectively.[11] Initially identified as a positive acute phase reactant (protein), C-reactive protein (CRP) is released into the bloodstream in response to hepatocyte inflammation. It is a sign of several illnesses and the continuous inflammatory process in our bodies.[12] About 60% of serum proteins are albumin, which is made in the liver.[13] Clinically, circulating CRP levels are used to forecast the likelihood of cardiovascular events and to help choose treatments for people at intermediate risk based on more precise risk assessment.[14]

The liver and blood cells produce serum total protein (TP), a complex mixture of several proteins, including albumin and globulin.[15] A vital protein, albumin serves a variety of physiological purposes. Low serum albumin levels may indicate the degree of inflammation and nutritional condition. Globulin, a significant component of total plasma protein, is involved in the inflammatory process and is a gauge for the degree and timing of inflammation.[16] Globulin, which comprises numerous inflammatory-related proteins, rises as inflammation progresses. To provide a more accurate picture of the body's nutritional and inflammatory condition, the albumin/globulin ratio (AGR) considers both albumin and globulin.[17] To our knowledge, this is the first study to evaluate the combined role of apelin, CRP, and serum protein parameters in predicting blood pressure using structural equation modeling in a Middle Eastern hypertensive population.

Aim

This study aims to show why CRP and apelin levels differ significantly in hypertensive individuals compared to healthy controls by monitoring the levels of these bioactive parameters in patients with hypertension. Understanding these potential interactions may provide insights into the mechanisms underlying hypertension and contribute to understanding blood pressure and developing novel therapeutic strategies.

Material and methods

Study population

The present study employed a cross-sectional observational design conducted between August 2024 to March 2025. Two hundred adult participants in the age range (35 to 65) years were enrolled in the study and divided into two groups, also all patient were middle income families and same local area. The participants were recruited using convenience sampling strategy from the Internal Medicine Consultant of Al-Basrah Teaching Hospital, Al-Basrah Governorate Southern Iraq. Prior to their participation in the study, all participants provided written informed consent, as required by the University of Basrah's Human and Animal Ethics Committee in Iraq (No. 2024/112). All human contact methods described in this study were approved by the University of Basrah's Human and Animal Ethics Committee in Iraq (No. 2024/112).

One hundred people with hypertension (HTN), consisting of 60 females and 40 males, are included in the study. The duration of hypertension was determined from the medical history of the affected patients, ranging from 5–15 years. All patients were prescribed various antihypertensive medications like alpha-blockers,

beta-blockers, angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists (ARBs). In addition, the duration of taking medicine ranged between 5–15 years. Also, 100 participants, aged between 35 and 65 years, were selected as the control group (healthy people). This group is comprised of 58 females and 42 males. All patients treated by an internal medicine consultant and diagnosed with hypertension were included in the trial.

Criteria of exclusion

Study included patients and controls total of 300 aged 35-65 years who were able to provide inform consent. One hundred patients and controls were subjected to exclusion because they had diabetic mellitus, thyroid disease, alcoholism, pregnancy, liver disease, kidney disease, tumors, or heart disease, also those taking medications that could influence the study outcomes.

Laboratory tests

Fasting blood samples were collected using disposable plastic syringes; about 5 mL of venous blood was extracted from patients, placed in a gel tube, centrifuged, and the serum was kept at (-20°C) until analysis. The biochemical parameters were measured as follows: Albumin and total proteins were measured by spectrophotometer (EMC-11S-V, Germany). C-reactive protein and apelin were measured using an enzyme-linked immunosorbent assay (ELISA) /Multiskan FC/Thermoscientific (Elabscience, USA). Elabscience Human CRP Elisa kit and Elabscience Human APL (Apelin) Elisa kit were used. Furthermore, Triglycerides, Cholesterol, HDL and LDL were measured spectrophotometrically.[18]

ELISA kit details

Elabscience® Human APLN(Apelin) ELISA Kit, Catalog No: E-EL-H0456, Sensitivity 37.5 pg/mL, Detection Range 62.5–4000 pg/mL, Repeatability Coefficient of variation (CV%) is<10%.

Elabscience® Human CRP ELISA Kit, Catalog No: E-EL-H0043, Sensitivity 0.23 ng/mL, Detection Range 0.39–25 ng/mL, Repeatability Coefficient of variation (CV%) is<10%.

Sample collection

Data were collected through a closed questionnaire that included 12 questions about concepts like (Name, Age, other chronic diseases, thyroid disease, duration of hypertension disease, taking medications, place of residence, marital status, height, weight and smoking). Body mass index (BIM) was calculated by dividing the weight (kg) by height (m^2)[19] using equation 1.

$$BMI\ \left(\frac{kg}{m^2}\right) = \frac{weight\ (kg)}{height\ (m^2)} \qquad (1)$$

Statistical analysis

The Statistical Package for the Social Sciences (SPSS), version 24, was used to describe statistical calculations to determine the study's mean and standard deviation (SD). Paired t-tests and ANOVA were employed for statistical comparisons; probability less than 0.05 was used as the statistical significance level. The chi-square test was used to evaluate frequency tables, while the independent sample t-test was used to assess mean and standard deviation tables. The association between the studied markers is ascertained using the Pearson correlation coefficient.

Principal Component Analysis (PCA) was applied to reduce the dimensionality of the dataset and highlight key variables explaining the most significant variance. Multivariate analysis of covariance (MANCOVA) was used to assess the effects of multiple dependent variables while controlling for confounding variables such as age and gender. Structural equation modelling (SEM) was employed to examine the complex relationships between observed and latent variables, specifically how CRP influences systolic blood pressure while accounting for other physiological markers. Cox Proportional Hazards regression was used for long-term follow-up to analyze the time spent on specific events such as cardiovascular events.

Results

Demographics and baseline characteristics

The demographic characteristics and baseline clinical data of the study participants are summarized in Table 1.

Table 1. Demographic characteristics of controls and patients

Parameter	Controls (n=100)	Patients (n=100)	p
Age (years, mean±SD)	47.1±6.3	48.6±7.4	<0.01
Gender (Male: Female)	42:58	40:60	0.74
BMI (kg/m²)	22.38±2.55	29.90±3.29	<0.001
Systolic BP (mmHg, mean)	118.2±7.5	160.4±15.6	<0.001
Diastolic BP (mmHg, mean)	78.1±5.2	95.8±7.8	<0.001

The patient group's mean age was significantly higher ($p<0.01$). Systolic and diastolic blood pressures were also significantly elevated in the patient group ($p<0.001$). No significant difference was observed in the gender distribution between the two groups ($p=0.74$).

Key biochemical parameters, including CRP, apelin, albumin, total protein, and globulin levels, were compared between the control and patient groups. These results are displayed in Table 2.

Total protein and globulin levels were significantly higher in the patient group ($p<0.001$), which could reflect a compensatory response to inflammation CRP levels, an inflammatory marker, were significantly elevated in the patient group, suggesting higher inflam-

matory activity. Apelin levels were reduced in patients, pointing to potential metabolic.[20] Both systolic and diastolic blood pressures were significantly higher in the patient group compared to the control group (p<0.001). On the other hand, people with hypertension, once they start blood pressure medication, cannot stop. Moreover, the patients were not committed to dietary issues, had low activity levels and were stressed. The medications they were taking impacted sodium and potassium ion levels but did not affect blood proteins; therefore, they were unlikely to have influenced the parameters measured in the study, such as albumin and globulin. This highlights the elevated cardiovascular risk in the patient cohort.[21,22]

Table 2. Biochemical parameter comparison

Parameter	Controls (mean±SD)	Patients (mean±SD)	p	T-test	Cohen's d	95% CI (approx.)
CRP (mg/L)	1.35±0.51	7.52±4.21	<0.001	-9.85	2.06	(1.64, 2.47)
Apelin (pg/mL)	2873.4±572.8	2386.2±401.7	<0.001	8.68	0.97	(0.40, 1.53)
Albumin (g/dL)	5.17±0.632	3.92±0.235	<0.001	15.30	2.79	(2.06, 3.53)
Total protein (g/dL)	6.1±1.2	7.2±1.4	<0.001	-6.53	0.83	(0.25, 1.41)
Globulin (g/dL)	1.8±0.9	3.4±1.0	<0.001	-17.61	1.65	(1.03, 2.27)
A/G ratio	3.37±2.61	1.04±0.22	<0.001	-7.35	1.57	(0.96, 2.17)
Triglycerides (mg/dL)	121.5±30.2	138.8±45.6	0.01	-2.61	0.44	(-0.03, 0.91)
Cholesterol (mg/dL)	180.4±25.6	200.3±32.8	<0.001	-3.94	0.66	(0.09, 1.23)
HDL (mg/dL)	55.3±12.1	48.9±11.7	0.0021	3.14	0.54	(0.01, 1.07)
LDL (mg/dL)	102.7±20.5	118.4±25.3	0.038	-3.98	0.67	(0.10, 1.24)

Multiple linear regression (MLR)

MLR was performed to examine the combined effects of multiple independent variables (e.g., CRP, apelin) on dependent variables (e.g., systolic BP, diastolic BP). The results of this regression analysis are shown in Table 3.

Table 3. MLR analysis (systolic BP as dependent variable)

Independent Variable	Coefficient (β)	Standard error	p
CRP	0.25	0.05	<0.001
Apelin	-0.15	0.06	0.02

CRP was found to have a strong positive association with systolic blood pressure (β=0.25, p<0.001), suggesting that higher CRP levels contribute to increased blood pressure. In contrast, apelin showed a negative relationship (β=-0.15, p=0.02), indicating a potential protective effect against elevated blood pressure.

Multivariate analysis of covariance (MANCOVA)

The results of *MANCOVA* are summarized in Table 4. The MANCOVA results confirmed that controlling for age and gender is crucial in understanding the effects of inflammatory and metabolic variables on blood pressure. All variables, including CRP and apelin, significantly affected both blood pressure and biochemical markers. The significant influence of age and gender emphasizes the importance of considering demographic factors when interpreting these relationships.

Table 4. MANCOVA results for blood pressure and biochemical parameters

Dependent variable	F-statistic	p
Systolic BP	12.45	<0.001
Diastolic BP	9.83	0.002
CRP	10.56	<0.001
Apelin	5.78	0.015

Principal component analysis (PCA)

The results of the PCA, showing the distribution of participants along the first two principal components, are summarized in Table 5.

Table 5. PCA results for participant distribution (controls vs. patients)

Principal component 1 (PC1)	Principal component 2 (PC2)	Group
2.35	1.47	Control
1.92	1.28	Control
2.74	1.59	Patient
3.05	2.12	Patient
2.33	1.45	Control

The first two components explained a substantial portion of the variance in the data (65%), effectively distinguishing between the control and patient groups. Patients tended to cluster in the lower right quadrant, reflecting higher inflammatory markers and altered blood pressure. These results underscore the effectiveness of PCA in separating groups based on biochemical and demographic factors.

Cluster analysis

K-means clustering was applied to group participants based on their demographic and biochemical profiles. The clustering analysis revealed two distinct groups with significant differences in inflammatory markers and blood pressure. The clustering results are summarized in Table 6.

Table 6. K-means clustering of participants (control vs. patient)

Cluster number	Group	CRP (mg/L)	Systolic BP (mmHg)	Apelin (pg/mL)
1	Control	1.35±0.51	118.2±7.5	2873.4±572.8
2	Patient	7.52±4.21	160.4±15.6	2386.2±401.7

The K-means clustering analysis confirmed that the control and patient groups could be clearly distinguished based on their biochemical and demographic characteristics. Controls showed lower CRP levels, systolic blood pressure, and higher apelin compared to pa-

tients. This further supports the utility of these variables for classifying participants into meaningful subgroups.

Structural equation modelling (SEM)

The results of SEM are shown in Table 7.

Table 7. SEM results for inflammatory markers and systolic blood pressure

Path	Coefficient (β)	p
CRP ⟶ Systolic BP	0.45	<0.001
Apelin ⟶ Systolic BP	-0.20	0.03
CRP ⟶ Apelin	-0.10	0.05

The SEM analysis demonstrated a direct and significant path from CRP to systolic blood pressure (β=0.45, p<0.001), suggesting that higher CRP levels directly contribute to increased blood pressure. Apelin was found to mediate the relationship between CRP and systolic blood pressure, with a significant negative path (β=-0.20, p=0.03), indicating that apelin may mitigate some of the effects of CRP on blood pressure.

Survival analysis

The model identified CRP and systolic blood pressure as significant predictors of survival time. The results are summarized in Table 8.

Table 8. Cox proportional hazards regression analysis

Variable	Hazard ratio (HR)	95% CI	p
CRP	1.75	1.15 – 2.65	0.005
Systolic BP	1.52	1.25 – 1.85	<0.001

CRP (HR=1.75, p=0.005) and systolic blood pressure (HR=1.52, p<0.001) were identified as significant predictors of cardiovascular events, highlighting their role in determining long-term health outcomes.

Visualizing differences in biochemical and physiological parameters: a comparison between controls and patients

A heat map (Fig. 1 and 2) compares some of the biochemical and physiological parameters of the control and patient groups: age; blood pressure, including systolic and diastolic; CRP, meaning C-reactive protein; apelin; albumin; total protein; globulin. In patients, CRP and blood pressure levels were significantly higher, reflecting enhanced inflammation and risk of CVD. Also, proteins like total protein and globulin were higher, probably due to a compensatory immune response or adaptation to stress. In controls, CRP levels were lower, as was blood pressure, while the levels of apelin were higher since all these factors are related to a good metabolic profile. Red represents higher values, and blue represents lower values from the cool, warm color map, making both groups easily distinguishable. The heat map is an obvious and straightforward view of physiological and biochemical changes between the control and patient groups, underlining inflammatory and cardiovascular health status for the differentiation of the two cohorts. It, therefore, becomes useful knowledge in underlying health status for each group, improving understanding of factors that influence disease progression.

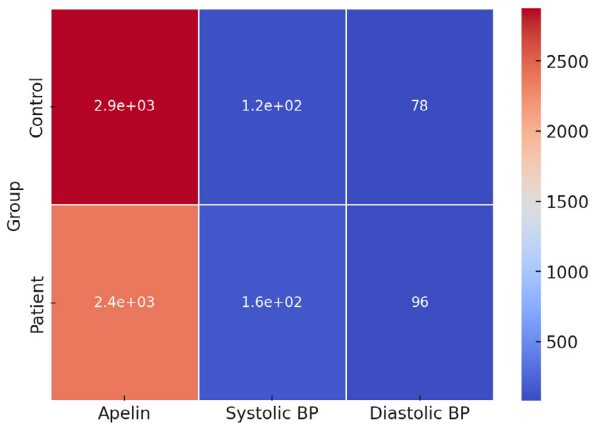
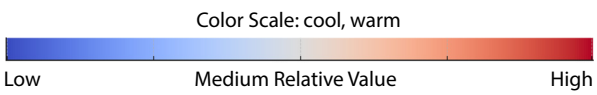


Fig. 1. Heat map of apelin and blood pressure parameters – visualizing apelin levels in control and patient groups and systolic and diastolic blood pressure



The heat map illustrates significant inflammatory and protein-related biomarkers differences between the control and patient groups. Patients exhibit elevated CRP levels, as shown in Figure 2, indicating heightened inflammation and decreased albumin, total protein, and globulin levels, suggesting altered protein metabolism and immune response.

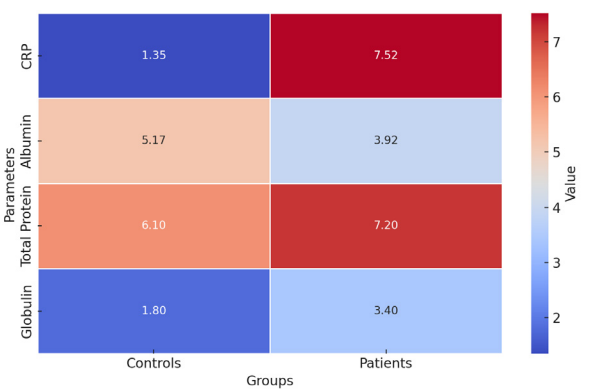
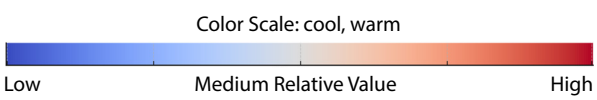


Fig. 2. Heat map of CRP, albumin, total protein, and globulin levels



Discussion

One of the more striking trends that the study exhibited was the prevalence of CRP in the patient group. Evidence by Ridker[23] suggests that CRP is an acknowledged systemic inflammatory marker which contributes significantly to the risk of CVD. Therefore, the finding of higher levels of CRP in the patient group coincided with evidence that the inflammatory process is central to the pathophysiology of cardiovascular diseases. This explains why our study shows such a high positive correlation between CRP and systolic blood pressure, at r=0.78. This further reiterates the need to monitor CRP levels among patients at risk for cardiovascular events.

The other significant outcome of the current study is the reduced level of apelin in the patient group. Apelin is a peptide hormone with various physiological roles in regulating blood pressure, cardiac function, and maintaining healthy vessels.[24] Proof supporting low levels of apelin in the patient group indicates that it positively affects cardiovascular functions. From this, one may assume that a low level of apelin in cardiovascular patients, especially in chronic conditions, could further deteriorate the conditions of such patients. Apelin may represent a potential target for future investigation.

The significantly decreased albumin-to-globulin ratio in the patient group tallies with observations suggesting altered protein metabolism and immune system activation during chronic disease.[25] However, a decreased A/G ratio would signal a shifted balance of plasma proteins due to chronic inflammation or activation of the immune system. This result again underlines the value of protein metabolism as a marker of systemic inflammation and immune response and further illustrates the complex pathophysiology of chronic diseases.

The PCA successfully separated the control and patient groups based on their biochemical and physiological profiles. PCA is a technique for dimensional reduction used for complex data; the first two components reflected a high amount of variation in the dataset, which separated the two groups successfully.[26] Patients were clustered in the lower right quadrant, reflecting a high level of inflammatory markers with a concomitant blood pressure alteration, confirming PCA's usefulness in identifying key variables for disease outcome prediction.

K-means clustering was applied to segregate the participants according to their demographic and biochemical characteristics into two marked clusters corresponding to controls and patients[27], who identified subgroups of the disease using cluster analysis based on a set of similar features. Clear-cut discrimination of controls and patients in our clustering analysis further extends the opportunity of the clustering methodology in the search for subgroups based on inflammation, metabolic dysfunction, and cardiovascular risk.

Our study's high hazard ratio obtained for CRP and systolic blood pressure suggests that these biomarkers may have a good predictive value for estimating the probability of cardiovascular events in patients and again support them as significant risk factors.[28,29]

The study results indicate noteworthy changes in inflammatory and nutritional plasmatic-related markers between patients and HC. High levels of CRP and lower levels of albumin in patients reflect the severity of inflammation and malnutrition, which is consistent with prior studies indicating that a high CRP/albumin ratio is associated with adverse clinical outcomes and increased mortality in critically ill patients.[30]

Furthermore, the lower serum concentrations of apelin in patients may demonstrate, similar to the studies mentioned above, that apelin is considered a regulatory peptide with anti-inflammatory and vasoprotective effects and represents endothelial dysfunction and compromised cardiovascular homeostasis.[31,32] Higher globulin concentrations and lower A/G ratio in patients may also reflect systemic inflammation and/or hepatic dysfunction, as these findings are well established as changes in protein concentrations in hypertensive and metabolic disorders.[33] These findings are consistent with previous literature and reaffirm that inflammation, protein metabolism, and vascular pathology are interconnected issues. While previous studies have examined the individual roles of CRP or apelin in cardiovascular disease, our study is novel in its integrative approach, combining biomarker profiling with advanced multivariate statistical modeling to explore potential mechanistic pathways in hypertension

Conclusion

This study highlights the significant role of inflammation and metabolic dysfunction in differentiating between control and patient groups. The findings underscore the importance of CRP, apelin and systolic blood pressure biomarkers in predicting cardiovascular risk and disease progression. The application of advanced statistical techniques, including multiple linear regression, PCA and K-means clustering, has provided more profound insights into the complex relationships between these biomarkers. These results contribute to the growing body of literature on the role of inflammation and metabolic dysfunction in cardiovascular health and emphasize the potential of these biomarkers in disease stratification, risk prediction, and therapeutic interventions.

Declarations

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The authors stated that they did not receive any funding for this work.

Author contributions

Conceptualization, Z.A.M. and E.Q.J.; Methodology, Z.A.M.; Software, U.H.R.; Validation, U.H.R., E.Q.J. and Z.A.M.; Formal Analysis, Z.A.M.; Investigation, Z.A.M.; Resources, Z.A.M.; Data Curation, E.Q.J.; Writing – Original Draft Preparation, Z.A.M.; Writing – Review & Editing, U.H.R., E.Q.J.; Visualization, Z.A.M.; Supervision, U.H.R., E.Q.J.; Project Administration, U.H.R., E.Q.J.; Funding Acquisition, Z.A.M.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

The data of participants were collected from the Internal Medicine Consultant of Al-Basrah Teaching Hospital, Al-Basrah Governorate Southern Iraq.

Ethics approval

All human contact methods described in this study were approved by the University of Basrah's Human and Animal Ethics Committee in Iraq (No. 2024/112).

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ORIGINAL PAPER

Evaluating tumor necrosis factor-alpha and interleukin 17 as plasma biomarkers for rheumatoid arthritis diagnosis

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ABSTRACT

Introduction and aim. Rheumatoid arthritis (RA) is a complex chronic inflammatory autoimmune disease with significant global health implications. In this study, our aim was to evaluate the clinical utility of plasma tumor necrosis factor-alpha (TNF-α) and interleukin-17 (IL-17) along with immunological parameters as diagnostic biomarkers for patients with RA.

Material and methods. A case-control study was conducted involving 75 RA cases and 75 age- and sex-matched controls. Plasma levels of TNF-α and IL-17 were measured using ELISA kits. Statistical analyses included receiver operating characteristic (ROC) curve analysis and correlation tests to evaluate the diagnostic accuracy, predictive value, and associations with disease activity parameters.

Results. RA patients exhibited significantly elevated TNF-α (275.5±99.9 vs. 46.2±8.4 pg/mL, p<0.001) and IL-17 (313.8±95.4 vs. 42.2±10.96 pg/mL, p<0.001) compared to controls. Patients with a family history of RA had higher TNF-α (p=0.019) and IL-17 (p=0.03) levels. ROC analysis revealed perfect diagnostic accuracy for both biomarkers (100% sensitivity and specificity) at cut-offs >65.2 pg/mL (TNF-α) and >83 pg/mL (IL-17).

Conclusion. There was a positive correlation between serum levels of IL-17 and TNF-α. Therefore, these biomarkers distinguish rheumatoid arthritis patients from healthy controls.

Keywords. anti-cyclic citrullinated peptide, biomarkers, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by immune-mediated inflammation of synovial joints, which leads to progressive cartilage and bone destruction, functional impairment, and potential multi-organ involvement.[1] This condition represents a complex interplay of genetic predisposition, environmental triggers, and dysregulated immune responses, resulting in the production of autoantibodies that target citrullinated peptides and immunoglobulins.[2] RA is characterized by symmetrical inflammatory polyarthritis, predominantly affecting the small joints of the hands and feet before progressing to larger joints.[3,4] This inflammatory cascade results in a hallmark clinical triad of pain, swelling, and morning stiffness lasting more than 30 minutes.[5]

RA affects a substantial proportion of the global population, with epidemiological studies generally indicating a population prevalence ranging from 0.5% to 1%.[6] Multiple

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studies have documented an increasing trend in the prevalence of RA in the recent decades. The age-standardized global prevalence rate increased by 14.1% between 1990 and 2020, with other studies reporting increases of 0.37%, 14.1%, and 6.4% from 1990 to 2019, 2020, and 2017, respectively.[5] According to systematic reviews, the worldwide average point prevalence is estimated to be 0.51%, whereas the period prevalence is slightly higher, at 0.56%. However, these figures mask the significant variations across the different populations and methodologies used for case identification.[7] Thus, the global burden of RA is considerable. In 2020, an estimated 17.6 million people were living with RA worldwide, with an age-standardized global prevalence rate of 208.8 cases per 100,000. This represents a 14.1% increase since 1990, indicating a rising disease burden, despite advances in treatment.[8] Furthermore, it has been demonstrated that the inflammatory marker C-reactive protein (CRP) increases the synthesis of proinflammatory cytokines, but its specificity is poor.[9] Among serological markers, rheumatoid factor (RF) and anti-citrullinated protein antibodies are particularly prominent. RF, a classical autoantibody discovered over eight decades ago, has been the cornerstone of RA diagnostics and research. Although its utility has been debated due to its presence in other autoimmune and inflammatory conditions, recent advancements have shed new light on its role, mechanisms, and potential implications in the pathophysiology and treatment of RA.[10] Cytokines are also thought to play an important role in causing inflammation, joint destruction, and extra-articular manifestations associated with RA. Therefore, the role of biomarkers in clinical diagnosis and decision-making is crucial. However, traditional biomarkers have low specificity and sensitivity in diagnosing infections in patients with RA, and specific infection markers are urgently needed.[9] In this study, we discuss tumor necrosis factor-alpha (TNF-α) and interleukin-17 (IL-17) as biomarkers and their potential clinical applications in RA.

TNF-α is a central mediator of RA pathogenesis, driving synovial inflammation, cartilage degradation, and systemic complications.[11] As a pleiotropic cytokine, TNF-α orchestrates a cascade of immunological and structural changes that define the progression.[12] TNF-α activates synovial fibroblasts, inducing the overproduction of matrix metalloproteinases (MMPs) and cathepsins, enzymes responsible for degrading collagen and proteoglycans in cartilage and bone.[13] This enzymatic activity leads to progressive joint erosion, a hallmark of RA. Concurrently, TNF-α promotes synovial hyperplasia and angiogenesis, facilitating the formation of invasive pannus tissue that exacerbates joint damage.[14] IL-17 is a proinflammatory cytokine family comprising six members (IL-17A–F), with IL-17A and IL-17F being the most studied.[15] Produced by Th17 cells, γδ T cells, and innate lymphoid cells, IL-17 coordinates host defense against pathogens but also drives immunopathology in autoimmune diseases such as RA.[16] Its dual role in balancing protective immunity and inflammatory damage makes it a critical focus in understanding RA pathogenesis.[17] IL-17 is a linchpin of RA pathogenesis, orchestrating synovial inflammation, bone erosion, and systemic complications.[18]

Aim

This study evaluated plasma TNF-α and IL-17 levels as diagnostic biomarkers of RA. We examined their correlation with RF/anti-CCP levels, discriminatory capacity against healthy controls, and predictive validity for adverse joint outcomes.

Material and methods

This case-control study was conducted between November 2024 and January 2025 at the Marjan Teaching Hospital in Babylon Province, Iraq, and included 75 RA patients and 75 age- and sex-matched healthy controls. The RA diagnosis was confirmed by a joint consultant physician through clinical examination and diagnostic criteria, including RF assessment. RA diagnosis was confirmed by a consultant rheumatologist using the 2010 ACR/EULAR classification criteria, incorporating clinical joint examination, seropositivity for RF, and symptom duration. All patients fulfilled ≥6 points on this validated scoring system. The patient groups were recruited from rheumatology clinics, and demographic and clinical data (e.g., age, sex, family history, comorbidities, treatment regimens, residential status, and smoking habits) were collected using structured questionnaires. The 75 healthy individuals in the control group, systematically selected to mirror the age and sex distributions of the patient group for comparability, were recruited through both hospital staff volunteers and community advertising with age/sex matching criteria. All potential controls underwent screening via a self-reported questionnaire to verify the absence of a personal or familial history of RA or other autoimmune disorders, excluding individuals with current inflammatory symptoms, and eliminating those using immunomodulatory drugs, ensuring that they had no autoimmune conditions. The exclusion criteria for both groups were incomplete medical histories or refusal to participate. Ethical approval (document number 1887, date: November 10, 2024) and informed consent were obtained prior to data collection and adherence to institutional guidelines.

A priori power analysis (G*Power 3.1) determined that 68 participants per group would provide 90% power (α=0.05, two-tailed) to detect clinically relevant effect sizes (d=0.7) in cytokine levels between RA and controls.[19] To account for subgroup analyses and potential exclusions, we enrolled 75 participants per group (n=150).

Blood collection

A venous blood sample (5 mL) was collected from both the patients and control group in a gel tube under ster-

ile conditions. After centrifugation at 15000 rpm for 10 min, the serum was separated and 0.4 mL was placed in Eppendorf tubes. All samples were labeled with a serial number and the person's name, immediately frozen by deep freezing in the hospital for further processing, and refreezing was avoided.

Biochemical measurement

The analytical tools employed in this study include a range of specialized diagnostic kits sourced from reputable international manufacturers. A set of immunoassay kits produced by BT LAB (China) was used, including the Human IL-17 ELISA kit (Lot No: 202412021), TNF-α kit (Lot No: 202501007), and ANTI-CCP kit (Lot No: 202412021). These kits are based on ELISA and are designed to detect various immunological markers. Additionally, CRP (Lot No: AAUGH02EX) and RF (Lot No: CPUHC05Z) kits from Chroma (Korea), which rely on rapid immunodiagnostic technology, were used. Complete blood count (CBC) analysis was conducted using equipment from Horiba (France) (Lot No: _241028I1), which is known for its high accuracy in routine hematological assessments. Together, these tools enhance the precision of the laboratory results and support the reliability of the data collected throughout the study.

Statistical analysis

GraphPad Prism® software (version 9.3.1, Boston, AM, USA) was used for statistical analyses to compare demographic and clinical parameters between RA patients and healthy controls. The chi-square test and independent samples t-test were employed, and Pearson's correlation coefficient was used for statistical analysis. In addition, normality of continuous variables was assessed using Shapiro-Wilk tests: all biomarkers (TNF-α, IL-17, RF, etc.) normality assumptions ($W<0.90$, $p<0.05$) and biomarkers were normally distributed ($W>0.95$, $p>0.1$).

Results

Table 1 presents the demographic characteristics and biomarker profiles of the 75 RA patients and 75 healthy controls. The RA cohort exhibited a marginally higher mean age (42±13.2 years) compared to controls (38.9±5.3 years; p=0.08), though this difference was not statistically significant. Sex distribution was comparable between the groups, with 35% of males in the RA group versus 38% in controls (p=0.2). A family history of RA has been reported in 32% of RA patients. BMI was significantly elevated in RA patients (28.7±2.9 kg/m²) relative to controls (24.6±2.1 kg/m²; p=0.005). Biomarker analysis revealed distinct profiles: RA patients demonstrated lower mean WBC counts (7.3±2.3 vs. 8.3±2.3 $\times 10^9$/L; p=0.06) and higher hemoglobin levels (12.9±1.9 vs. 12.0±1.4 g/dL; p=0.04) compared to controls. Markers of inflammation and autoimmunity, including RF: 16.4±7.4 vs. 9.8±2.9 IU/mL CRP: 10.2±6.8 vs. 5.2±1.7 mg/dL), anti-cyclic citrullinated peptide (anti-CCP: 2.3±0.68 vs. 0.2±0.05 pg/mL), TNF-α: 275.5±99.9 vs. 46.2±8.4 pg/mL), and IL-17: 313.8±95.4 vs. 42.2±10.96 pg/mL), were all significantly elevated in RA patients (p<0.001).

Table 1. Demographic and clinical characteristics of study participants

Demographic and biomarker		Healthy group n=75	RA n=75	p
Age (years), mean±SD		38.9±5.3	42±13.2	0.08[a]
Gender	Male, n (%)	28 (38%)	26 (35%)	0.2[b]
	Female, n (%)	47 (62%)	49 (65%)	
Family history	Yes, n (%)	-	24 (32%)	
	No, n (%)	-	51 (70%)	
BMI (kg/m²), mean±SD		24.6±2.1	28.7±2.9	0.005[a]
WBC(x10⁹/L), mean±SD		8.3±2.4	7.3±2.3	0.06[a]
HB (g/dL), mean±SD		12.0±1.4	12.9±1.9	0.04[a]*
RF (IU/mL), mean±SD		9.8±2.9	16.4±7.4	<0.001[a]
CRP (mg/dL), mean±SD		5.2±1.7	10.2±6.8	<0.001[a]
Anti-CCP (pg/mL), mean±SD		0.2±0.05	2.3±0.68	<0.001[a]
TNF-α (pg/mL), mean±SD		46.2±8.4	275.5±99.9	<0.001[a]
IL-17 (pg/mL), mean±SD		42.2±10.96	313.8±95.4	<0.001[a]

n – number of cases, SD – standard deviation,
[a] – independent sample t-test. [b] – Chi-square

Table 2 presents a comparative analysis of serum inflammatory biomarker levels between patients with RA with (n=18) and without (n=42) family history of the disease. All biomarkers exhibited significantly higher mean concentrations in the family history group than those in the non-family history group. TNF-α levels were elevated in the family history cohort (309.6±122 pg/mL vs. 253.3±64.4 pg/mL; p=0.019), and IL-17 levels were significantly higher in this group (345.7±91 pg/mL vs. 298.4±83.8 pg/mL; p=0.03). Statistical significance (p<0.05).

Table 2. Inflammatory biomarker levels in RA patients stratified by family history (independent sample t-test)

Biomarkers		Family history n=24	Without family history n=51	p
TNF-α (pg/mL)	Mean±SD	309.6±122	253.3±64.4	0.019
IL 17 (pg/mL)	Mean±SD	345.7±91	298.4±83.8	0.03

Receiver operating characteristic (ROC) curve analysis assessed the diagnostic accuracy of TNF-α and IL-17 for RA (Fig. 1), revealing that both biomarkers achieved perfect discrimination with 100% sensitivity and 100% specificity at their optimal cut-off values (TNF-α>65.2 pg/mL, IL-17>83 pg/mL), resulting in perfect area under the curve (AUC) values of 100% (p<0.001 for each), where the 95% confidence interval for TNF-α sensitivity was (95.0–100) and for specificity was (89.4-100), while for IL-17 the sensitivity 95% CI was (94.3–100) and the specificity 95% CI was (89.57–100).

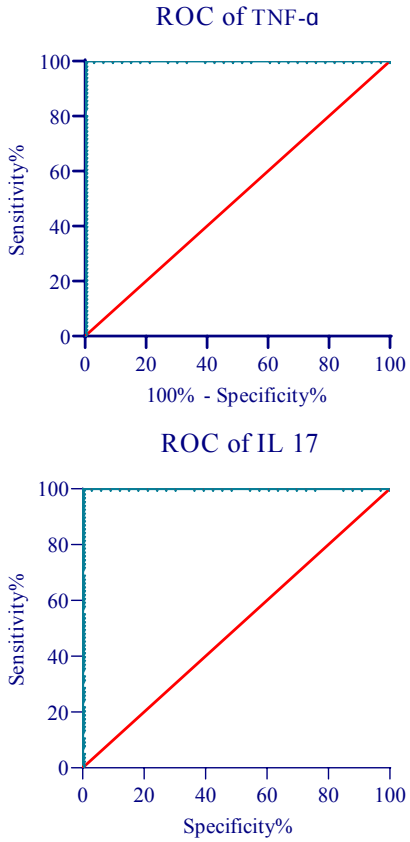

Fig. 1. The ROC curve for (TNF-α, IL-17)

A correlation matrix revealed a moderately positive association between TNF-α and RF (r=0.396, p=0.018, Fig. 2). Anti-CCP exhibited a weak positive correlation with TNF-α level (r=0.290, p=0.013). No significant correlations were observed between CRP or RF levels and the other variables (p>0.05).

In addition, although no statistically significant relationship was found between IL-17 and RF/anti-CCP, where IL-17 demonstrated exceptional diagnostic accuracy and was elevated in RA patients (particularly those with a family history), our correlation analysis revealed no significant association with RF or anti-CCP levels.

Discussion

RA exhibits pronounced sex disparity, with women being disproportionately affected across all populations studied. This condition predominantly impacts women, with a female-to-male ratio of around 3:1 being commonly seen.[20] More precise estimates from global burden of disease studies indicate a 2.45 female-to-male ratio of recurrence, confirming this substantial sex difference.[21] The sex disparity in the RA incidence was similarly pronounced, with a female-to-male incidence rate ratio of 2.3. Interestingly, this disparity appeared most pronounced among young adults, with the peak differential observed among individuals aged 20-29 years.[22] This age-specific pattern suggests potential hormonal influences on disease onset, and supports the hypothesis that reproductive factors may modulate RA risk in women. Although RA can manifest at any age, it demonstrates a characteristic age-dependent pattern of onset and prevalence. The peak in both prevalence and incidence typically occurs around the eighth decade of life,[22] although global data suggest a peak around the age of 60.[21] This age-related pattern has important implications for healthcare planning, particularly given the global demographic shift toward aging populations.

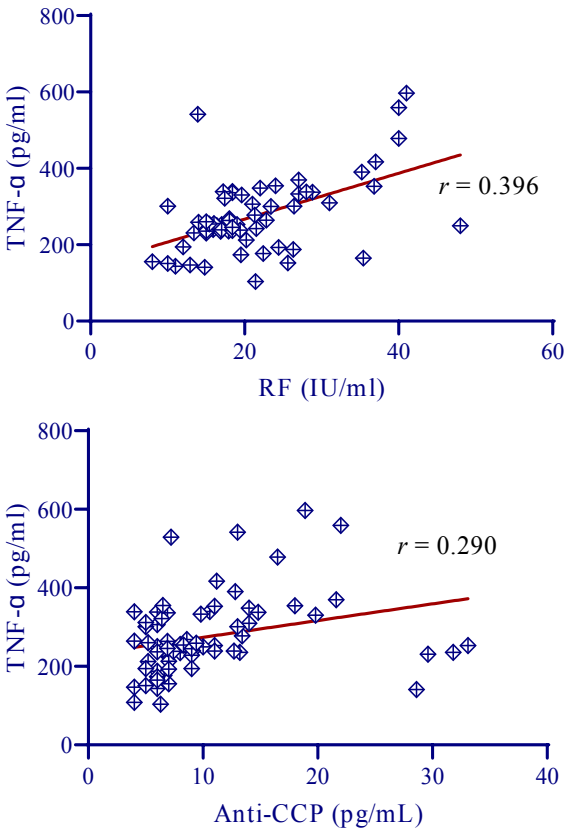

Fig. 2. Positive correlation between the TNF-α and different biomarkers RF and Anti-CCP

Research indicates that TNF-α, a proinflammatory cytokine, is markedly greater in RA patients than in healthy controls, showing a strong diagnostic potential. Additionally, research has shown that individuals with a relative history of RA exhibit higher TNF-α concentrations than those without a genetic predisposition, suggesting a hereditary component in disease-related inflammation. These findings align with a 2024 Iraqi study involving 63 newly diagnosed RA patients and 20 healthy controls, which reported significantly increased TNF-α levels in RA cohorts, particularly among severe cases compared to mild to moderate cases.[23] Notably, genetic predisposition may influence TNF-α expression, as patients with a family history of RA exhibit higher levels of TNF-α than those without, implicating hereditary factors in inflammatory dysregulation. Diagnostic evaluations

further support the clinical relevance of TNF-α. ROC curve analysis identified a cut-off of 4.25 pg/mL, yielding 76% sensitivity and 70% specificity for distinguishing patients with RA from controls. These findings collectively emphasize the role of TNF-α as a biomarker for RA diagnosis and progression monitoring.[24] Additional case-control studies examining TNF-α in RA, including a study involving 150 participants (119 RA and 31 healthy controls) conducted by Farrugia, demonstrate that RA patients' serum TNF-α levels were considerably greater than those of healthy people. TNF-α levels were substantially linked with age, BMI, and biological therapy.[25] Nonetheless, our results suggest that TNF-α may be a potential biomarker for the pathogenesis of RA.

Previous investigations have has indicated that older persons had higher plasma levels of TNF.[26–29] These findings suggest that TNF-α modulates joint degradation. These observations indicated that elevated systemic and local TNF-α levels are associated with RA.[30] Therefore, in our RA study, the relationship with serum TNF-α levels and RF anti-CCP was statistically significant. Collectively, these results suggest that TNF-α is not only a biomarker for RA, but also a key factor in disease progression and therapy response.[31]

IL-17, another key inflammatory mediator, was considerably increased in patients with RA as opposed to controls. Furthermore, TNF, IL-17 has demonstrated ideal diagnostic accuracy, distinguishing RA with complete sensitivity and specificity. Furthermore, patients with relatives with a history of RA also exhibited higher IL-17 levels, suggesting a role in genetic susceptibility. A study by Samaan et. al. in Egypt compared 45 cases with RA and 45 controls. RA patients' serum IL-17 levels were significantly higher than those of the control group, with a cut-off of >175 pg/mL predicting illness activity ranging from mild to high (DAS28 ≥3.2) with 100% sensitivity and specificity. This highlights IL-17 utility in arranging patients according to the severity of their conditions and monitoring therapeutic responses.[32] This study focused on the function of IL-17 in RA, revealing that serum IL-17 levels were elevated in patients with RA, suggesting a potential role of IL-17 in the pathogenesis of CarP-positive RA. These findings highlight IL-17 possible involvement in specific RA subgroups, as reported by Selimov et al.[33] Although these mechanisms bolster host defense, IL-17 dysregulation in RA exacerbates pathology by driving synovial inflammation (synovitis), bone destruction via osteoclastogenesis, and systemic complications, highlighting its dual role in protective immunity and inflammatory diseases.[34,35] Therefore, in this work, we looked at how IL-17, a significant Th1 cytokine generated by activated T cells, contributes to the activation of synovial fibroblasts in RA.

Based on these findings, we propose that the rise in these indicators may be due to interactions between the immune system and cells within the inflamed joints that stimulate the release of inflammatory cytokines, contributing to enhanced inflammation and tissue destruction.[36] Farag et al. found that serum IL-17 levels were significantly elevated in patients with RA compared to those in patients with osteoarthritis and controls. These elevated levels correlated positively with disease activity parameters, such as DAS-28, power Doppler ultrasound findings, and radiographic severity (Larsen score), suggesting IL-17's role in RA pathogenesis. The authors concluded that IL-17 may serve as a biomarker for disease activity and a potential therapeutic target.[37] Similarly, Nasef et al. demonstrated markedly increased IL-17 and Th17 cells in patients with RA relative to healthy controls, showing a strong correlation with DAS28. Following anti-rheumatic therapy, both IL-17 levels and Th17 cell frequencies decreased along with clinical improvement, further underscoring their pathogenic role.[38] A study by Atwa et al. on RA patients and healthy controls observed that serum IL-17 levels were 4.7-fold higher in patients with RA. IL-17 was positively relates with DAS28, and IL-17 predicted disease activity with 81.2% sensitivity and 75% specificity.[39] The positive relationship between TNF-α and both anti-CCP and RF stems from TNF-α-activating B cells producing these autoantibodies, which in turn contributes to a continuous inflammatory environment.

A recent study by Okutan et al. found that three novel inflammatory markers, pan-immune-inflammation value, systemic immune-inflammation index, and systemic inflammation response index, were significantly elevated in rheumatoid arthritis patients compared to healthy controls and showed positive correlations with disease severity is evaluated by DAS28 values.[40] ROC analysis revealed a perfect diagnostic accuracy (AUC=1.0). While statistically robust in our study, such perfect discrimination is uncommon in biomarker studies and may reflect stringent control selection, homogenous RA groups, and technical precision. Nevertheless, these results require validation in larger, heterogeneous populations, including patients with non-RA inflammatory conditions, to confirm their real-world applicability.

Study limitations and strengths

While TNF-α and IL-17 demonstrated perfect diagnostic accuracy (100% sensitivity/specificity) in distinguishing RA patients from healthy controls in this study, this finding is context-specific, and several limitations must be acknowledged. The cohort was relatively small (75 RA cases and 75 age/sex-matched controls) and sourced from a single Iraqi center, necessitating validation in larger multicenter studies across diverse populations to confirm generalizability. Crucially, the control group lacked patients with other inflammatory rheumatic diseases (e.g., SLE, psoriatic arthritis, osteoarthritis, and

spondyloarthropathies); therefore, the diagnostic performance and specificity of these biomarkers for differentiating RA from conditions with potentially elevated cytokine levels remain unknown and require assessment in mixed cohorts reflective of clinical practice. Furthermore, the RA patients were not stratified by disease activity (e.g., DAS28), seropositivity status, treatment history, factors that may influence cytokine levels, and diagnostic utility. Finally, longitudinal data are required to evaluate biomarker dynamics during development of the disease or in response to therapy.

Conclusion

This research showed the significance of TNF-α and IL-17 in patients with RA, highlighting their diagnostic utility. Elevated TNF-α and IL-17 levels effectively distinguished patients with RA from healthy controls, achieving exceptional diagnostic accuracy. It has emerged as an independent predictor of adverse joint outcomes, underscoring its unique role in RA pathogenesis. In addition, patients with a family history of RA exhibit heightened inflammatory biomarker levels, suggesting genetic and epigenetic influences on disease susceptibility. These findings highlight the pronounced dysregulation in inflammatory and autoimmune biomarkers in RA, underscoring their potential roles in disease pathogenesis and clinical differentiation from healthy individuals.

Declarations

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Author contributions

Conceptualization, A.H.A.A. and F.M.K.; Methodology, F.M.K.; Software, F.M.K.; Validation, A.H.A.A. and F.M.K.; Formal Analysis, F.M.K.; Investigation, A.H.A.A.; Resources, F.M.K.; Data Curation, F.M.K.; Writing – Original Draft Preparation, F.M.K.; Writing – Review & Editing, A.H.A.A.; Supervision, A.H.A.A.; Project Administration, A.H.A.A. and F.M.K.

Conflicts of interest

No conflicts of interest.

Data availability

The datasets utilized and analyzed in the present research are available from the author upon reasonable request.

Ethics approval

Before collecting the samples, a local ethics committee reviewed and authorized the research protocol, subject information, and consent form. This approval was granted by document number 1887, date: November 10, 2024.

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ORIGINAL PAPER

Evaluation of endothelin 1 and N-terminal pro-B-type natriuretic peptide in patients with rheumatoid arthritis

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ABSTRACT

Introduction and aim. Rheumatoid arthritis (RA) is a chronic disease characterized by synovial inflammation and joint destruction. This study evaluated the novel biomarkers vitamin D binding protein (VDBP), retinol-binding protein 4 (RBP4), N-terminal pro-B-type natriuretic peptide (NT-proBNP), and endothelin-1 (ET-1) for RA diagnosis. To our knowledge, this is the first study to simultaneously assess these biomarkers across different treatment stages in RA, linking systemic inflammation with subclinical cardiovascular involvement.

Material and methods. A case-control study enrolled 61 RA patients [G1 (newly diagnosed, untreated, n=10)], G2 [3 months csDMARDs, n=22], G3 [≥6 months biologic+csDMARDs, n =29]), 27 age/sex-matched healthy controls. The serum levels of VDBP, RBP-4, CRP, NT-proBNP, and ET-1 measured using enzyme-linked immunosorbent assay.

Results. All evaluated biomarkers were significantly elevated in RA patients compared to controls ($p<0.0001$): RBP-4 (49.172±21.935 vs. 14.006±3.988 ng/mL), VDBP (12.091±3.334 vs. 2.882±1.136 ng/mL), NT-proBNP (1341.787±626.068 vs. 11.452±3.260 pg/mL), ET-1 (14.246±4.031 vs. 3.932±1.422 pg/mL). Subgroup analysis revealed a significantly higher VDBP in newly diagnosed untreated patients (G1:14.455±4.126 ng/mL) than in treated groups (G2:11.379±2.632; G3:11.816±3.269 ng/mL; $p<0.05$). NT-proBNP peaked in G1 (1789.2±710.81 pg/mL), decreased in csDMARD-treated (G2: 1154.0±537.58 pg/mL), biologic-treated (G3: 1329.96±601.18 pg/mL) group.

Conclusion. VDBP, RBP-4, CRP are effective diagnostic biomarkers of RA. Significant elevations in NT-proBNP and ET-1 levels associated with cardiac complications, correlated with disease activity, and improved with therapy.

Keywords. endothelin 1, N-terminal pro-brain natriuretic peptide retinol–binding protein-4, vitamin D binding protein,

Introduction

Rheumatoid arthritis (RA) is defined as a chronic, systemic autoimmune pathology driven by inflammation that targets joints and may involve diverse extra-articular organs (e.g., cardiac, renal, pulmonary, gastrointestinal, ocular, dermal, and neurological systems).[1] Contemporary research indicates RA etiology involves an integrated contribution from genetic factors, epigenetic regulation, and environmental exposures (such as tobacco smoke, airborne particulates, and notably, the internal environment shaped by the microbiome).[2] Pathogenesis hinges on aberrant interactions within the innate and adaptive immune compartments.[3] These immune disturbances precipitate the generation of characteristic autoantibodies, including RF and antibodies against post-translationally modified proteins (AMPA, encompassing anti-citrullinated, anti-carbamylated, and anti-acetylated pro-

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tein antibodies), as well as the synovial infiltration of T and B lymphocytes.[4,5]

Vitamin D binding protein (VDBP) is a circulating multifunctional plasma protein that can greatly increase the chemotactic potential of neutrophil chemoattractant.[6] Recent studies suggest that DBP has previously underestimated, major, and central roles in inflammatory diseases such as RA.[7] A significant association was observed between RA and VDBP, as previously reported. VDBP can transport $1,25(OH)_2D_3$ between the blood and cell membranes.[8] While hepatic synthesis accounts for most VDBP, leading to its broad distribution in fluids like plasma, CSF, semen, saliva, and breast milk, it is also expressed locally at sites of RA pathology by immune cells and synoviocytes. Importantly, VDBP concentrations in the synovial tissue of RA patients have been shown to be significantly reduced, as previously reported.[9]

The second un routine marker that help diagnosis of RA is retinol-binding protein (RBP4) serves as the transporter for all-trans retinol, circulating in serum as a moderately tight 1:1 molar complex with the vitamin. RBP4 is mainly produced in the liver, with additional synthesis occurring in adipose tissue.[10] It circulates in the bloodstream and binds to transthyretin (TTR), a large protein that increases its molecular mass to approximately 80,000, thereby preventing its elimination through glomerular filtration.[11]

RBP4 is an acute-phase protein upregulated in RA. RBP4 is activated by cytokines, including interleukin (IL-1 and IL-6), and exhibits pro-inflammatory effects.[12] Additionally, RBP4 positively correlated with C-reactive protein levels. RBP4 has been identified as a predictor of atherosclerosis in RA patients. The relationship between elevated RBP levels and RA pathology remains unclear. Previous research has identified RBP4 as a potential biomarker for RA.[13] This study identified cardiac complications in patients with RA. One of the most important biomarkers, endothelin-1 (ET-1), was first identified in 1988 as a potent vasoconstrictor originally isolated from endothelial cells. Production is induced in various cell types by cardiovascular disease risk factors and during the progression of cardiovascular disease.[14] ET-1 induces positive inotropic and chronotropic effects, as well as hypertrophic activity in cardiomyocytes. Endothelin exerts its effects through the activation of two receptor subtypes, ET type A (ET_A)and ET type B (ET_B). Endogenous ET-1 may play a role in the progression of multiple cardiovascular diseases.[15]

The second cardiac parameter is the N-terminal pro-brain natriuretic peptide (NT-proBNP), a prohormone that is cleaved to release brain natriuretic peptide (BNP, also referred to as B-type natriuretic peptide). B-type natriuretic peptide (BNP) and its biologically inactive cleavage product, NT-proBNP, are recognized biomarkers for cardiovascular diseases and subclinical cardiac injury.[16] This study uniquely evaluates a panel of inflammatory and cardiac biomarkers – VDBP, RBP4, NT-proBNP, and ET-1 – in patients with RA across different treatment stages. Unlike previous research focusing on single markers, our approach allows simultaneous assessment of disease activity and cardiovascular risk, providing a more integrated view of systemic inflammation and cardiac involvement in RA.

Aim

The objective of this study was to ascertain the efficiency of the above novel rheumatoid biomarkers in the diagnosis and follow-up of the disease, as well as to estimate the cardiac complications that may be associated with RA by measuring novel cardiac biomarkers such as NT-proBNP and endothelin 1 in the sera of patients.

Material and methods

Design of the study

A case-control study enrolled 61 patients with RA aged 20–66 years and 27 age- and sex-matched healthy controls. RA patients were stratified into three groups: G1 (n=10): newly diagnosed patients meeting the ACR/EULAR 2010 criteria, with symptom duration <6 months, and no prior exposure to disease-modifying antirheumatic drugs (DMARDs) or biologics. G2 (n=22): Patients receiving conventional synthetic DMARDs (methotrexate or leflunomide) for 3±0.5 months, with stable dosing ≥1 month. G3 (n=29): Patients on combined biologic therapy (anti-TNF agents or rituximab) and csDMARDs for ≥6 months with a stable treatment regimen.

The exclusion criterion was age >70 years (to minimize age-related cardiac dysfunction). Comorbidities affecting NT-proBNP/ET-1 levels included cardiac: heart failure (NYHA Class II–IV), prior myocardial infarction, LVEF <50%, atrial fibrillation, significant valvular disease, or uncontrolled hypertension (>160/100 mmHg), renal: eGFR of <60 mL/min/1.73m², pulmonary hypertension (ECHO-estimated PASP >40 mmHg), persistent inflammatory disorders (e.g., systemic lupus erythematosus and vasculitis), active infections, psoriatic arthritis, immune-modifying medications, or any chronic conditions affect study outcomes. Additional exclusion criteria for controls included elevated C-reactive protein (CRP, >5 mg/L), positivity for rheumatoid factor (RF) or anti-citrullinated protein antibodies (ACPA), subclinical cardiovascular disease (NT-proBNP >125 pg/mL or abnormal ECG), history of cardiac/renal disorders, and RA-associated comorbidities (e.g., metabolic syndrome). Individuals failing to meet the inclusion criteria or presenting the above conditions were excluded to ensure sample homogeneity and result validity.

Data and blood collection

The study participants were recruited at Basra Teaching Hospital (Basra Governorate, Southern Iraq). Patient data were collected through structured interviews.

Rheumatologist-confirmed RA patients meeting the American College of Rheumatology 2010 classification criteria with positive inflammatory markers were enrolled. Age-and sex-matched healthy controls were selected according to predefined exclusion criteria to minimize confounding factors. Venous blood samples were collected from all participants in sterile serum separator gel tubes. The serum was subsequently isolated by centrifugation (3,000×g, 15 min) and aliquoted for batch analysis using ELISA and serological testing. The sample size was calculated using the G-power software.

Laboratory tests

All biomarkers, RBP4 (Cat. EH0266), VDBP (Cat. RDEEH20001), NT-proBNP (Cat. EH0350), CRP (Cat. ACN 210), and ET-1 (Cat. EH0648) were quantified using human-specific ELISA kits (Fine Test Company, China), according to the manufacturer's protocols. using a BioTek 800TS microplate reader (USA), and concentrations were calculated from standard curves, as specified in the respective kit instructions.

Statistical analysis

For tabulation and analysis, SPSS spreadsheet version 26.0 was used. Excel was used to display the important facts. For each cardiac and rheumatic parameter, means and standard deviations were calculated for the Control, Pretreatment, sDMARDs, and bDMARDs groups. The Kolmogorov test determined variable normality, which is a prerequisite for parametric and nonparametric analyses. Tables 1 and 2 show how the Mann-Whitney U test was used to compare the control and patient groups. For each biomarker, the Kruskal-Wallis test and post-hoc pairwise comparisons were used to compare the four groups (Tables 3 and 4). Spearman's rank correlation coefficient was used to assess the relationships between the biomarkers.

Ethics approval

The Ethics Committee approved the protocol on 17/12/2023, No 860, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants.

Results

This study conducted a comparative analysis of various biochemical markers between controls and patients. The ages of the participants in the control and patient groups were not significantly different (p=0.315). However, we identified significant elevations in several biomarkers among patients relative to controls (Table 1). Specifically, CRP, RBP4, and VDBP concentrations were all markedly higher in the patient cohort, with significance (p≤0.001).

Table 1. Biomarker values in the control and patient groups compared (Mann Whitney's test)

	Control (n=27)	Patients (n=61)	p
Age (years)	43.55± 8.06	46.57±9.65	0.315
CRP (mg/L)	0.756±0.109	13.097±18.702	<0.001
RBP4 (ng/mL)	14.006±3.988	49.172±21.935	<0.0001
VDBP (ng/mL)	2.882±1.136	12.091±3.334	<0.0001

Table 2 displays the significant differences in the biomarker levels between the two groups. NT-proBNP concentrations were substantially increased in patients (1341.787±626.068 pg/mL) compared to controls (11.452±3.260 pg/mL) (p<0.0001). Similarly, ET-1 concentrations were significantly higher in patients (14.246±4.031 pg/mL) than in controls (3.932±14.212 pg/mL; p<0.0001).

Table 2. Comparison of cardiac parameters between patients and controls (Mann-Whitney's test)

	Control (n=27)	Patients (n=61)	p
NT proBNP (pg/mL)	11.452±3.260	1341.787±626.068	<0.0001
ET1 (pg/mL)	3.932±14.212	14.246±4.031	<0.0001

Analysis across patient subgroups revealed distinct patterns (Table 3). CRP levels were lowest in controls (0.756 mg/L), differing significantly from subgroup G1 (45.600 mg/L; p<0.0001) and G2 (7.045 mg/L; p<0.0001), but not from G3 (6.479 mg/L). Control RBP4 levels were significantly lower than those in G1 (51.125 ng/mL), G2 (50.602 ng/mL), and G3 (47.413 ng/mL; all p<0.0001), though RBP4 did not differ significantly between patient subgroups. Similarly, VDBP concentrations were lowest in controls (2.882 ng/mL), differing significantly from all patient groups (G1: 14.455 ng/mL, G2: 11.379 ng/mL, G3: 11.816 ng/mL; all p<0.0001). VDBP levels in G1 were significantly higher than in G2 (p<0.0001) and G3 (p<0.0001), while G2 and G3 showed no significant difference.

Table 3. Comparison of rheumatic parameters between the study groups (Kruskal Wallis test)*

	Control (n=27)	G1 (n=10)	G2 (n=22)	G3 (n=29)
Age (years)	43.55±8.06 [a]	47.90±11.87[a]	47.09±10.84 [a]	45.72±8.04 [a]
CRP (mg/L)	0.756±0.109[a]	45.600±28.733[b]	7.045±4.673[b]	6.479±4.041[a]
RBP4 (ng/mL)	14.006±3.988[a]	51.125±22.733[b]	50.602±22.450[b]	47.413±21.926[b]
VDBP(ng/ml)	2.882±1.136[a]	14.455±4.126[b]	11.379±2.632[c]	11.816±3.269[c]

* Different superscript letters (a, b, c) denote significant differences (p<0.05) between groups based on post hoc multiple comparison testing.

Table 4 of the cardiac biomarkers indicated substantial elevations in these markers within the patient groups relative to the control group. The Control group exhibited markedly lower NT-proBNP levels (11.452

pg/mL) compared to the other groups, G1 (1789.2 pg/mL), and G2 (1154 pg/mL). On the other hand, the G3 group (1329.966 pg/mL) reported a significant value that was intermediate between the values of the G1 (1789.2±710.81 pg/mL) and G2 (1154.0±537.58 pg/mL) groups. Additionally, the lowest value of ET1 (3.932 pg/mL) was reported for the control group, which was significantly different from the G1 (12.235 pg/mL), G2 (13.987 pg/mL), and G3 (15.135 pg/mL) groups.

Table 4. Cardiac parameters in patients pre- and post-treatment compared to the control group (Kruskal Wallis test)*

	Control (n=27)	G1 (n=10)	G2 (n=22)	G3 (n=29)
Age (years)	43.55±8.06[a]	47.90±11.87[a]	47.09±10.84[a]	45.72±8.04[a]
NT-proBNP (pg/mL)	11.452±3.260[a]	1789.2±710.81[b]	1154.0±537.58[c]	1329.96±601.18[d]
ET1 (pg/mL)	3.932±1.422[a]	12.235±3.783[b]	13.987±3.931[b]	15.135±4.039[b]

* Different superscript letters (a, b, c) denote significant differences (p<0.05) between groups based on post hoc multiple comparison testing.

Table 5, shows significant positive correlations between each of the studied biomarkers: NT-proBNP, ET1, CRP, RBP4, and VDBP.

Table 5. Correlation between all study parameters in the patient groups

Parameters		ET1	CRP	RBP4	VDBP
NTproBNP	R	0.594	0.653	0.563	0.742
	p	<0.0001	<0.0001	<0.0001	<0.0001
ET1	R		0.530	0.558	0.643
	p		<0.0001	<0.0001	<0.0001
CRP	R			0.674	0.675
	p			<0.0001	<0.0001
RBP4	R				0.637
	p				<0.0001

Discussion

RA is an autoimmune disease that causes inflammation and has systemic, extra-articular and persistent effects. Numerous biomarkers have been investigated in RA, and they are important for diagnosis, treatment prognosis, disease activity monetarization, and prediction of the effectiveness of biological therapy. The novelty of our work lies in the combined evaluation of VDBP, RBP4, NT-proBNP, and ET-1 in a treatment-stratified RA population, enabling a concurrent analysis of inflammatory status and subclinical cardiovascular involvement. To our knowledge, no previous study has examined these biomarkers together in newly diagnosed, csDMARD-treated, and biologic-treated RA patients, thereby offering new perspectives on their potential utility for comprehensive disease monitoring and early detection of cardiovascular complications.

This study revealed notable increases in CRP levels among the patient groups when compared to the control group, as illustrated in Tables 1 and 3. Research conducted by Fathy et al. and Cooper et al. has shown that RA patients exhibit elevated CRP levels when compared to healthy controls, as RA is a persistent inflammatory immunological disorder, and CRP functions as an indicator of inflammation.[17,18]

RBP4 levels were significantly elevated in the RA patient groups compared to the control group, as shown in Tables 1 and 3. These results are consistent with those of previous studies by Allah et al. and Pazos-Pérez et al., who confirmed the high levels of RBP4 in RA.[19,20] Notably, there were no significant differences between the patient groups G1, G2, and G3. This means that there was no significant impact of disease severity or type of treatment on marker sensitivity. This is inconsistent with the results of a previous study by Wei et al., who reported that RBP4 levels were not affected by disease severity.[21] RBP4 levels may be elevated in RA patients in response to elevated levels of TNF-α, IL-6, and IL-1β, which can upregulate RBP4 production. RBP4 is primarily synthesized in the liver and its production may be influenced by systemic inflammation.[22] RA can lead to altered liver function, which affects the synthesis and clearance of RBP4.[23] RA is associated with high oxidative stress, which leads to increased tissue damage and immune cell activation. RBP4 has been linked to oxidative stress responses, and its elevation could be a compensatory mechanism for mitigating cellular damage.[24]

VDBP levels in this study showed significant elevations among the RA patient groups compared to the control group, as shown in Tables 1 and 3. G1 displayed the highest levels because this pretreatment group did not undergo any type of treatment. These results are consistent with those of a previous study by Obied and Sarhat, which reported low levels of VDBP in healthy people compared to RA patients.[25] This is because RA is a chronic inflammatory condition marked by increased concentrations of pro-inflammatory cytokines, which might prompt the liver to synthesize more VDBP, akin to acute-phase proteins.[26] Additionally, RA patients often experience altered vitamin D metabolism, with an increased demand for VDBP to transport vitamin D metabolites, especially due to chronic inflammation and immune dysregulation.[27] Many patients with RA have vitamin D deficiency, which might trigger a compensatory increase in VDBP to enhance vitamin D transport and availability. A study not far away by Delrue and Speeckaert previously suggested that VDBP serve as a novel indicator of inflammation.[28]

The present study showed significant elevations in the cardiac biomarker ET-1 level in RA patient groups compared to the controls, as shown in Tables 2 and 4.

Consistent with these findings, prior research by Cienfuegos et al. and Yang et al. indicates elevated ET-1 levels in RA patients relative to controls.[29,30] Elevated levels of ET-1 in patients with RA compared to healthy controls may be attributed to chronic inflammation and cytokine stimulation that are characterized in RA, which stimulates the production of ET-1 in vascular endothelial.[31] ET-1 acts as a vasoconstrictor, while nitric oxide (NO) acts as a vasodilator. In rheumatic diseases, oxidative stress increases, reducing the availability of NO and leading to an increased vasoconstrictive effect of ET-1.[32] ET-1 may play a key role in the pathophysiology of rheumatoid disease, including arthritis.[33]

The estimation of NT-proBNP levels revealed significant elevations in the RA patient groups compared to the controls, as shown in Tables 2 and 4. The results of this study are in agreement with the results of previous studies by Zhu et al. and Raadsen et al. indicated high levels of NT-proBNP in RA patients.[34] Notably, the G1 demonstrated the highest NT-proBNP levels, with a statistically significant elevation relative to G2 and G3, which means that the treatment overcame the results. Several studies by McKechnie et al. and Hassan et al. have explored the relationship between NT-proBNP levels and RA.[35,36] Elevated NT-proBNP levels in RA patients can indicate subclinical heart failure, a condition that is common in RA patients due to chronic inflammation affecting the heart, as mentioned previously by Shamsi et al. this is in agreement with a recent study.[37] In a study by Heslinga et al., in four Dutch centers with patients with early RA showed a significant reduction in NT-proBNP after 6 months of anti-rheumatic treatment. Such a decrease in NT-proBNP levels is not limited to patients in remission, suggesting that suppression of inflammation is sufficient to reduce NT-proBNP levels in patients with RA.[38]

Furthermore, our study showed a significant positive correlation between all the study biomarkers, as shown in Table 5. This means that inflammation, which is a characteristic of RA, may lead to cardiovascular complications in patients with RA.

Study limitations

This study has several limitations that should be acknowledged. The limited sample size could constrain generalizability and impede biomarker validation, compounded by a single-center, cross-sectional design focused solely on Iraqi patients, which limits insights into biomarker dynamics over time (such as monitoring treatment response or disease progression). Therefore, larger longitudinal studies and validation in diverse populations are essential to confirm biomarker utility for monitoring, prognosis, and broader clinical application.

Conclusion

Based on the study findings, VDBP, RBP-4, and CRP serve as effective diagnostic biomarkers for rheumatoid arthritis, exhibiting significant elevations across patient groups compared to controls. Furthermore, significantly increased NT-proBNP and ET-1 levels in RA patients indicate substantial cardiac complications associated with the disease. The strong positive correlations observed between all studied biomarkers suggest that systemic inflammation characteristic of RA contributes to these cardiovascular sequelae.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The Ethics Committee approved the protocol on 17/12/2023, No 860, and the study was conducted in accordance with the Declaration of Helsinki.

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ORIGINAL PAPER

Neuroprotective effects of *Vernonia amygdalina* and *Moringa oleifera* in alloxan-induced diabetic Wistar rats

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ABSTRACT

Introduction and aim. Diabetes mellitus is a metabolic disorder that affects multiple organs, including the hippocampus, a key region involved in memory. This study aimed to investigate the neuroprotective and antidiabetic effects of *Vernonia amygdalina* and *Moringa oleifera* in an alloxan-induced diabetic rat model.

Material and methods. Thirty-five adult Wistar rats were randomized into seven groups and treated with aqueous extracts of *V. amygdalina*, *M. oleifera*, their combination, or glibenclamide for 30 days following alloxan-induced diabetes. Fasting blood glucose (FBG), hippocampal acetylcholinesterase (AChE) activity, cognitive performance (Morris Water Maze test) and histopathological changes in the hippocampus were evaluated.

Results. Alloxan significantly increased FBG (20.68±1.04 mmol/L), AChE activity (40.40±0.40 nmol/mL), and escape latency (51.75±4.39 sec), and reduced hippocampal cell density. Treatment with *V. amygdalina* and *M. oleifera* reduced FBG (8.29±0.93 mmol/L), AChE activity (34.50±0.30 nmol/mL), and escape latency (3.39±0.45 sec), and improved hippocampal histoarchitecture.

Conclusion. *V. amygdalina* and *M. oleifera* demonstrated neuroprotective and antidiabetic effects in diabetic rats. These results support their potential as adjunct agents to prevent diabetes-induced cognitive dysfunction.

Keywords. acetylcholinesterase, diabetes mellitus, hippocampus, *Moringa oleifera, Vernonia amygdalina*

Introduction

Diabetes mellitus is a significant global health concern, with its complications significantly impairing the health of affected individuals, making it a worldwide concern.[1,2] It is a metabolic disorder caused by both aberrant insulin secretion and disrupted insulin action.[3,4] The hormone insulin, which is released by the islets of Langerhans' pancreatic β-cells, enables cells to absorb

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glucose and utilize it as fuel; however, excess glucose in the blood stream leads to hyperglycemia which in turn impairs health.[5] Although diabetes mellitus exists in several forms, type 2 diabetes mellitus (T2DM) is the most common among middle-aged and older adults. Early-onset T2DM is linked with extended periods of hyperglycemia, rapid disease progression, significant insulin resistance, and declining beta-cell function, all of which impair glycemic control and heighten the likelihood of complications.[6]

Interestingly, T2DM is increasing exponentially worldwide, largely due to physical inactivity, sedentary lifestyles, and poor dietary habits.[7] The International Diabetes Federation estimates indicate the number of people aged 20-79 living with diabetes will surge from over 530 million in 2021 to more than 780 million by 2045.[8] It has also been estimated that a person with diabetes mellitus has higher chances (1.5 times more prone) to experience cognitive dysfunction than a normal healthy person.[9] In Nigeria, a study carried out in Kano and other states estimated that T2DM caused impaired cognitive function.[10-13] In another study, it was estimated that 40% of the T2DM patients had cognitive impairment.[14]

Cognition is closely linked to the brain, which comprises various structures, including the hippocampus, which is widely recognized for its critical role in memory and learning. It functions as a memory control center, directing the storage and retrieval of memories in appropriate brain regions for long-term use.[15,16] However, the hippocampus is particularly vulnerable to metabolic diseases like diabetes mellitus because of its complex anatomical makeup.[17,18] Hyperglycemia is the metabolic syndrome component most strongly linked to cognitive impairment, a growing comorbidity of diabetes mellitus.[19,20] This impairment is caused by injury to the hippocampus as well as shrinkage and disruption of cholinergic transmission brought about by the key factors of diabetes mellitus: chronic hyperglycemia, and oxidative stress.[21] Extreme hyperglycemia and oxidative stress modify the hippocampus's neuronal membrane and structure, as well as its capacity to receive neural impulses, which alters the signal pathways needed for memory.[22,23]

The search for remedies for hippocampal degeneration and memory impairment has gained prominence in recent years. In order to manage diabetes mellitus and prevent these associated complications, tight glycemic control must be achieved and maintained.[24] For this reason, World Health Organization (WHO) suggested the creation of oral hypoglycemic medicines from medicinal plants as a form of herbal treatment for diabetes mellitus.[25] *Moringa oleifera* (Lam) and *Vernonia amygdalina* are two examples of medicinal plants that have been used for centuries because of their therapeutic and dietary value in the management of diabetes mellitus.[26] *M. oleifera* belongs to the Moringaceae family, found abundantly in the tropics and widely used in folklore and traditional medicine. Because of the numerous health benefits of the plant's various parts, hence its reputation as the miracle tree.[27] According to reports, the leaves of this plant contain high levels of micro-nutrients such as potassium, iron, vitamins, and vital amino acids as well as numerous phytochemicals including flavonoids, phenols, alkaloids, nitrile glycoside, and tannins.[28-31]

Likewise, *V. amygdalina* commonly called bitter leaf is native to tropical regions. The Asteraceae family including *V. amygdalina* are best identified due to their bitter leaves caused by the availability of anti-nutritional elements such as alkaloids, saponins, glycosides, and tannins.[32] They are also packed with essential micronutrients, including vitamins and minerals, and a good source of dietary fiber, rendering them vital for human nutrition.[33] The plant is consumed locally as food and is also used for medical purposes such as suppression of cancer, neuroprotective effects, hepato-protective effects, and other several purposes.[34-36]

Aim

This study focuses on investigating the protective effect of *V. amygdalina* and *M. oleifera* against diabetes-induced neurodegeneration and acetylcholinesterase (AChE) activity in the hippocampus and cognitive decline in adult Wistar Rats.

Material and methods

Study location

This study was conducted in the Department of Anatomy, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus.

Collection, identification and preparation of plant materials

M. oleifera and *V. amygdalina* were procured from Nkwo Market, a well-known market in Nnewi town, Anambra State. Identification and authentication of the specimens were conducted at the Department of Botany, Nnamdi Azikiwe University, with the herbarium numbers NAUH-17B and NAUH-20B respectively. The leaves were washed thoroughly to remove dust particles. They were air-dried and pulverized. The aqueous extracts of both *M. oleifera* and *V. amygdalina* were separately prepared according to the method described by Thilza et al.[37] The extracts were freeze-stored at 4°C in a refrigerator (Model: HTF-319, China) until use. The extracts were made into varying doses on each administration day and administered using orogastric tube according to the treatment group doses and animal's body weight.

Procurement and handling of experimental animals

Thirty-five adult Wistar rats, with weights ranging from 130g to 170g were sourced and housed at the College of

Health Sciences Animal House, Nnamdi Azikiwe University, for two weeks to acclimatize according to standard ethical procedure (NAU/CHS/NC/FBMS/472). The animals had access to rat chow and water ad libitum throughout the duration of the experiment. The animal welfare and health status were monitored throughout the experiment, following the guidelines set by the Federation of European Laboratory Animal Science Associations.[38]

Induction of diabetes

Alloxan diabetes induction was done as described by Ibrahim et al.[39] All rats, except those in the control group (Group A), received intraperitoneal injections of alloxan monohydrate at a dose of 150 mg/kg body weight. Alloxan was prepared by dissolving alloxan monohydrate in sterile normal saline solutions. To prevent alloxan-induced hypoglycemia, rats were provided with a 10% glucose solution for 24 hours after alloxan injection.[40,41] Diabetes was confirmed 72 hours post-alloxan administration by assessing fasting blood glucose levels in overnight-fasted rats using a glucometer (Accu-Chek Active Performa, Roche, Germany). Rats were classified diabetic if their blood glucose levels, measured from tail punctures, were >200 mg/dL (11 mmol/L); levels were checked daily for 5 consecutive days. Treatment began 5 days after alloxan injection and lasted for 30 days. Alloxan was used at a moderate dose to induce partial β-cell dysfunction, replicating the impaired insulin production characteristic of late-stage T2DM. This model reflects the key features of T2DM, including insulin resistance and β-cell exhaustion.

Experimental design

A total of 35 adult Wistar rats were randomly allocated into 7 groups (A-G) of 5 rats each.

Group A: each control rat received only water and rat chow

Group B: each diabetic control rat received only water and rat chow

Group C: alloxan-induced diabetic rats were administered 500 mg/kg body weight aqueous leaf extract of *V. amygdalina* and *M. oleifera* once daily.

Group D: alloxan-induced diabetic rats were administered 700 mg/kg body weight aqueous leaf extract of *V. amygdalina* and *M. oleifera* once daily.

Group E: alloxan-induced diabetic rats were treated once daily with the standard antidiabetic drug glibenclamide (5 mg/kg body weight).[42]

Group F: alloxan-induced diabetic rats were administered 350 mg/kg body weight aqueous leaf extract of *V. amygdalina* once daily.

Group G: alloxan-induced diabetic rats were administered 350 mg/kg body weight aqueous leaf extract of *M. oleifera* once daily.

Determination of fasting blood sugar (FBS)

FBS was determined four times during the course of the study. The blood glucose level was determined after induction and also on the 7th day, 15th day and 30th day. This was measured by pricking the tail with a lancet after cleaning with 10% alcohol, applying the blood to a test strip in a glucometer (Accu-Chek Active Performa), and obtaining a direct reading in mmol/L.[43]

Determination of spatial memory with Morris Water Maze test

Spatial memory was assessed via the Morris Water Maze test according to Stewart and Morris.[44] The Morris water maze setup included a 1m diameter pool with 30 cm deep water, divided into four quadrants (NE, SE, SW, NW). A submerged platform was placed in one quadrant, with external visual cues guiding the rats. Rats underwent 24-hour training, where they were placed in different quadrants to find the hidden platform within 60 seconds, with 15-minute intervals. Training continued until they could find the platform in under 15 seconds. During the test, the water was made opaque, the platform taken out, and the rats placed in different quadrants. The duration for rats to locate the platform's previous position was measured as escape latency.

Termination of experiment

After the experiment, the Wistar rats were fasted for 24 hours, then euthanized by decapitation following chloroform anesthesia, and their brains harvested. One cerebral hemisphere was preserved in 10% formal saline for histopathological analysis while the other hemispheres were kept in the refrigerator until ready for biochemical analysis.

Brain homogenate preparation

The cerebral hemispheres for biochemical analysis were homogenized with 10 mL of ice-cold 0.01 M phosphate buffer at pH 7.4. The homogenate was centrifuged at 7,000 rpm for 15 minutes in the cold medium and the supernatant obtained was stored at 4°C for biochemical evaluation.

Determination of brain AChE activity

The AChE assay was performed using a modified protocol, with a reaction mixture containing brain homogenate, phosphate buffer (0.01M, pH 7.4), and 100 µl of 5,5-dithiobis-2-nitrobenzoic acid.[45] A baseline reading was taken before adding acetylthiocholine to start the reaction, and absorbance changes were monitored at 412 nm at 2-minute intervals for 10 minutes.

Histopathological analysis

The cerebral hemispheres were immersed in 10% formal saline for fixation at room temperature for 24 hours.

The hippocampus was excised from each for histological assessment using hematoxylin and eosin (H&E) and done by a blind observer. Tissues were dehydrated in alcohol (70%, 80%, 90%, and absolute) for one hour each and cleared in xylene (2 changes; 30 minutes each). The cleared tissues were impregnated in molten paraffin wax, allowed to cool and a paraffin block formed and ready for sectioning using rotary microtome. Tissues were mounted on glass slides aided with albumin of egg and the section (5 μm) immersed in water bath at 50–55°C temperature to remove wrinkles. Water was drained off the slides and placed inside the incubator fix sections on the slide and dry. Tissues were then stained with hematoxylin and eosin technique, viewed under a compound light microscope (Zeedo microscope) and photomicrographs taken from each group and labelled using Photoscape v3.7.

Measurement of the neuron density in the CA1 area

The neuron numbers of the hippocampus in the CA1 area were counted under a compound microscope (Zeedo microscope) at 400× magnification. An accompanying software of the microscope, USB 2.0 camera viewer was used in the counting process to determine the thickness in the CA1 area.

Statistical analysis

IBM Statistical Package for Social Sciences version 23 for Windows (IBM Incorporation, Armonk, New York, USA) was used for data analysis, and the results expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by post Hoc LSD was applied for determining the significance. Level of significance was established at p≤0.05.

Results

Effect of aqueous leaf extracts of M. oleifera and V. amygdalina on FBG

The FBG level was evaluated in the treatment groups after the administration of *M. oleifera* and *V. amygdalina*. The results in table 1 showed a significant elevation (p<0.05) in blood glucose level at day 1 in groups B, C, D, E, F, and G compared to A, which is indicative of the successful induction of diabetes mellitus by the alloxan. This is consistent across days 7, 15, and 30, which is indicative of sustained diabetes mellitus in the Wistar rats. However, treatment of the Wistar rats with *M. oleifera* and *V. amygdalina* resulted in a significant reduction (p<0.05) in FBG level across the groups when compared to the diabetic control (group B). This reduction in the FBG level of *M. oleifera* and *V. amygdalina* treated groups are comparable to the glibenclamide treated group (group E), suggesting hypoglycemic effects of the extracts.

Table 1. Effect of aqueous leaf extracts of *M. oleifera* and *V. amygdalina* on FBG level at day 1, 7, 15 and 30 in diabetic adult Wistar rats*

	FBG day 1 (mmol/L)	FBG day 7 (mmol/L)	FBG day 15 (mmol)	FBG day 30 (mmol/L)
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Group A (control)	4.60±0.29 [b]	4.50±0.31 [b]	4.42±0.22 [b]	4.82±0.322 [b]
Group B (diabetic control)	20.68±1.04 [a]	21.07±0.85 [a]	23.40±2.06 [a]	25.50±1.16 [a]
Group C (DM+500 mg/kg of *M. oleifera*+*V. amygdalina*)	22.45±2.28 [a,b]	23.18±2.08 [a,b]	19.15±0.43 [a,b]	12.60±3.98
Group D (DM+700 mg/kg of *M. oleifera*+*V. amygdalina*)	20.30±0.67 [a,b]	21.02±0.85 [a,b]	17.68±1.82 [a,b]	8.29±0.93 [a,b]
Group E (5 mg/kg of glibenclamide)	20.47±1.20 [a,b]	20.30±1.28 [a,b]	12.73±2.59 [a,b]	7.57±1.22 [a,b]
Group F (350 mg/kg of *V. amygdalina*)	18.97±0.65 [a,b]	38.15±18.88	19.40±0.45 [a,b]	9.23±1.08 [a,b]
Group G (350 mg/kg of *M. oleifera*)	20.17±1.43 [a,b]	21.18±1.16 [a,b]	19.95±0.60 [a,b]	10.44±0.08 [a,b]

* data was analyzed using ANOVA followed by post Hoc LSD multiple comparison and values were considered significant at considered significant at p≤0.05, SEM – standard error of mean. [a] – p≤0.05 compared with control (group A), [b] – p≤0.05 compared with diabetic control (group B)

Effect of aqueous leaf extracts of M. oleifera and V. amygdalina on cognitive function

An evaluation of cognitive status in the diabetic Wistar rats after treatment with *M. oleifera* and *V. amygdalina* using Morris Water Maze test is shown in Table 2. Induction of diabetes mellitus significantly increased the time spent before finding the escape platform in MWMT across all the treatment groups suggesting cognitive decline. Treatment with *M. oleifera* and *V. amygdalina* significantly decreased (p<0.05) the time spent in finding the platform in all the treatment groups suggesting the anti-diabetic and nootropic effects of the extracts.

Table 2. Effect of aqueous leaf extracts of *M. oleifera* and *V. amygdalina* on Morris Water Maze test in diabetic adult Wistar rats[a]

	Escape latency (secs) initial	Escape latency (secs) day 1	Escape latency (secs) day 30
	Mean±SEM	Mean±SEM	Mean±SEM
Group A (control)	1.91±0.40	4.81±0.32*	2.65±0.67*
Group B (diabetic control)	1.77±0.52	51.75±4.39*	17.33±4.39*
Group C (DM+500 mg/kg of *M. oleifera*+*V. amygdalina*)	1.84±0.33	24.40±9.23	7.13±2.49
Group D (DM+700 mg/kg of *M. oleifera*+*V. amygdalina*)	1.59±0.32	9.23±1.08*	3.39±0.45*
Group E (5 mg/kg of glibenclamide)	1.76±0.15	7.58±1.23*	2.93±0.47*
Group F (350 mg/kg of *V. amygdalina*)	1.58±0.24	8.32±0.91*	4.43±0.59*
Group G (350 mg/kg of *M. oleifera*)	1.53±0.15	9.92±01.01*	5.37±0.38*

[a] data were analyzed using t-test, and values considered significant at p≤0.05, SEM – standard error of mean, * – p≤0.05 indicates statistical significance

Effect of aqueous leaf extracts of M. oleifera and V. amygdalina on AChE activity

The Ache activity in the hippocampus was evaluated after treatment with *M. oleifera* and *V. amygdalina*. As shown in Table 3, the Ache activity was significantly lower in group A compared to B (p=0.03). Groups C, D, E, F, and, G had significantly lower AChE activity compared to group B. Also, AChE showed a significantly higher activity levels in groups B, C, D, E, F, and, G compared to A.

Table 3. Effect of aqueous leaf extracts of *M. oleifera* and *V. amygdalina* on AChE (Ache) activity in diabetic adult Wistar rats

	AChE (nmol/mL) Mean±SEM
Group A (control)	21.25±0.85 [b]
Group B (diabetic control)	40.40±0.40 [a]
Group C (DM+500 mg/kg of *M. oleifera*+*V. amygdalina*)	37.35±0.95 [a, b]
Group D (DM+700 mg/kg of *M. oleifera*+*V. amygdalina*)	34.50±0.30 [a, b]
Group E (5 mg/kg of glibenclamide)	31.20±1.20 [a, b]
Group F (350 mg/kg of *V. amygdalina*)	35.00±0.80 [a, b]
Group G (350 mg/kg of *M. oleifera*)	36.10±0.30 [a, b]

* data was analyzed using ANOVA followed by post Hoc LSD multiple comparison and values were considered significant at p≤0.05, SEM: standard error of mean, [a] – p≤0.05 compared with control (group A), [b] – p≤0.05 compared with diabetic control (group B)

Histopathological evaluation

The histoarchitecture of the hippocampus of each group was evaluated and the photomicrographs presented in Figure 1. Histopathological section of the hippocampus of the diabetic control (group B) revealed change in the cell density with average thickness in the CA1 area reaching 68.73 µm suggesting loss of neurons (neurodegeneration) whereas *M. oleifera* and *V. amygdalina* treated Wistar rats maintained higher cell densities in the CA1 areas suggesting neuroprotective activity.

Discussion

This study evaluated the combined effects of *M. oleifera* and *V. amygdalina* on the hippocampus of alloxan-induced diabetic adult Wistar rats. Both plants significantly lowered fasting blood glucose levels, demonstrating anti-hyperglycemic effects comparable to glibenclamide. The results indicate that both plant extracts, whether administered alone or in combination, are effective in controlling hyperglycemia. The hypoglycemic effects of the extracts could be a result of their bioactive phytochemicals with antioxidant activity.[46-48] These antioxidant activities may promote more insulin production by the remaining functional pancreatic beta cells, facilitate the repair of beta cells damaged by alloxan, or enhance glucose uptake and metabolism across various organ systems. Kempferol, quercetin and tannins have been identified as very potent antioxidants and they are all found in the plant materials used.[49-51] Findings by Momoh and his colleagues indicate that both leaf extract of *M. oleifera* and *V. amygdalina* have immense anti-hyperglycemic effect when administered to diabetic rats.[52]

Aqueous leaf extracts of *M. oleifera* and *V. amygdalina* attenuate AChE activity, thereby enhancing acetylcholine levels in the hippocampus and inadvertently enhancing cognitive performance, marked by decreased escape la-

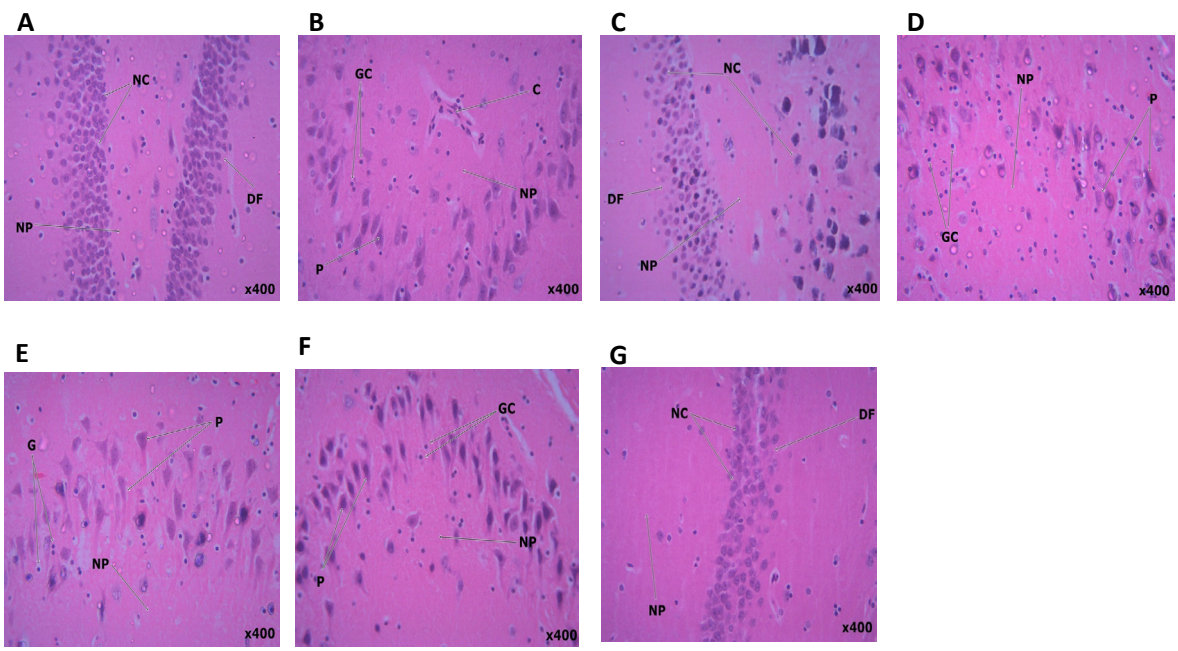

Fig. 1. Effects of *M. oleifera* and *V. amygdalina* on histopathological changes induced by diabetes on the hippocampus of adult Wistar rats. A: control group, B: diabetic control, C: DM+500 mg/kg of *M. oleifera*+*V. amygdalina*, D: DM+700 mg/kg of *M. oleifera*+*V. amygdalina,* E: 5 mg/kg of Glibenclamide, F: 350 mg/kg of *V. amygdalina,* and G: 350 mg/kg of *M. oleifera*

tency in the Morris water test compared to the diabetic control (group B). The significant decrease in AChE activity leads to a corresponding increase in hippocampal acetylcholine, as less is metabolized by the enzyme. This confirms the extracts' ability to enhance synaptic acetylcholine availability, modulate cholinergic neurotransmission, and function as potent AChE inhibitors comparable to established drugs. Csernansky et al. and Muthuraju et al., revealed that down-regulation of AChE activities using AChE inhibitors such as physostigmine and galantamine restored learning and memory deficits and increased acetylcholine level.[53,54] Oladele and colleagues, in their study also found that administration of methanolic leaf extracts of *V. amygdalina* significantly decreased the activity of AChE in different regions of the brain.[35]

Additionally, patients with diabetes mellitus often exhibit impaired attention, memory, general intelligence, and slower information processing.[6,18,21] These findings support the present study, which observed increased time in the Morris water test and significantly elevated AChE activity in the diabetic control group compared to Group A. However, administration of *M. oleifera* and *V. amygdalina* extracts significantly reduced both the time spent in the Morris Water Maze test and AChE activity in the treatment groups, except at the lower dose (group C), which showed no significant time reduction from baseline but a significant reduced time compared to the diabetic control. These findings align with those of Rahmath et al. as well as Kirisattayakul et al., supporting the nootropic effects of the extracts.[55,56]

Neurodegeneration in the hippocampus is closely linked to memory impairment, and diabetes mellitus has been shown to cause neuronal death in this region, likely due to oxidative stress and lipid peroxidation.[18,57] In this study, average thickness in the CA1 region was used to estimate neuronal density. Histopathological analysis revealed reduced CA1 thickness in the diabetic control group (68.73 μm) compared to group A (79.61 μm). However, treatment with *M. oleifera* and *V. amygdalina* extracts conferred neuroprotection, with CA1 thickness values of 71.46 μm, 74.39 μm, 76.31 μm, 73.79 μm, and 73.44 μm in groups C through G, respectively, indicating improved neuronal density relative to the diabetic control. These findings are supported by Sutalangka et al. and Aguwa et al., who reported that *M. oleifera* and *V. amygdalina* extracts enhance spatial memory and reduce hippocampal neurodegeneration.[58-60] The neuroprotective effects observed may be attributed to the antioxidant phytochemicals, such as flavonoids and tannins, which scavenge free radicals and inhibit lipid peroxidation and neuronal cell death.

Overall, co-administration of *M. oleifera* and *V. amygdalina* leaf extracts significantly improved fasting blood glucose levels, reduced AChE activity, enhanced cognitive function, and mitigated neurodegeneration, comparable to the effects of the standard antidiabetic drug glibenclamide. Between the two, *V. amygdalina* demonstrated greater efficacy, likely due to its higher flavonoid and tannin content, compounds known to support cognitive function and lower blood glucose levels.[61,62]

Study limitations and recommendations

This study evaluated only one neurotransmitter enzyme, AChE; therefore, the findings should be validated by assessing additional neurotransmitter enzymes. Furthermore, the use of more specific histological stains would provide greater insight into the underlying neuronal changes.

Conclusion

Our result confirms the age long traditional and therapeutic value of *V. amygdalina* and *M. oleifera* in the treatment of diabetes mellitus and its associated complications. It presents empirical and compelling evidence that diabetes mellitus adversely affects the hippocampus and cognition, and that administration of aqueous extracts of *V. amygdalina* and *M. oleifera* protected against neurodegeneration, cognitive decline, and attenuated AChE activity. This proves the neuroprotective effect of these plant extracts and makes them relevant for use in preventing and managing neurodegeneration and other neurological disorders associated with diabetes mellitus.

Declarations

Funding

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Author contributions

Conceptualization, C.A.A. and D.N.E.; Methodology, U.C.O. and D.N.E.; Software, B.N.O.; Validation, U.C.O. and P.M.D.; Formal Analysis, B.N.O.; Investigation, C.A.A.; Resources, P.M.D. and M.F.O.; Data Curation, P.M.D.; Writing – Original Draft Preparation, C.A.A. and U.C.O.; Writing – Review & Editing, B.N.O. and M.F.O.; Visualization, D.N.E.; Supervision, U.C.O. and D.N.E.; Project Administration, B.N.O.; Funding Acquisition, C.A.A.

Conflicts of interest

The authors declare no competing interests.

Data availability

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

Ethical approval

The protocol for the study was approved by the Ethics Committee, Faculty of Basic Medical Sciences, Nnamdi Azikiwe Universitty, Nnewi Campus and was assigned the reference number NAU/CHS/NC/FBMS/472.

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ORIGINAL PAPER

Association between handgrip strength and cognitive function in older adults living with cancer

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ABSTRACT

Introduction and aim. Handgrip strength (HGS) serves as a key indicator of muscle performance and may reflect cognitive status in older cancer patients. We examined whether handgrip strength was associated with cognitive function in this group of patients.

Material and methods. For this study, a cross-sectional design was used, analyzing data from patients aged 60 years and older diagnosed with cancer, collected through Comprehensive Geriatric Assessments (CGAs). The information included demographics, HGS levels, and Mini-Mental State Examination (MMSE) scores.

Results. Among 352 participants (average age 75.7 years), HGS showed a moderate positive correlation with cognitive function, stronger in females ($r=0.36$, $p<0.001$) than in men ($r=0.22$, $p=0.005$). Each 1 kg increase in HGS was associated with a 0.29-point increase in MMSE scores in women and 0.13 points in males.

Conclusion. Higher HGS appears to better cognitive outcomes in older adults with cancer. As muscle strength is potentially modifiable, future research should explore whether HGS-targeted interventions could preserve or enhance cognitive health. Implementing regular handgrip strength assessments in geriatric oncology could help to detect patients who are vulnerable to cognitive decline.

Keywords. aging, cancer, cognition, handgrip strength, sarcopenia

Introduction

Currently, it is estimated that over 57 million people worldwide are living with cognitive impairment, and this number is projected to increase to 153 million by 2050.[1] Such cognitive decline in older adults can result in a significant global economic burden, with estimates suggesting losses of up to $14.5 trillion USD between 2020 and 2050.[2] Among older adults, cognitive impairment is most frequently attributed to conditions such as Alzheimer's disease, vascular dementia, frontotemporal dementia, and Lewy bodies. However, cognitive dysfunction may also arise in the context of cancer and its treatments – a condition known as cancer-related cognitive impairment (CRCI).

CRCI refers to a variety of cognitive symptoms that are believed to be the result of the effects of systemic malignancies and/or their therapies, even when the central nervous system is not directly involved.[3] These symptoms can be self-reported or objectively identified through validated neuropsychological assessments

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and often include reduced attention, slower processing speed, and language retrieval difficulties.[4] CRCI can appear at different points in the cancer journey, including prior to diagnosis, during treatment, or in survivorship. Reported prevalence rates range from 23% to 31%, depending on the timing and type of assessment.[5]

Several risk factors have been associated with CRCI, including specific types of cancer (e.g. hormone-related tumors), chemotherapeutic agents ("chemo brain"), cranial irradiation, anti-hormonal treatments, genetic predisposition (e.g. *APOE, COMT, BDNF* polymorphisms), and various psychosocial influences such as depression, anxiety, fatigue and lack of social support.[7]

Among evidence-based strategies to reduce the risk of dementia, promoting regular physical activity is strongly recommended. Physical activity is associated not only with improved overall health, but also with better cognitive outcomes in aging populations.[8]

Comprehensive Geriatric Assessment (CGA) is a multidisciplinary approach used to assess the complex needs of older adults, particularly those with chronic illnesses such as cancer. It includes functional and cognitive assessments, among others, and is valuable for guiding treatment decisions and optimizing care plans in older cancer patients.[9] Since muscle strength, often measured through handgrip strength (HGS), is positively linked to physical activity, and physical activity is inversely associated with cognitive decline, an emerging area of research focuses on the association between muscle strength, particularly handgrip, and cognitive outcomes in aging cancer populations.[10] To date, only one published study has specifically investigated this association in older cancer patients. Our study aimed to address this gap in the literature by examining the correlation between handgrip strength and cognitive performance in a Latin American population of older people diagnosed with cancer.[11]

Aim

This study aimed to evaluate the relationship between handgrip strength and cognitive function among older adults with cancer treated in a Peruvian healthcare setting.

Material and methods

Design

We carried out a cross-sectional observational study within the Geriatrics Department at Almenara Hospital in Lima, Peru. We retrospectively reviewed data from electronic medical records corresponding to comprehensive geriatric evaluations (CGA) performed between January 2023 and February 2025. A nonprobability, convenience sampling method was applied to select eligible participants.

Inclusion criteria were: (a) adults aged 60 years or older, hospitalized or seen in outpatient settings; (b) confirmed cancer diagnosis; and (c) CGA request issued by the oncology team.

Exclusion criteria included: (a) people who were illiterate or unable to complete the Mini-Mental State Examination (MMSE) due to conditions such as visual or hearing impairment, or any other factor affecting test reliability; (b) patients with hand-related musculoskeletal disorders, such as rheumatoid arthritis, that could affect grip strength; and (c) those diagnosed with tumors or brain metastases of the primary central nervous system.

Peru's national education system is mandatory for children aged six and includes six years of primary school and five years of secondary education. University-level programs typically last between three and seven years. Pre-school education was not considered in this study.

Cognitive Function Assessment

Cognitive function was assessed with the Spanish version of the Mini-Mental State Examination (MMSE).[12] A score below 24 points was considered indicative of cognitive impairment. This tool has been recommended by the International Cognition and Cancer Task Force (ICCTF) for evaluating domains most commonly affected by cancer treatments.[13,14] The MMSE is widely validated and has been used in previous studies involving cancer patients.[15]

Handgrip Strength Assessment

Handgrip strength (HGS) was evaluated using a Jamar hydraulic hand dynamometer, adhering to the standardized Southampton protocol.[16] Participants were seated with their forearm resting on the armrest, the wrist neutrally at the edge of the armrest, and the feet flat on the floor. During the test, patients were instructed to 'squeeze *as hard as possible until told to stop*', with timing guided by when the dynamometer needle stopped rising. Two to three measurements were taken per hand, on alternating sides. For analysis, the highest value recorded from either hand. Probable sarcopenia was defined using the criteria of the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) criteria: <27 kg for men and <16 kg for women.[17]

Comprehensive Geriatric Assessment (CGA)

The CGA was conducted by trained geriatricians and encompassed multiple domains, including physical function, mobility, nutritional status, cognition, mood, comorbid conditions, and social support. Functional status was measured using the Katz Index of Basic Activities of Daily Living (BADL).[18] Nutritional screening was performed using the Short Form (MNA-SF).[19] Depressive symptoms were identified via a documented diagnosis, current use of antidepressants or semi-structured interviews based on DSM-IV criteria.[20] The burden was quantified with the Cumulative Illness Rating Scale for

Geriatrics (CIRS-G).[21] Additional variables collected included demographic and clinical information such as age, sex, body mass index (BMI), and cancer diagnosis.

Statistical analysis

We used descriptive statistics to characterize the study population. Categorical variables were reported as counts and percentages, while continuous data were summarized using means and standard deviations or medians and ranges, depending on their distribution. Pearson correlation coefficients were calculated to assess the relationship between handgrip strength and MMSE scores. The interaction between sex and handgrip strength was statistically significant; therefore, all analyses were stratified by sex. Multivariate linear regression models were built to adjust for potential confounders, including age, educational attainment, BMI, depression, nutritional status, and comorbidity burden (CIRS-G). All statistical analyses were conducted using Epi Info version 7.2.

Ethical considerations

Ethical approval for the study was granted by the Research Ethics Committee of the Almenara Hospital in Lima, Peru (approval code: 80-CIEI-OIyD-GRPA-ESSALUD-2023, dated 27 March 2023). All patient information was treated confidentially and in accordance with ethical standards.

Results

In total, 352 patients who met the eligibility criteria were included in the analysis. The mean age was 75.7 years (SD: 7.2), with 169 (48.0%) males and 183 (52.0%) females. Males had significantly more years of formal education (p<0.05), while depression symptoms were more commonly observed among females (p<0.05) (Table 1).

The distribution of cancer types was as follows: colorectal (23.0%), stomach (9.1%), prostate (8.5%), lung and pancreatic (both 7.4%), breast (6.8%), biliary tract (5.7%), liver (5.1%), non-Hodgkin lymphoma (4.8%), skin (4.8%), head and neck (4.0%), endometrial, ovarian and kidney cancers (each 2.6%), multiple myeloma (1.4%), leukemia (1.2%), bladder (0.6%) and other types (2.5%).

Cognitive impairment, defined as an MMSE score below 24, was present in 24.8% of the total sample. The prevalence was almost identical between sexes: 24.9% in men and 24.7% in women (p=0.545). Probable sarcopenia, based on the strength cutoffs, was significantly more common among men (67.5%) than females (51.4%) (p=0.0022) (Table 1).

Linear regression analysis demonstrated that for each 1 kg increase in HGS, MMSE scores increased by an average of 0.29 points in women (95% CI: 0.18–0.40; p<0.001) and 0.13 points in men (95% CI: 0.04–0.23; p=0.005). Pearson's correlation coefficients indicated a moderate positive association between HGS and cognitive scores in females (r=0.36, p<0.001) and a weaker but significant correlation in men (r = 0.22, p = 0.005).

Table 1. Patient characteristics[a]

	Total (n=352)	Male (n=169; 48%)	Female (n=183; 52%)	p
Age (years), mean (SE)	75.7 (7.2)	76.3 (7.3)	75.1 (7.2)	NS
Handgrip strength (kg), mean (SE)	19.1 (7.2)	23.7 (6.6)	14.9 (4.6)	<0.001
Probably sarcopenia n (%)*		114 (67.5%)	94 (51.4%)	<0.001
MMSE, mean (SE)	24.8 (3.9)	24.9 (4.1)	24.7 (3.7)	NS
Cognitive disorder (MMSE <24), n (%)	105 (29.8%)	47 (27.8%)	58 (31.7%)	NS
Body mass index (BMI) (kg/m^2), mean (SE)	24.7 (4.6)	24.6 (4.2)	24.9 (5.0)	NS
Years of education (years), mean (SE)	9.7 (4.4)	10.4 (4.2)	9.2 (4.5)	<0.05
Malnutrition MNA SF score (<8); n(%)	112 (31.8)	58 (34.3)	54 (29.5%)	NS
Depression (criteria DSM IV), n (%)	52 (14.8%)	18 (10.7%)	34 (18.6)	NS
CIRS-G Total score >10, n (%)	28 (8.0)	12 (7.1%)	16 (8.7%)	NS
CIRS-G Total score mean (SD)	5.6 (3.1)	5.7 (2.9)	5.5 (3.2)	NS
Katz Index of ADL (score < 6/6), n (%)	101 (28.7%)	47 (27.8%)	54 (29.5%)	NS

[a] * – handgrip strength: <27 kg in men and <16 kg in women, SE – standard deviation, MMSE – Mini-Mental State Examination, CIRS-G – Cumulative Illness Rating Scale-Geriatric, ADL –Activities of Daily Living

Table 2. Correlation between hand grip strength and cognitive function in older adult patients living with cancer: adjusted multivariate regression analysis in female patients[a]

Variable	Coefficient	95% Confidence	Limits	Std Error	F-test	p
Handgrip strength (kg)	0.193	0.082	0.305	0.057	11.654	<0.001
Years of education	0.259	0.153	0.365	0.054	23.2714	<0.001
CIRS-G Total score	-0.285	-0.448	-0.122	0.082	11.9605	NS
Depression (criteria DSM IV) (True/False)	-1.598	-2.836	-0.36	0.627	6.4876	NS
Body mass index (BMI)	-0.065	-0.171	0.041	0.054	1.4605	NS
Malnutrition (MNA SF score <8)	0.027	-0.172	0.226	0.101	0.0726	NS

[a] CIRS-G Cumulative Illness Rating Scale-Geriatric

Table 3. Correlation between hand grip strength and cognitive function in older adult patients living with cancer: adjusted multivariate regression analysis in male patients[a]

Variable	Coefficient	95% Confidence	Limits	Std Error	F-test	p
Handgrip strength (kg)	0.114	0.03	0.198	0.042	7.2164	<0.05
Years of education	0.324	0.199	0.449	0.063	26.2789	<0.001
CIRS-G Total score	-0.145	-0.34	0.049	0.098	2.1793	NS
Depression (criteria DSM IV) (True/False)	-3.027	-4.816	-1.238	0.906	11.1642	<0.05
Body mass index (BMI)	0.046	-0.098	0.191	0.073	0.3985	NS
Malnutrition (MNA SF score <8)	0.164	-0.067	0.395	0.117	1.9682	NS

[a] CIRS-G Cumulative Illness Rating Scale-Geriatric

Multivariate linear regression further confirmed these associations. Among female participants, both handgrip strength ($p<0.001$) and education ($p<0.001$) were independently associated with MMSE scores (Table 2). In male patients, handgrip strength ($p<0.05$), educational level ($p<0.001$), and presence of depression ($p<0.05$) were significant predictors of cognitive performance (Table 3).

Discussion

In a cohort of older Peruvian adults with cancer, this study found a positive correlation between handgrip strength (HGS) and cognitive function. To our knowledge, this is the first investigation to examine this relationship in a Latin American population of geriatric oncology.

Although only one prior study has directly addressed this topic, carried out in the United States with the National Health and Nutrition Examination Survey (NHANES)[11] – our findings are largely consistent. That earlier study by Yang et al. demonstrated a significant link between muscle strength and cognitive outcomes, but only in female participants. In contrast, our analysis revealed a statistically significant correlation in both men and females, although the strength of the association was greater in females. Several methodological differences may explain the observed discrepancies. For example, Yang et al. used the Animal Fluency Test (AFT)[22] and the Digit Symbol Substitution Test (DSST)[23], which assess executive function and processing speed. Our study utilized the Mini-Mental State Examination (MMSE), which evaluates a broader set of cognitive domains. Moreover, the US study included only certain types of cancer (eg breast, prostate, colon, and cervical), while our cohort encompassed a wider variety of malignancies. The cut-off values for sarcopenia also varied; our study used the EWGSOP2 criteria (<27 kg for men, <16 kg for women), while Yang et al. applied higher thresholds. The stronger correlation observed in women may be partly explained by two key factors: a higher prevalence of probable sarcopenia and a lower average level of formal education compared to men. Both factors are known to influence cognitive vulnerability and reserve.

Previous research in non-cancer populations has also reported a consistent association between reduced grip strength and lower cognitive scores, particularly on the MMSE.[24] Some authors have suggested that HGS can serve as a clinical indicator of cognitive decline in older adults.[25] Physiologically, the link between muscle function and cognition may be mediated by mechanisms such as myokine release, inflammatory signaling, or neurovascular changes.[26,27,28,29] For example, individuals with lower HGS have been shown to exhibit higher volumes of cerebral white matter hyperintensities, commonly associated with cognitive impairment.[30] Additionally, studies have demonstrated that unilateral handgrip exercises can influence brain connectivity and reduce reaction times in the contralateral limb, possibly through increased cortical excitability and altered GABAergic inhibition. These findings support the hypothesis of a bidirectional "muscle-brain" interaction.[31]

Cognitive reserve, the brain's resilience against damage, is shaped by factors such as education, socioeconomic status and engagement in cognitive and physical activities.[32] In our sample, women had lower education levels and higher rates of sarcopenia, which can reflect a lower cognitive reserve compared to men. These characteristics, combined with an older average age and diverse cancer diagnoses, make this population particularly vulnerable to cognitive decline.[33]

Recognizing the association between HGS and cognition can have practical implications. Interventions that target muscle strength, such as resistance training, may not only enhance physical function but also support cognitive health in older cancer patients.

Study limitations

This study has several limitations that must be considered. First, its retrospective and observational design prevents the establishment of causality between handgrip strength and cognitive performance. Furthermore, we lacked baseline data on cognitive function or muscle strength before cancer diagnosis or treatment, which limits the ability to assess longitudinal changes. Inclusion of patients with various cancer types of cancers may have introduced heterogeneity, as different malignancies and treatment regimens could have differential impacts on cognitive and physical function. Lastly, information regarding specific cancer therapies received by participants (eg chemotherapy, radiotherapy, hormonal therapy) was not available, which could represent a confounding factor.

Conclusion

In general, the findings revealed a meaningful positive correlation between handgrip strength and cognitive performance in older people with cancer, indicating that lower muscle strength may be associated with diminished cognitive abilities in this group. Given that the strength of the handgrip is a modifiable factor, future prospective studies should explore whether targeted interventions to improve muscular function could also benefit cognitive outcomes. Incorporating routine grip strength into geriatric oncology practice may help identify patients at risk of cognitive decline and guide comprehensive care strategies.

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Declarations

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Author contributions
Conceptualization, T.J.O.; Methodology, R.R.O.; Validation, E.C.M.; Formal Analysis, T.J.O.; Investigation, E.C.M.; Resources, T.J.O.; Data Curation, E.C.M.; Writing – Original Draft Preparation, T.J.O.; Writing – Review & Editing, T.J.O. and R.R.O.; Visualization, T.J.O.; Supervision, R.R.O.; Project Administration, T.J.O.

Conflicts of interest
The authors declare that they have no conflicts of interest.

Data availability
Due to institutional privacy regulations, the datasets used and analyzed in this study are not publicly accessible. Nevertheless, the corresponding author may provide them on a justified request and with the necessary ethical clearance.

Ethics approval
Ethical approval for this study was obtained from the Institutional Research Ethics Committee of Almenara Hospital in Lima, Peru (approval No. 80-CIEI-OI-yD-GRPA-ESSALUD-2023; March 27, 2023). All data was managed in accordance with national ethical regulations.

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ORIGINAL PAPER

Sex differences in hypertension prevalence and risk factors in India – a comparative study based on National Family Health Survey IV and V

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ABSTRACT

Introduction and aim. Hypertension is increasing universally, mainly in developing countries like India. This study analyzed sex differences in hypertension using data from National Family Health Survey (NFHS) rounds IV and V, with a focus on prevalence trends and associated risk factors.

Material and methods. This study utilized data from NFHS rounds IV and V, focusing on males and females aged 15–49 years. The samples included 103,525 males and 667,258 females in NFHS-IV, and 93,267 males and 695,707 females in NFHS-V. Univariate, bivariate, and multivariate analytical techniques were employed to address the study's objectives.

Results. The NFHS-IV and V data revealed a notable increase in hypertension prevalence across India, with significantly higher odds observed among individuals in the older reproductive age group (45–49 years) for both sexes. Hypertension occurred 4.85 times higher among male in the age group 45–49 compared to 15-24 age group, which rose to 5.23 in NFHS-V. Among female, the odds increased from 5.39 in NFHS-IV to 6.40 in NFHS-V. Remarkably, illiterate male showed lower odds of hypertension linked to their educated peers in both survey rounds, while female with only primary education showed higher odds. Regional disparities were also evident, with both male and female from the Northeast showing elevated odds – particularly female, who had an odds ratio of 1.47 in NFHS-IV.

Conclusion. The observed sex-specific variations in hypertension and its risk factors indicate a need for public health strategies to designed for each gender. Tailored interventions addressing education, lifestyle behaviors, and regional disparities are essential to effectively manage and prevent hypertension in India's diverse population.

Keywords. hypertension, India, NFHS, prevalence, risk Factors

Introduction

The global hypertension problem, often called high blood pressure (HBP), is responsible for many cases of cardiovascular disease, stroke, kidney failure, and premature death. HBP is repeatedly called the "silent killer" since it is not diagnosed until the condition develops very serious. Hypertension claims the lives of nearly 9.4 million individuals each year and most significant modifiable factors for both sickness and death around the globe.[1] In low- and middle-income countries, adult hypertension cases worldwide were expected to exceed 1.2 billion in 2021.[2] The prevalence of hypertension is rising steadily in India due to the country's aging population, stress, sedentary lifestyles, poor dietary habits, and

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speedy urbanization. The prevalence of high blood pressure in India is estimated at about 30%.[3] Factors such as access to healthcare, regional disparities, and socioeconomic status differences all contribute to this growing trend.[4]

Worldwide, there is growing interest in the disparities between male and female in the etiology, pathophysiology, and management of hypertension. Biological, hormonal, and behavioral factors manifest hypertension in both males and females. For example, due to the protective properties of estrogen, the premenopausal female usually has lower blood pressure than males of the same age. Based on previous studies in post-menopausal women, this condition is likely to opposite, and elderly females are more likely to have hypertension.[5] Some studies have shown that lifestyle factors like smoking and drinking affect hypertension in males compared to females, while females may face obstacles when it comes to accessing and receiving healthcare, particularly in rural areas.[6,7]

A study has shown that higher levels of salt sensitivity and arterial stiffness raise the risk of cardiovascular disease in postmenopausal females.[8] Research has demonstrated that sex hormones control endothelial, oxidative stress, and inflammation – all of which are important pathways in the etiology of hypertension.[9] Furthermore, sex-specific gene expression profiles that affect blood pressure regulation have been discovered by recent genetic and epigenetic research. This implies that the biological causes of hypertension differ between the sexes.[10]

Social and behavioral elements are just as significant as biological ones. Males are more likely than females to smoke, drink too much alcohol, and not exercise, all of which raise the risk of hypertension.[11] However, because of sociocultural norms, low health literacy, and gender bias in clinical settings, females – particularly those in low- and middle-income countries – frequently face a systemic lack of accessing health care, under-diagnosis, and undertreatment.[12,13]

A global review by Wang et al. showed that women are just as likely or more likely than males to have bad cardiovascular outcomes, but they are less likely to get antihypertensive treatment that follows guidelines, especially in older people.[14] The World Health Organization's INTERHEART study and the STEPS survey also found that females in many areas, including South Asia and sub-Saharan Africa, are less likely to know about high blood pressure and how to treat and control it. This shows that health strategies need to be more gender-sensitive.[9,15]

Studies highlighted these gender-specific trends in hypertension, emphasizing the need for sex-specific strategies in prevention and management.[8,9] In the Indian context, socio-cultural factors, delayed childbearing, hormonal transitions, and rising obesity rates among urban female are influencing the shifting hypertension burden. Additionally, the combined effect of genetic predisposition and environmental exposures is now recognized as a critical driver of hypertension risk in both sexes.[16,17]

Moreover, recent advancements in precision medicine and global health research highlight the importance of integrating sex as a biological variable in both epidemiological surveillance and clinical practice.[18-20] The Lancet Commission on Female and Cardiovascular Disease emphasized that the historical under representation of females in cardiovascular research has led to gaps in diagnostic accuracy and therapeutic effectiveness for female patients.[21] Tailored interventions, such as community-based blood pressure screening programs that address women's unique health needs – including reproductive health milestones like pregnancy-induced hypertension – are gaining importance. In India, initiatives such as the National Program for Prevention and Control of Cancer, Diabetes, Cardiovascular Diseases, and Stroke (NPCDCS) offer a platform to incorporate such gender-sensitive approaches.[22] However, for these efforts to succeed, there must be a stronger emphasis on disaggregated data analysis, targeted health messaging, and improved access to antihypertensive care for females across diverse socio-economic backgrounds.

Evaluating variations in hypertension prevalence in India can be done using surveys like the National Family Health Survey (NFHS). The latest datasets from 2015–2016 and 2019–2021 give the blood pressure levels and associated risk factors in male and female between the ages of 15 and 49. These datasets are strong, but there is not adequate research on the evaluation of hypertension prevalence and its risk factors in both rounds together by sex. Knowledge about these differences is necessary for making public health interventions that work for the targeted population. Both males and females come across different sets of problems, including the way they eat, stress, using tobacco or alcohol, and unequal healthcare. Moreover, gender can affect decisions about treatment and make it more difficult to manage high blood pressure.

Aim

This study aims to compare the occurrence of hypertension and hypertension linked factors in Indian males and females by using two rounds of NFHS-IV and V datasets to understand socio-demographic and behavioral factors and offer sex-sensitive strategies for hypertension prevention and management in an Indian context.

Material and methods

The NFHS data is a comprehensive, multi-phase survey implemented by the Ministry of Health and Family Welfare (MoHFW) in India and overseen by the International Institute of Population Sciences (IIPS), Mumbai. This analysis draws on data from the fourth (2015–2016) and fifth (2019–2021) rounds of the NFHS. As part of the Demographic and Health Survey (DHS) program, the NFHS gathers important data on health, fertility, family planning, mortality, and nutritional status across the Indian population.[23]

Sampling design

Both NFHS-IV and NFHS-V employed a two-stage stratified sampling method. Villages were in rural areas (primary sampling units (PSUs)), whereas census enumeration blocks (CEBs) were used in urban areas. In the primary stage, a predetermined number of PSUs were nominated from each stratum using probability proportional to size (PPS) sampling. In the second stage, 22 households were systematically and randomly selected from each PSU to join in the survey. NFHS-IV covered 640 districts in 29 states and 7 union territories, collecting data from 699,686 females aged between 15 and 49 years and 101,839 males aged 15 to 54 years. NFHS-V expanded the survey to 707 districts in 28 states and 8 union territories, surveying 724,115 females and 112,122 males in the same age group. The analysis used individual recoding files for males and females separately and combined them to examine sex differences. Only respondents with complete blood pressure measurements and valid demographic covariates were included in the final analysis. For this analysis, males aged 15–49 years were selected to match the female age group, enabling better age comparison between sexes. The samples comprised 103,525 males and 667,258 females in NFHS-IV, and 93,267 males and 695,707 females in NFHS-V (Fig. 1). Both surveys provide estimates at the state and district level, disaggregated by rural or urban residence and other key socio-demographic factors. Structured questionnaires were administered through computer-assisted personal interviews (CAPI) by trained enumerators. The NFHS tools include four core questionnaires: Household, Woman's, Man's, and Biomarker. More information refers to NFHS-IV and V reports.[23]

Blood pressure measurement and inclusion criteria

Blood pressure measurements were performed as part of the biomarker component of the survey using an Omron digital automated blood pressure monitor (model HEM-8712 in NFHS-IV and newer model in NFHS-V). Trained researchers followed a standardized protocol: respondents were asked to rest in a seated position for at least five minutes. Three measurements were then taken at approximately five-minute intervals. The average of the last two measurements was used for analysis, in accordance with WHO guidelines and the DHS protocol. [1,23] In particular, the NFHS measurement protocol also complies with the methodological standards of the International Society of Hypertension's "Measurement Month in May" initiative (2017–2019), which aims to standardize hypertension screening worldwide and improve data equivalence across populations.[24]

Variable descriptions

Outcome variable

For each participant, blood pressure (BP) was measured three times at five-minute intervals using an OMRON digital monitor by trained health personnel. The mean of the last two readings was taken as the ultimate BP value. Participants were considered hypertensive if their average systolic BP was ≥140 mmHg, their average diastolic BP was ≥90 mmHg, or if they reported current use of prescribed antihypertensive medication. For analytical purposes, a binary variable was generated, with hypertensive individuals coded as one and not a hypertensive individuals coded as zero in both NFHS-IV and NFHS-V.[23]

Independent variables

In this study, we included socio-demographic variables as covariates to categorize key risk factors related with hypertension. Age groups were divided into four groups: 15–24, 25–34, 35–44, and 45–49 for males and females in NFHS-IV and NFHS-V. Additional socio-demographic variables measured such as education level (primary, no education, secondary, or higher), place of residence (rural or urban) and occupation (unemployed, employed, or engaged in agriculture). Marital status was classified as unmarried or married, while religious affiliation included Hindus, Muslims, or others. Caste categories included Scheduled Castes/Scheduled Tribes (SC/ST), Other Backward Classes (OBC), and others. Wealth status was measured using the Wealth Index, categorized into five groups: poorest, poorer, middle, richer, and richest. Additionally, lifestyle factors incorporated into the analysis included alcohol consumption (yes or no), tobacco use (yes or no), and geographical region (central, north, east, northeast, west, and south).

Statistical analysis

The analysis incorporated univariate, bivariate, and multivariable logistic regression methods. Descriptive analysis was used to summarize participant appearances. Bivariate analyses assessed the relationship between explanatory variables and outcome variable, using Pearson's Chi-square test for associations and the z-test to compare differences between NFHS-IV and NFHS-V.

To examine predictors of hypertension, a multivariable binary logistic regression model was applied, with results presented as adjusted odds ratios (AORs) and 95% confidence intervals (CIs). Sampling weights were accounted for in all analyses, and statistical significance was set at a two-tailed p-value<0.05. Data analysis was performed using IBM SPSS version 19.0.

Bivariate technique

A cross-tabulation was projected to recognize the occurrence of prevalence of high blood pressure in each characteristic and Pearson's χ2 test was estimated to know the association of factors with hypertension. The following formula has been used to estimate Pearson's chi-square test in this study.

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i} \text{ with } (r-1)(c-1) \text{ degrees of freedom}$$

Where,

O_i = observed number of samples

E_i = expected number of samples

The z-test for two independent samples is estimated to know the change of prevalence from round 4 to round 5. The following formula has been used to estimate z-test for two independent samples in this study.

$$z = \frac{x_1 - x_2}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}} \text{ with } (n_1 + n_1 - 2) \text{ degrees of freedom}$$

Where,

$\overline{x}_1$ = sample average of the first sample

$\overline{x}_2$ = sample average of the second sample

SD_1^2 = sample variance of the first sample

SD_2^2 = sample variance of the second sample

n_1 = total number of observations in first samples

n_2 = total number of observations in second samples

Multivariate technique

Binary logistic regression was employed to estimate the likelihood of hypertension across different categories of explanatory variables. In this model, the dependent variable was binary (hypertensive vs. non-hypertensive), while the independent variables included both categorical and continuous predictors. The following binary logistic regression framework was applied in this study.

$$log\left(\frac{p}{1-p}\right) = \alpha + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n$$

Let $f(x) = \alpha + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n$

Where,

$$p = \frac{e^{f(x)}}{1 + e^{f(x)}} \quad \& \quad q = 1 - p$$

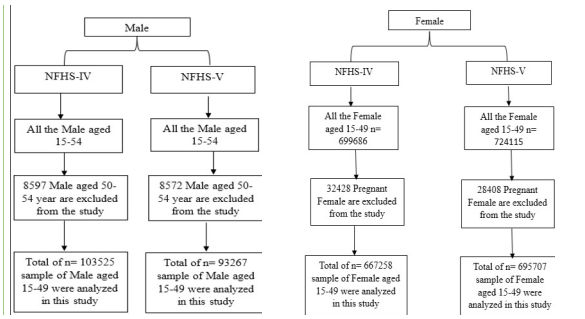

Fig. 1. Assortment of study participants from both NFHS IV and V for male and female samples

Results

The prevalence of hypertension in India has increased from NFHS-IV to NFHS-V for both males and females. In NFHS-IV, 15.5% of males and 12.2% of females had hypertension, which rose to 16.4% for males and 13.3% for females in NFHS-V (Fig. 2). Background characteristics of males and females from NFHS-IV and NFHS-V are presented in Table 1.

The highest increases were observed in the 35–44 and 45–49 age groups. Among females aged 35–44, the prevalence rose from 19.8% to 21.3%, while in the 45–49 age group, it increased from 28.1% to 31.0%. Similar patterns were observed among males in both rounds. For instance, in the 35–44 age group, the prevalence increased from 24.3% in Round IV to 26.7% in Round V; and in the 45–49 group, it rose from 30.2% to 33.7%. Hypertension prevalence also increased among both males and females in urban and rural areas. In urban areas, it rose from 13.4% to 14.6% among females and from 18.8% to 19.8% among males. In rural areas, it increased from 12.4% to 13.4% among females and from 15.5% to 17.3% among males. Increases were also observed across education levels and lifestyle factors. Among males with no formal education, the prevalence rose from 17.3% to 20.2%, while among females in the same category, it increased from 16.2% to 19.3% (Table 2 and Table 3).

The analysis of hypertension among males and females in India reveals significant associations with various demographic and socioeconomic characteristics. For males, age emerged as a critical factor, with those aged 45–49 displaying the highest adjusted odds ratio (AOR) of hypertension (AOR=4.85, p<0.001, 95% C.I: 4.47–5.25) in NFHS-IV and (AOR=5.23, p<0.001, 95% C.I 4.81–5.68) in NFHS-V. Education also played a role, as illiterate males had lower odds of hypertension (AOR=0.81, p<0.001, 95% C.I: 0.75–0.87in NFHS-IV and AOR=0.85, p<0.001, 95% C.I: 0.79–0.92 in NFHS-V) compared to their higher educated counterparts. Geographically, males in the Northeast exhibited higher odds (AOR=1.29, p<0.001, 95% C.I: 1.21–1.08 in NFHS-IV and AOR=1.08, p<0.001, 95% C.I:

1.01–1.16 in NFHS-V). Married males were more likely to have hypertension (AOR=1.22, p<0.001, 95% C.I: 1.15–1.30 in NFHS-IV and AOR=1.21, p<0.001, 95% C.I: 1.14–1.28 in NFHS-V). Additionally, unemployed and agriculture occupation of males showed lower odds (AOR=0.91, p<0.001, 95% C.I: 0.85–0.96 and AOR=0.89, p<0.001, 95% C.I: 0.86–0.93 in NFHS-IV and (AOR=0.81, p<0.001, 95% CI: 0.75–0.87 and AOR=0.88, p<0.001, 95% C.I: 0.84–0.92 in NFHS-V). Also, the poorest males had reduced odds of hypertension (AOR=0.63, p<0.001, 95% C.I: 0.58–0.68 in NFHS-IV and AOR: 0.72, p<0.001, 95% C.I: 0.66–0.77 in NFHS-V) compared to the richest. Alcohol consumption was significantly associated with increased odds of hypertension among males (AOR: 1.34, p<0.001, 95% C.I: 1.29–1.39 in NFHS-IV and AOR=1.44, p<0.001, 95% C.I: 1.38–1.50 in NFHS-V) (Table 4).

Among females, those aged 45–49 had the highest odds of hypertension (AOR=5.39, p<0.001, 95% CI: 5.21–5.57 in NFHS-IV and AOR=6.40, p<0.001, 95% C.I: 1=6.18–6.63 in NFHS-V). Notably, rural females initially had higher odds (AOR=1.05, p<0.001, 95% C.I: 1.03–1.07 in NFHS-IV), but this trend reversed in NFHS-V (AOR=0.98, p<0.001, 95% C.I: 0.98–1.00). Muslim females showed increased odds in NFHS-IV (AOR=1.22, p<0.001, 95% C.I: 1.19–1.25), while females from other religious groups had higher odds in NFHS-V (AOR=1.22, p<0.001, 95% C.I: 1.22–1.25). Females who have a primary education exhibited elevated odds of hypertension (AOR=1.28, p<0.001, 95% C.I: 1.24–1.33 in NFHS-IV and AOR=1.42, p<0.001, 95% C.I: 1.29–1.39 in NFHS-V). Regionally, women in the Northeast had higher odds in NFHS-IV (AOR: 1.47, p<0.001, 95% C.I: 1.43–1.51), higher risk were shifted to the Central region in NFHS-V (AOR: 1.11, p<0.001, 95% C.I: 1.09–1.14). Married females also had significantly higher odds (AOR=1.16, p<0.001, 95% C.I: 1.12–1.19 in NFHS-IV and AOR=1.24, p<0.001, 95% C.I: 1.20-1.28 in NFHS-V). Alcohol consumption was consistently linked to a higher risk of hypertension (AOR=1.43, p<0.001, 95% C.I: 1.37–1.49 in NFHS-IV and AOR=1.41, p<0.001, 95% C.I: 1.35–1.48 in NFHS-V) (Table 5).

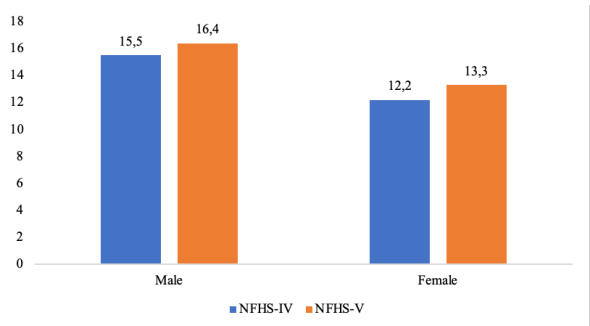

Fig. 2. Prevalence of hypertension among males and females in India, NHFS IV and V

Table 1. Background characteristics of males and females in NFHS-IV and NFHS-V

Variables	NFHS-IV		NFHS-V	
	Male	Female	Male	Female
	n (%)	n (%)	n (%)	n (%)
Age-group				
15–24	35712 (34.5)	230412 (34.5)	31070 (33.3)	227011 (32.6)
25–34	30791 (29.7)	198657 (29.8)	27652 (29.6)	206660 (29.7)
35–44	25851 (25)	165591 (24.8)	23712 (25.4)	178024 (25.6)
45–49	11171 (10.8)	72598 (10.9)	10833 (11.6)	84012 (12.1)
Residence				
Urban	32771 (31.7)	196917 (29.5)	24211 (26)	173942 (25)
Rural	70754 (68.3)	470341 (70.5)	69056 (74)	521765 (75)
Religion				
Hindu	77115 (74.5)	496025 (74.3)	70608 (75.7)	525869 (75.6)
Muslim	14437 (13.9)	89340 (13.4)	11317 (12.1)	86140 (12.4)
Others	11973 (11.6)	81893 (12.3)	11342 (12.2)	83698 (12)
Caste				
SC/ST	36883 (37.7)	239513 (35.9)	35496 (40)	263550 (40)
OBC	40181 (41.1)	260754 (39.1)	35991 (40.6)	266321 (40.4)
Others	20666 (21.1)	166991 (25)	17230 (19.4)	128850 (19.6)
Education				
No education	12593 (12.2)	188407 (28.2)	9980 (10.7)	162651 (23.4)
Primary	12684 (12.3)	84010 (12.6)	10169 (10.9)	81896 (11.8)
Secondary	61706 (59.6)	318853 (47.8)	56197 (60.3)	354007 (50.9)
Higher	16542 (16)	75988 (11.4)	16921 (18.1)	97153 (14)
Region				
North	22855 (22.1)	134495 (20.2)	19565 (21)	142028 (20.4)
Central	25964 (25.1)	175810 (26.3)	21452 (23)	163067 (23.4)
East	15864 (15.3)	119723 (17.9)	13887 (14.9)	112963 (16.2)
Northeast	13360 (12.9)	94145 (14.1)	13540 (14.5)	98754 (14.2)
West	11434 (11)	54157 (8.1)	10606 (11.4)	69481 (10)
South	14048 (13.6)	88928 (13.3)	14217 (15.2)	109414 (15.7)
Marital status				
Unmarried	39869 (38.5)	171704 (25.7)	36754 (39.4)	181211 (26)
Married	63656 (61.5)	495554 (74.3)	56513 (60.6)	514496 (74)
Wealth index				
Poorest	17035 (16.5)	125566 (18.8)	18151 (19.5)	142912 (20.5)
Poorer	21584 (20.8)	141850 (21.3)	20823 (22.3)	153749 (22.1)
Middle	22604 (21.8)	140571 (21.1)	19928 (21.4)	145760 (21)
Richer	21516 (20.8)	132927 (19.9)	18494 (19.8)	134521 (19.3)
Richest	20786 (20.1)	126344 (18.9)	15871 (17)	118765 (17.1)
Consumes tobacco				
No	67173 (64.9)	610220 (91.5)	57234 (61.4)	651086 (93.6)
Yes	36352 (35.1)	57038 (8.5)	36033 (38.6)	44621 (6.4)
Consumes alcohol				
No	71296 (68.9)	650548 (97.5)	69552 (74.6)	682532 (98.1)
Yes	32229 (31.1)	16710 (2.5)	23715 (25.4)	13175 (1.9)
Occupation				
Unemployed	24170 (23.4)	24170 (23.4)	18870 (20.3)	18870 (20.3)
Employee	49916 (48.3)	49916 (48.3)	45411 (48.8)	45411 (48.8)
Agriculture	29242 (28.3)	29242 (28.3)	28758 (30.9)	28758 (30.9)
Body mass index				
Normal	59880 (61.2)	387734 (59.2)	24253 (57.4)	401557 (59.8)
Underweight	19166 (19.6)	146439 (22.4)	8903 (21.1)	121992 (18.2)
Overweight and obese	18751 (19.2)	120983 (18.5)	42269 (21.5)	148195 (22.1)

Table 2. Prevalence of hypertension and its association with selected background characteristics of male in NFHS-IV and NFHS-V[a]

Variables	NFHS-IV		NFHS-V		z-test
	Prevalence (%)	χ^2 (p-value)	Prevalence (%)	χ^2 (p-value)	
Age group					
15–24	6.7	5217.1	6.9		1.024
25–34*	15.7	(<0.05)	16.6	5688.5	2.95
35–44*	24.3		26.7	(<0.05)	6.122
45–49*	30.2		33.7		5.569
Type of place of residence					
Urban*	18	99.9	19.8	74.2	5.412
Rural*	15.5	-0.05	17.3	(<0.05)	9.088
Religion					
Hindu*	16	241.4	17.8	326.9	9.216
Muslim	14.2	(<0.05)	13.6	(<0.05)	1.383
Other*	20.9		22.8		3.509
Caste					
SC/ST*	16.7	107.9	18.8	48.8	7.391
OBC*	15	(<0.05)	16.9	(<0.05)	7.143
Others	18.2		18.8		1.497
Educational level					
No education*	17.3	150	20.2	132.8	5.529
Primary*	17.4	(<0.05)	20.9	(<0.05)	6.664
Secondary*	15.2		16.9		7.937
Higher	18.9		18.1		1.884
Region					
North	18.2	825	18.3		0.266
Central*	12.8	(<0.05)	17.3		13.588
East*	12.8		14.3	381.0	3.766
North East*	22.3		21.3	(<0.05)	1.986
West*	16.2		15		2.455
South*	18.1		20.9		5.945
Marital status					
Unmarried*	8.7	2723.3	9.1	3192.7	1.942
Married*	21.1	(<0.05)	23.7	(<0.05)	10.782
Occupation					
Unemployed*	9.4	1172.7	8.2		4.378
Employed*	19.3	(<0.05)	21.1	1578.9	6.91
Agriculture*	16.8		19.2	(<0.05)	7.524
Wealth index combined					
Poorest*	12.3	609.1	15.3	233	8.173
Poorer*	13.7	(<0.05)	16.7	(<0.05)	8.604
Middle*	16.2		18.1		5.182
Richer	18.5		19.1		1.531
Richest*	20.2		21.1		2.107
Consumes tobacco					
No*	15.7	59.9	16.4	249.0	3.35
Yes*	17.5	(<0.05)	20.4	(<0.05)	9.96
Consumes alcohol					
No*	14.1	806.2	15.3	1288.7	6.358
Yes*	21.2	(<0.05)	25.7	(<0.05)	12.369
Body mass index[$]					
Normal	14.2	609.1	21.9	796.6	-25.544
Underweight	6.7	(<0.05)	17.3	(<0.05)	-24.109
Overweight and obese	31.6		34.2		-4.320

[a] * – significant change in proportion variations among NFHS-IV and NFHS-V (using z-test) at 5% level of significance, % – indicates percentage change from NFHS-IV to V, χ^2 – indicate Chi-square value, ST – Scheduled Tribe, SC – Scheduled Caste and OBC – Other Backward Class, $ – based on the matched cases in NFHS-V dataset

Table 3. Prevalence of hypertension and its association with selected background characteristics of female in NFHS-IV and NFHS-V[a]

Variables	NFHS-IV		NFHS-V		z-test
	Prevalence		Prevalence		
Age-group					
15–24*	4.7		4.20		8.20
25–34	10.4	37404.4	10.40	49379.4	0
35–44*	19.8	(<0.05)	21.30	(<0.05)	10.88
45–49*	28.1		31.00		12.56
Place of residence					
Urban*	13.4	131.3	14.60	166.5	10.50
Rural*	12.4	(<0.05)	13.40	(<0.05)	14.85
Religion					
Hindu*	11.9		13.20	741.4	19.84
Muslim*	14.1	1054.2	13.40	(<0.05)	4.25
Other*	15.6	(<0.05)	16.70		6.08
Caste					
SC/ST*	12.7	610.1	13.70	207	10.47
OBC*	11.6	(<0.05)	13.00	(<0.05)	15.47
Other*	14.2		14.70		3.83
Education					
No education*	16.2	4315.7	19.30	8790.9	23.93
Primary*	15	(<0.05)	17.80	(<0.05)	15.40
Secondary*	10.8		11.40		7.82
Higher*	9.4		9.00		2.85
Region					
North*	13.3	2807.1	14.40	662.9	8.37
Central*	10.8	(<0.05)	13.50	(<0.05)	24.01
East*	11.1		12.00		6.78
North East*	17.4		15.50		11.24
West	12.4		12.60		1.05
South*	12.8		13.70		5.88
Marital status					
Unmarried*	5.1	11870.9	04.50	17248.9	8.32
Married*	15.3	(<0.05)	16.90	(<0.05)	21.88
Wealth index					
Poorest*	11.1	744.2	12.10	678.3	8.08
Poorer*	12	(<0.05)	13. 00	(<0.05)	8.21
Middle*	12.5		13.90		11.07
Richer*	13.8		14.50		5.19
Richest*	14.1		15.20		7.69
Consumes tobacco					
No*	12.3	845.6	13.30	1267.9	16.81
Yes*	16.5	(<0.05)	19.30	(<0.05)	11.52
Consumes alcohol					
No*	12.4	1182.7	13.50	1036.5	18.91
Yes*	21.4	(<0.05)	23.20	(<0.05)	3.70
Body mass index					
Normal*	11.1	23490.2	11.80	23797.7	9.76
Underweight	7	(<0.05)	7	(<0.05)	0
Overweight	25.7		25.80		0.59

[a] * – significant difference in percentage change between NFHS-IV and NFHS-V (using z-test) at 5% level of significance, % – indicates percentage change from NFHS-IV to V, χ^2 – indicate Chi-square value, SC – Scheduled Caste, ST – Scheduled Tribe, OBC – Other Backward Class, $ – based on the matched cases in NFHS-V dataset

Table 4. Results of multivariable logistic regression to measure the effect of covariates on hypertension among Indian males, NFHS-IV and NFHS-V[a]

Variables	Male			
	NFHS–IV		NFHS–V	
	Exp(B) (95% CI)	p	Exp(B) (95% CI)	p
Age group				
15–24	1		1	
25–34	2.08 (1.94–2.22)	<0.05	2.08 (1.94–2.24)	<0.05
35–44	3.55 (3.3–3.82)	<0.05	3.63 (3.37–3.92)	<0.05
45–49	4.85 (4.47–5.25)	<0.05	5.23 (4.81–5.68)	<0.05
Place of residence				
Urban	1		1	
Rural	1.03 (0.98–1.07)	0.26	0.97 (0.92–1.01)	0.15
Religion				
Hindu	1		1	
Muslim	0.95 (0.9–1.02)	0.14	0.8 (0.75–0.86)	<0.05
Other	1 .00 (0.94–1.07)	0.90	1.24 (1.17–1.32)	<0.05
Caste				
SC/ST	1		1	
OBC	0.93 (0.89–0.97)	<0.05	0.93 (0.89–0.97)	<0.05
Other	1.00 (0.95–1.05)	0.93	1.00(0.95–1.05)	0.93
Education				
Higher	1		1	
No education	0.81 (0.75–0.87)	<0.05	0.85 (0.79–0.92)	<0.05
Primary	0.86 (0.8–0.92)	<0.05	0.94 (0.87–1.01)	0.09
Secondary	0.87 (0.82–0.91)	<0.05	0.96 (0.91–1.01)	0.13
Region				
North	1		1	
Central	0.73 (0.69–0.77)	<0.05	1.07 (1.01–1.13)	<0.05
East	0.71 (0.67–0.76)	<0.05	0.79 (0.74–0.84)	<0.05
North East	1.29 (1.21–1.38)	<0.05	1.08 (1.01–1.16)	<0.05
West	0.86 (0.81–0.92)	<0.05	0.81 (0.76–0.87)	<0.05
South	0.89 (0.84–0.95)	<0.05	1.07 (1.01–1.14)	<0.05
Marital status				
Unmarried	1		1	
Married	1.22 (1.15–1.3)	<0.05	1.21 (1.14–1.28)	<0.05
Occupation				
Employed	1		1	
Unemployed	0.91 (0.85–0.96)	<0.05	0.81 (0.75–0.87)	<0.05
Agriculture	0.89 (0.86–0.93)	<0.05	0.88 (0.84–0.92)	<0.05
Wealth index				
Richest	1		1	
Poorest	0.63 (0.58–0.68)	<0.05	0.72 (0.66–0.77)	<0.05
Poorer	0.68 (0.63–0.72)	<0.05	0.79 (0.73–0.84)	<0.05
Middle	0.81 (0.76–0.86)	<0.05	0.85 (0.8–0.9)	<0.05
Richer	0.95 (0.9–1)	<0.05	0.91 (0.86–0.97)	<0.05
Consumes tobacco				
No	1		1	
Yes	0.95 (0.92–0.99)	0.02	0.97 (0.93–1.01)	0.11
Consumes alcohol				
No	1		1	
Yes	1.34 (1.29–1.39)	<0.05	1.44 (1.38–1.5)	<0.05

[a] CI – confidence interval, ST – Scheduled Tribe, SC – Scheduled Caste, OBC – Other Backward Class

Table 5. Results of multivariable logistic regression to measure the effect of covariates on hypertension among Indian females in India, NFHS-IV and NFHS-V[a]

Variables	NFHS–IV		NFHS–V	
	Exp(B) (95% CI)	p	Exp(B) (95% CI)	p
Age group				
15–24	1		1	
25–34	1.78 (1.73–1.84)	<0.05	1.91 (1.85-1.98)	<0.05
35–44	3.46 (3.35–3.56)	<0.05	3.93 (3.8-4.06)	<0.05
45–49	5.39 (5.21–5.57)	<0.05	6.4 (6.18-6.63)	<0.05
Place of residence				
Urban	1		1	
Rural	1.05 (1.03–1.07)	<0.05	0.98 (0.96-1)	0.05
Religion				
Hindu	1		1	
Muslim	1.22 (1.19–1.25)	<0.05	1.06 (1.03-1.08)	<0.05
Other	0.99 (0.96–1.01)	0.37	1.22 (1.19-1.25)	<0.05
Caste				
SC/ST	1		1	
OBC	0.94 (0.92–0.96)	<0.05	0.95 (0.94-0.97)	<0.05
GM	1.02 (1–1.04)	0.07	0.99 (0.97-1.01)	0.28
Education				
Higher	1		1	
No education	1.26 (1.22–1.3)	<0.05	1.38 (1.33-1.42)	<0.05
Primary	1.28 (1.24–1.33)	<0.05	1.42 (1.37-1.47)	<0.05
Secondary	1.16 (1.12–1.19)	<0.05	1.23 (1.19-1.26)	<0.05
Region				
North	1		1	
Central	0.88 (0.86–0.91)	<0.05	1.11 (1.09-1.14)	<0.05
East	0.91 (0.89–0.94)	<0.05	0.93 (0.9-0.95)	<0.05
North East	1.47 (1.43–1.51)	<0.05	1.03 (1-1.06)	0.08
West	0.96 (0.93–0.99)	<0.05	0.89 (0.86-0.91)	<0.05
South	0.87 (0.85–0.89)	<0.05	0.82 (0.8-0.84)	<0.05
Marital status				
Unmarried	1		1	
Married	1.16 (1.12–1.19)	<0.05	1.24 (1.2-1.28)	<0.05
Wealth index				
Richest	1		1	
Poorest	0.99 (0.96–1.03)	0.72	0.88 (0.86-0.91)	<0.05
Poorer	0.98 (0.95–1.01)	0.15	0.94 (0.91-0.97)	<0.05
Middle	0.96 (0.94–0.99)	0.01	0.98 (0.95-1)	0.1
Richer	1.02 (1–1.05)	0.12	1 (0.97-1.03)	0.98
Consumes tobacco				
No	1		1	
Yes	0.93 (0.91–0.95)	<0.05	1.06 (1.03-1.09)	<0.05
Consumes alcohol				
No	1		1	
Yes	1.43 (1.37–1.49)	<0.05	1.41 (1.35-1.48)	<0.05
Body mass index				
Normal	1		1	
Underweight	0.75 (0.73–0.77)	<0.05	0.79 (0.77-0.81)	<0.05
Obese	2.19 (2.16–2.23)	<0.05	2.05 (2.02–2.09)	<0.05

[a] CI – confidence interval, ST – Scheduled Tribe, SC – Scheduled Caste, OBC – Other Backward Class

Discussion

The findings from NFHS-IV and NFHS-V about hypertension trends in India highlight a concerning public health issue, with significant rises in prevalence among both males and females. Hypertension rates increased from 12.2% in NFHS-IV to 13.3% in NFHS-V, highlighting a broader trend that reflects the growing global concern over non-communicable diseases (NCDs) in developing countries.[1,24] Hypertension, a leading risk factor for cardiovascular diseases (CVDs), stroke, and kidney disease, has reached alarming levels – particularly in older populations. These findings align with global patterns, where hypertension prevalence increased with age, especially among individuals aged 35–49 years, as seen in both this study and existing worldwide literature.[1,24] This trend reflects deeper societal changes that are influencing lifestyle and health behaviors.

The rising prevalence of hypertension across all age groups is particularly concerning, with a notable increase among individuals aged 35–44 and 45–49 years. Our findings, consistent with existing literature, reveal that middle-aged adults are more susceptible to hypertension due to a combination of factors such as psychological stress, unhealthy dietary habits, physical inactivity, and environmental conditions.[25-28] However, these trends are not even across the sexes. Men in these age groups often exhibit higher hypertension rates, potentially related to more alcohol consumption, tobacco use, and occupational stress. In contrast, women – particularly those in the post-reproductive age group – may experience increased hypertension risk due to hormonal changes, rising obesity, and limited access to preventive healthcare.

The increase in hypertension prevalence in both urban and rural populations is a significant finding. In urban areas, prevalence rose from 13.4% in NFHS-IV to 14.6% in NFHS-V, while in rural areas, it increased from 12.4% to 13.4% over the same period. This urban-rural disparity reflects complex lifestyle and environmental dynamics and gender-related dynamics. Although urbanization often improves access to healthcare, it also brings lifestyle deviations such as increased consumption of processed foods, decreased physical activity, and elevated stress levels – all of which contribute to the increasing prevalence of hypertension.[28,29] However, the influence of these factors can vary by gender; for example, urban women may face unique challenges balancing work, household responsibilities, and limited opportunities for physical activity, increasing their vulnerability to hypertension. In contrast, rural populations – especially women – often confront additional barriers, including lower health literacy, limited autonomy in health decision-making, inadequate hypertension management, and insufficient healthcare infrastructure. Studies show that individuals in rural areas often experience delayed diagnosis and poor treatment adherence, with women disproportionately affected by these challenges, exacerbating hypertension-related health outcomes.[23,26,30]

The increasing prevalence of hypertension is strongly influenced by socioeconomic factors, such as income and educational attainment, with notable sex differences in how these factors affect hypertension risk. Hypertension prevalence was significantly higher among individuals with lower education levels, for both males and females; however, the impact tends to be more pronounced among women. For instance, the prevalence of hypertension among females with no education rose from 16.2% in NFHS-IV to 19.3% in NFHS-V, representing a strong correlation between education, awareness, and the prevention of hypertension. Lower educational attainment in women often limits their access to health information, decision-making autonomy, and healthcare utilization, thereby increasing their vulnerability to undiagnosed and poorly managed hypertension. In contrast, men with low education levels may experience higher hypertension prevalence partially due to increased occupational stress and lifestyle factors such as tobacco and alcohol use. Hypertension prevalence also increased among males with only primary education or no education, underscoring the importance of community outreach and tailored health education initiatives that address the different needs and challenges faced by men and women in managing hypertension.[24,31]

Higher rates of hypertension in both males and females were closely associated with lifestyle choices, particularly alcohol and tobacco use. Tobacco uses significantly raises blood pressure levels, as reflected in the data showing an increase in hypertension prevalence from 16.5% in NFHS-IV to 19.3% in NFHS-V.[4] Smoking accelerates the onset of hypertension by increasing vascular resistance and causing endothelial damage.[32] The importance of addressing modifiable risk factors is further underscored by the positive correlation between alcohol consumption and hypertension, which rose from 21.4% in NFHS-IV to 23.2% in NFHS-V. Research consistently shows that reducing alcohol and tobacco use through lifestyle interventions can substantially lower hypertension prevalence and improve public health outcomes.[28]

Regional variations in hypertension prevalence also interact with sex differences, influencing risk patterns differently for males and females. For example, lifestyle, dietary habits, and healthcare access can vary significantly by region and gender, affecting hypertension rates. In some regions, cultural norms and gender roles may limit women's access to healthcare or healthy food options, increasing their vulnerability to hypertension. Conversely, men in certain areas might face higher occupational stress or greater exposure to risk behaviors

such as tobacco and alcohol use, which elevate hypertension risk. These regional and gender-specific dynamics highlight the necessity for tailored interventions that consider both geographic and sex-based factors to effectively address hypertension across India.[29,30]

Urgent public health action is required due to the rising prevalence of hypertension in India, especially among older adults, people living in rural areas, and those with certain lifestyle factors like obesity, alcoholism, and tobacco use. Early detection, lifestyle modification, and improved access to treatment should be the main goals of interventions, particularly in underprivileged rural areas. Public health initiatives that highlight the importance of routine health screenings and hypertension prevention can play a crucial role in reducing its burden on the Indian population. While biological sex is a key factor in hypertension risk, it is equally important to recognize that sex often intersects with gender-based disparities in healthcare access and health-seeking behavior. These sociocultural barriers, particularly affecting female, may influence awareness, diagnosis, and management of hypertension, and must be measured in designing inclusive and equitable intervention strategies.

Conclusion

The NFHS-IV and NFHS-V data show that the prevalence of hypertension is rising in India, especially among middle-aged people, those living in rural areas, and those with certain risk factors like obesity, tobacco use, and low levels of education Notably, educational status influences hypertension risk differently for males and females—while illiterate males show lower odds, females with primary education are at greater risk, suggesting possible gaps in awareness and health-seeking behavior. Additionally, regional disparities, such as higher odds among individuals from the Northeast – especially females – indicate the importance of region-specific and gender-sensitive public health responses. These findings call for differentiated hypertension prevention and management strategies that address the unique social, educational, and regional determinants across genders.

Declarations

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Author contributions

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

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

The datasets analyzed during the current study are available in the Demographic and Health Survey repository (https://dhsprogram.com/data/).

Ethics approval

The study performed a secondary analysis and there was no identifiable information about the respondents. Hence, ethical approval was not required.

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ORIGINAL PAPER

Serum leptin and glucagon-like peptide-1 levels in diabetic patients with end-stage renal disease on hemodialysis – a cross-sectional study in Iraqi patients

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ABSTRACT

Introduction and aim. Diabetic kidney disease (DKD), a common complication of type 2 diabetes mellitus, is the leading cause of end-stage renal disease (ESRD). This study aimed to evaluate differences in serum leptin and glucagon-like peptide-1 (GLP-1) levels between diabetic ESRD patients undergoing hemodialysis and healthy controls.

Material and methods. A cross-sectional observational study was conducted involving 65 participants: 31 type 2 diabetic patients with ESRD on hemodialysis and 34 healthy controls. Serum leptin and GLP-1 concentrations were measured using enzyme-linked immunosorbent assays.

Results. Patients with ESRD exhibited significantly higher serum leptin levels (1.7±1.0 ng/mL) compared to controls (1.4±0.7 ng/mL; p=0.001), and significantly lower GLP-1 levels (19.6±11.2 pmol/L vs. 37.0±25.7 pmol/L; p=0.001).

Conclusion. Elevated leptin levels and reduced GLP-1 concentrations in diabetic ESRD patients suggest a potential role of these biomarkers in renal injury and metabolic regulation. The findings highlight the therapeutic promise of GLP-1 receptor agonists in this population.

Keywords. Chronic kidney disease, diabetic kidney disease, end-stage renal failure, glucagon-like peptide 1, hemodialysis, leptin

Introduction

The global prevalence of diabetes mellitus (DM) has dramatically increased, affecting approximately 11% of the world's population by 2021. A significant complication of diabetes, particularly type 2 diabetes mellitus (T2DM), is diabetic kidney disease (DKD), also known as diabetic nephropathy (DN), which develops in 20–50% of T2DM patients.[1] DKD is a leading cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) worldwide, significantly contributing to morbidity and mortality. It progressively impairs kidney function, beginning with glomerular hyperfiltration and advancing through stages marked by microalbuminuria and declining glomerular filtration rate (GFR),[2] ultimately leading to ESRD. CKD, defined as kidney damage or reduced function for over three months with health consequences, is cause, GFR (stages G1–G5), and albuminuria (A1–A3) levels (CGA classification).[3]

Leptin is an endocrine hormone secreted primarily by adipose tissue. It is involved in multiple metabolic processes, including regulation of body weight, food intake, and energy balance, as well as inflammatory and hemostatic pathways that contribute to cardiovascular and hypertensive disorders.[4] The relationship between leptin and renal disorders has been the subject of numerous investigations. Elevated leptin levels have been

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associated with hyperglycemia and increased blood pressure, the latter likely mediated by enhanced sympathetic nervous system activity, as well as renal impairment. Patients with CKD, particularly those with DKD, often exhibit higher serum leptin levels due to decreased renal clearance and increased adipose tissue synthesis.[5] Leptin promotes renal inflammation and fibrosis through pathways including transforming growth factor-beta (TGF-β) and other signaling methods, leading to glomerulosclerosis and proteinuria, hallmark features of DKD. Consequently, leptin is considered a potential marker for renal injury.[6] Elevated leptin levels have also been associated with endothelial dysfunction, which is characterized by increased collagen synthesis and mesangial cell hypertrophy, both of which can exacerbate kidney injury.[6,7]

The glucagon-like peptide-1 (GLP-1) is an essential hormone for controlling hunger and glucose metabolism. Because of its various physiological effects, GLP-1 has drawn interest for its medicinal potential in treating obesity, T2DM, and other disorders. The primary function of GLP-1 is the incretin effect, which helps maintain glucose homeostasis by increasing insulin release in response to meals.[8] Additionally, it decreases food intake, delays stomach emptying, and affects gut motility, all of which help with weight management and diabetes treatment. Beyond its metabolic roles, GLP-1 exhibits neuroprotective and cardioprotective effects, reduces inflammation, and influences cognitive functions such as memory and learning.[9] There is growing recognition of the link between GLP-1 hormone levels and CKD, especially concerning the protective role of GLP-1 receptor agonists (GLP-1 RAs) in CKD patients. It has been demonstrated that GLP-1 RAs reduce the incidence of major adverse kidney events in patients with CKD and type 2 diabetes by 18%.[10] To our knowledge, this study is among the first to assess the correlation between serum leptin and GLP-1 levels in diabetic patients with ESRD undergoing hemodialysis within the Iraqi population. Although prior research has examined these biomarkers across diverse populations, there is a shortage of data specifically from Iraq and analogous underrepresented areas. Our study offers region-specific insights into the hormonal profiles of this patient cohort, which may vary due to genetic, environmental, and lifestyle factors distinctive to this population. These results enhance the comprehension of the metabolic and inflammatory pathways associated with DKD in Iraqi patients and underscore the potential for formulating more individualized treatment approaches, including the application of GLP-1 receptor agonists, specifically designed to meet the requirements of this population. Ultimately, this research addresses a deficiency in the literature and advocates for the incorporation of diverse populations in forthcoming biomarker and therapeutic studies.

Aim

This study examines the potential differences in leptin and GLP-1 hormone concentration and their sensitivity and specificity for DKD patients on hemodialysis in Iraqi patients. This study offers a novel addition among the few undertaken in Al-Basrah, Iraq, offering population-specific insights into biomarker behavior in an underrepresented community.

Material and methods

This study was designed as an observational, cross-sectional analysis aimed at comparing leptin and GLP-1 levels in diabetic ESRD patients and healthy controls. This study was conducted in Basrah Teaching Hospital from January 2024 to April 2024. Demographic and laboratory data were collected. A total of 65 patients were included, of whom (n=31) end-stage renal disease patients with diabetes mellitus on hemodialysis were classified and defined according to the National Kidney Foundation KDOQI definition for chronic kidney disease.[11] Patients with an estimated glomerular filtration rate (eGFR) <15 mL/min/1.73 m² and were receiving maintenance hemodialysis. eGFR was computed by using the Modification of Diet in Renal Disease (MDRD) equation:[12]

$$\text{eGFR} = 186 \times [\text{serum Cr}] - 1.154 \times \times [\text{age}] - 0.203 \times [0.742 \text{ if women}]$$

Which is known to be more accurate. Patients undergoing chronic hemodialysis were eligible to participate in the study if they agreed to do so, and control participants were healthy volunteers without diabetes or kidney disease (n=34), with median ages of 57.5±7.6 and 41±10.6 years, respectively. The control group was younger on average (41±10.6 years) compared to the ESRD group (57.5±7.6 years). This age difference was statistically significant ($p<0.0001$). The lack of age matching represents a potential confounding factor and is acknowledged as a limitation of this study. Written consent was obtained from all participants. Serum samples from the participants were collected and stored at –80°C until analysis. Each group has made the following biomarkers: blood pressure, random blood sugar (RBS), glycated hemoglobin A1c (HbA1c), urea, creatinine, and eGFR. Leptin and GLP-1 levels were detected by using enzyme-linked immunosorbent assay kits (BT LAB, China), which have a sensitivity of 0.021 ng/mL and an inter-assay coefficient of variation of <10%, and a sensitivity of 1.27 pmol/L with an inter-assay coefficient of variation of <10%, respectively. The study protocols received approval from the ethics committees of the Basrah Teaching Hospital and the Basrah Dialysis Center.

Ethics approval

The study received approval from the Ethics Committee of the College of Pharmacy at the University of Basrah. (Protocol No. 248, dated December 15, 2023) and the Basrah Health Directorate (Protocol No. 74, dated January 27, 2024). All procedures were performed in compliance with the Declaration of Helsinki and any institutional rules. Informed written consent was acquired from all subjects before enrollment. Participants were notified of the study's objective and their entitlement to withdraw at any moment without repercussions, and the confidentiality of their personal data.

Inclusion criteria

Patients aged 30 to 70 of both genders suffering from renal failure, diabetic mellitus, and hypertension, were involved in the study.

Exclusion criteria

Pregnant women, breastfeeding, smokers, thyroid dysfunction, patients with a history of chronic liver disease, hepatitis type B and C, malignancy, rheumatoid arthritis, patients on dipeptidyl peptidase 4 (DPP-4) inhibitors agents, GLP-1 agonists agents, immunosuppressant agent, disease-modifying antirheumatic drug, kidney transplantation, and stroke were excluded from participating in this study.

Statistical analysis

The data were defined as means and medians. A t-test analysis (unpaired test) was conducted to compare continuous variables. Normality of data distribution was calculated using the Shapiro–Wilk test. Group comparisons were performed using a two-tailed unpaired t-test for normally distributed variables. A p-value <0.05 was considered statistically significant. Regression analysis was not performed due to sample size limitations, which is a limitation of this study. Also, no formal power calculation was performed due to the exploratory nature of this study.

Results

The ESRD group was significantly older than the control group (57.5±7.6 years vs. 41.0±10.6 years; $p < 0.001$). This age difference was not controlled for in the analysis and is acknowledged as a potential confounding factor. urea in the disease group shows significantly higher levels as compared to the control group (123.9±73.6 mg/dL vs 20.6±6.7 mg/dL, p<0.0001). Creatinine levels are significantly higher in the disease group compared to the control group (7±2.6 mg/dL vs 0.8±0.2 mg/dL, p<0.0001). The eGFR value in the disease group is significantly lower than in the control group (8.9±4 mL/min/1.73 m^2 vs 106.1±22.7 mL/min/1.73 m^2, p<0.0001). the HbA1c (%), the disease group shows significantly higher HbA1c% levels as compared to the control group (8.2±1 vs 5.3±0.6%, p<0.0001). The systolic blood pressure (SBP) levels in the disease group show significantly higher systolic values as compared to the control group (147.7±15.8 mmHg vs 121.1±3 mmHg, p<0.0001). The diastolic blood pressure (DBP) levels in the disease group are significantly higher than those in the control group (86.9±10.7 mmHg vs 80.7±1.9 mmHg, p<0.031). Leptin levels in the disease group show significantly higher leptin values as compared to the control group. (1.7±1ng/mL vs 1.4±0.7 ng/mL, p<0.001). Concerning GLP-1 levels, the disease group 1 shows significantly lower systolic value as compared to the control group (19.6±11.2 pmol/L vs 37±25.7 pmol/L, p<0.001, Table 1).

Table 1. Laboratory data (expressed as mean±SD)

Parameters	Disease group (n=31)	Control group (n=34)	p-value	Mean difference (95% CI)	Cohen's d
Urea (mg/dL)	123.9±73.6	20.6±6.7	<0.0001	+103.3 (77.3 to 129.3)	2.03
Creatinine (mg/dL)	7±2.6	0.8±0.2	<0.0001	+6.2 (5.28 to 7.12)	3.44
eGFR (mL/min/1.73 m^2)	8.9±4	106.1±22.7	<0.0001	-97.2 (-105.0 to -89.4)	-5.84
HbA1c (%)	8.2±1	5.3±0.6	<0.0001	+2.9 (2.49 to 3.31)	3.56
RBS (mg/dL)	205.5±91	106.7±11.5	<0.0001	+98.8 (66.5 to 131.1)	1.56
Systolic (mmHg)	147.7±15.8	121.1±3	<0.0001	+26.6 (20.95 to 32.25)	2.39
Diastolic (mmHg)	86.9±10.7	80.7±1.9	0.031	+6.2 (2.38 to 10.02)	0.83
Leptin (ng/mL)	1.7±1	1.4±0.7	0.001	+0.3 (-0.12 to 0.72)	0.35
GLP-1 (pmol/L)	19.6±11.2	37±25.7	0.001	-17.4 (-26.9 to -7.9)	-0.86

Leptin was significantly correlated with serum creatinine and p=0.005, eGFR with p=0.015, GLP-1 with p=0.001, SBP with p=0.002, and DBP with p=0.025. The other data, age, HbA1c, RBS, and urea, were not significantly correlated with leptin, as shown in Table 2.

Table 2. Correlation table between serum leptin and metabolic, renal, and hemodynamic parameters in patients

Parameters	Leptin	
	r	p
Age	0.156	0.078
HbA1c%	0.057	0.519
RBS (mg/dL)	-0.050	0.578
Urea (mg/dL)	0.093	0.293
Creatinine (mg/dL)	0.246	0.005
eGFR (mL/min/1.73 m^2)	-0.213	0.015
GLP-1 (pmol/L)	0.295	0.001
Systolic blood pressure (mmHg)	0.275	0.002
Diastolic blood pressure (mmHg)	0.197	0.025

Discussion

The principal functions of the kidneys are the elimination of waste products and the regulation of water balance, and the management of blood pressure. ESRD is a severe condition necessitating prompt intervention, including dialysis or kidney transplantation.[13]

A decline in various filtration markers, such as eGFR, is consistently associated with increased ESRD

risk. As in the current study, an elevation in biomarkers related to renal functions, including serum urea, serum creatinine, and a decrease in eGFR, as seen in Table 1, in the disease group, leads to ESRD. These findings are consistent with Rebholz et al.[14] Declines in eGFR were consistently associated with progression to ESRD.

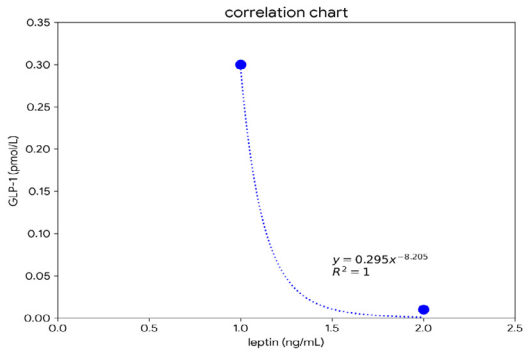

Fig. 1. Correlation chart between leptin and GLP-1

Hypertension is a significant risk factor for ESRD, with various studies highlighting its impact on kidney health. In this current study, elevation in blood pressure in the disease group contributes to renal deterioration, which agrees with the current findings. That contrasts with Kim et al., who found that higher hypertension exposure (systolic BP≥140 mmHg) was linked to increased ESRD risk.[15] Studies conducted at several sites reveal a clear relationship between hypertension and elevated leptin levels.[16,17]

Factors including hyperglycemia, inflammation, and fibrosis affect the declining renal function in DKD and the progression to ESRD.[18]

In this study, patients in the disease group with high levels of random blood sugar and HbA1c have ESRD and are on hemodialysis. This agrees with the results of the investigations of Oluboyo and Azeez et al.[19,20] However, these studies included a larger number of patients and included other renal markers, such as uric acid and cystatin C. Chronic hyperglycemia leads the kidneys to undergo structural and functional alterations. In contrast, CKD has the potential to impair glucose metabolism, which may lead to the development of diabetes and insulin resistance.

Higher leptin levels in the disease group are associated with an increased cardiovascular risk profile, consistent with previous findings in ESRD populations. Hence, the kidneys can undergo pathogenic alterations. These alterations can cause more protein to seep into the filtration. Leptin's direct profibrotic actions on glomeruli suggest a possible interaction between leptin and glomerular endothelial/mesangial cells; furthermore, the kidney's augment expression of type IV collagen results from increased leptin levels.[5]

On the other hand, low GLP-1 levels in the disease group are noteworthy because they correspond with the progression of renal impairment. This finding is consistent with the study by Diwesh Chawla et al. 2023.[21] Which also reports a low level of GLP-1. GLP-1, which is predominantly produced in the gastrointestinal tract, plays a protective role in kidney function, primarily through its receptor (GLP-1R).

A low level of GLP-1 is owing to its fast breakdown by DPP-4. The study, Lebherz et al.[22] disagreed with our research, which states that GLP-1 levels are significantly elevated in critically ill patients and those with end-stage renal disease compared to healthy participants; this increase can be attributed to both enhanced GLP-1 secretion and reduced clearance, particularly in inflammatory conditions and chronic kidney disease. In our study, the level of GLP-1 in the control group was higher than in the disease group, but in the study of Cao et al.[23] the comparison of (GLP-1) hormone levels between healthy controls and disease patients revealed substantial differences. Healthy people often have greater GLP-1 levels, which are essential for glucose metabolism and kidney protection. Meal intake boosts their strong GLP-1 response, improving insulin secretion and glucose homeostasis. In contrast, the disease group frequently has low GLP-1 levels, contributing to metabolic and renal problems. This is consistent with the results of this current study. Healthy people often have greater GLP-1 levels, which is compatible with this study's results.

Leptin has significant correlations with GLP-1 (r=0.295, p=0.001), as in Table 2, moderately and significantly supports a metabolic-hormonal interplay between the adipose and incretin systems. Leptin and GLP-1 both play roles in energy balance, appetite, and insulin dynamics.

Research suggests that leptin stimulates GLP-1 secretion and regulates its impact on insulin secretion and appetite control. A study by Tomasik et al. 2020 indicates that leptin may serve as an advantageous biomarker for predicting GLP-1 activity.[24]

It should be noted that the disease group was older on average than the control group. Age is known to influence several metabolic and inflammatory biomarkers, including leptin and GLP-1, and may partially explain the observed differences. While our statistical analysis aimed to account for these differences, residual confounding cannot be excluded.

Study limitations

This study has several limitations. The sample size was quite limited, which may have restricted the generalizability of the findings to larger groups. Second, the control and disease groups were not age-matched, potentially introducing confounding effects. Third, the cross-sectional design prevents any inference of caus-

al relationships between the measured biomarkers and disease status. Finally, the absence of multivariate analyses restricts our ability to adjust for potential covariates, such as comorbidities or medication use, that may have influenced the results.

Conclusion

This cross-sectional study demonstrated that patients with diabetic ESRD undergoing hemodialysis had significantly higher serum leptin levels and lower GLP-1 concentrations compared to healthy controls. These findings support previous evidence suggesting that leptin may contribute to kidney injury and comorbidities, while GLP-1 may offer protective renal effects. Despite the limited sample size and absence of age-matching between groups, the results highlight the potential relevance of these biomarkers in the pathophysiology of DKD. Further longitudinal and interventional studies are needed to evaluate the prognostic and therapeutic implications of leptin and GLP-1, particularly in the context of GLP-1 receptor agonist therapy in CKD patients.

Declarations

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Author contributions

Conceptualization, H.H.F., R.D.S. and J.K.H.; Methodology, J.K.H.; Software, R.D.S.; Validation, H.H.F., R.D.S. and J.K.H.; Formal Analysis, R.D.S.; Investigation, J.K.H.; Resources, R.D.S.; Data Curation, H.H.F.; Writing – Original Draft Preparation, H.H.F.; Writing – Review & Editing, J.K.H.; Visualization, H.H.F.; Supervision, R.D.S.; Project Administration, J.K.H.; Funding Acquisition, H.H.F.

Conflicts of interest

All authors declare that there are no conflicts of interest in this article.

Data availability

All data are available from the corresponding author and will be made available upon request.

Ethics approval

The study received approval from the Ethics Committee of the College of Pharmacy at the University of Basrah (Protocol No. 248, dated December 15, 2023) and the Basrah Health Directorate (Protocol No. 74, dated January 27, 2024).

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ORIGINAL PAPER

Exploring the diagnostic potential of micro-RNA-320 and anti-Müllerian hormone in women with polycystic ovary syndrome – a case-control study

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ABSTRACT

Introduction and aim. Polycystic ovary syndrome (PCOS) is a multifactorial endocrine disease manifested with hormonal imbalance, insulin resistance (IR), and reproductive dysfunction. Due to its heterogeneous clinical presentation, diagnosis remains challenging. MicroRNA-320a-3p and anti-Müllerian hormone (AMH) have recently emerged as promising biomarkers. This study aimed to assess their diagnostic potential in PCOS women.

Material and methods. A case-control research was performed in 90 women aged 18–40 years, including 45 women with PCOS and 45 age- and body mass index-matched healthy individuals. Hormonal and metabolic markers were measured using standard immunoassays, and the expression of microRNA-320a-3p was quantified using real-time polymerase chain reaction.

Results. Women with PCOS demonstrated statistically higher concentrations of luteinizing hormone (9.45±6.0 vs 5.09±2.2 mIU/mL), increased luteinizing hormone to follicle-stimulating hormone ratio (1.64±0.87 vs 0.76±0.3), and elevated fasting blood glucose (105.5±14.7 vs 94.3±13.5 mg/dL), all with $p<0.001$. Contrary to expectations, insulin and homeostatic model assessment for IR values were lower in the PCOS group, possibly reflecting a predominance of non-obese phenotypes. AMH levels were also reduced (2.27±1.0 vs 3.34±1.1 ng/mL, $p<0.001$). Expression of microRNA-320a-3p was significantly downregulated (0.61±1.27 vs 2.81±5.03-fold, $p=0.0009$). MicroRNA-320a-3p expression correlated positively with luteinizing hormone levels and the luteinizing hormone to follicle-stimulating hormone ratio, while AMH was associated with IR. The combined use of both markers improved diagnostic differentiation between groups.

Conclusion. MicroRNA-320a-3p and AMH show promise as diagnostic biomarkers in polycystic ovary syndrome. Their integration with traditional clinical markers may enhance diagnostic accuracy and provide deeper insight into the pathophysiological complexity of the disorder.

Keywords. anti-Müllerian hormone, insulin resistance, microRNA-320, polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) a prevalent endocrine disease among females during their reproductive years (has affected at least 5-10% of women globally), and it has a global prevalence of approximately 4-20%. The features of PCOS include polycystic ovarian morphology, persistent anovulation and hyperandrogenism. PCOS is a multifaceted disorder, with a multitude of

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factors (hormonal, metabolic and genetic) contributing to its development and associated long-term outcomes, including cardiovascular diseases, infertility and type 2 diabetes (T2D). Although the pathophysiological mechanisms associated with PCOS are well-studied, there continues to be a challenge with diagnosing the disorder due to the heterogeneity of clinical findings and laboratory results; therefore, researchers will need to identify new, valid, and reliable biomarkers for improved diagnostic accuracy and the understanding of disease processes.[1,2]

Insulin resistance (IR) is a common physiological finding in patients diagnosed with PCOS, resulting in significant, compensatory hyperinsulinemia, further contributing to hormonal dysregulation, increased hyperandrogenism, and infertility.[3] IR has been noted previously to play a major role in the pathophysiologic processes of PCOS, impacting over 75% of individuals diagnosed with PCOS.[2]

Micro-ribonucleic acid-320 (miRNA-320) has recently gained attention as a proposed molecular marker for various types of reproductive and metabolic disorders. As a regulator of gene expression, miRNA-320 plays an essential role in the regulation of glucose metabolism, IR, and ovarian function, which are all processes involved in the pathophysiology of PCOS.[4] The anti-Müllerian hormone (AMH) has also been investigated as a potential diagnostic marker for PCOS, reflecting the level of follicular arrest and dysfunction in PCOS patients.[5] The other metabolic and hormonal markers that are important in the endocrinology and metabolic profile of PCOS are designed (luteinizing hormone (LH), follicle-stimulating hormone (FSH), insulin, and fasting blood glucose (FBG)), which play critical roles in explaining hormonal and metabolic disturbances in PCOS. The typical hormonal diagnostic markers for PCOS are the LH to FSH ratio, whereas IR and hyperinsulinemia are metabolic features of PCOS that have a significant bearing on the progression of PCOS and the impact of PCOS treatment.[6,7]

To our knowledge, this is the first research determine the combination of diagnostic accuracy of miRNA-320a-3p and anti-AMH in patients with PCOS. Previous studies have primarily aimed on either AMH or miRNA-320a-3p, whereas this current study examines ambidirectionally for hormonal and metabolic parameters to produce an overall assessment of PCOS. It also uncovers some unexpected patterns, such as lower levels of AMH and insulin. which challenge conventional understanding and highlight phenotypic heterogeneity.

Aim

The aim of this research is to compare the performance of microRNA-320a-3p and AMH with traditional hormone and metabolic markers for diagnosing women with PCOS. A case-control design was utilized to ascertain whether these biomarkers provide greater accuracy when used to diagnose women with PCOS. Additionally, the study will identify the relationship of these biomarkers to metabolic and endocrine aberrations, which may facilitate their integration into standard clinical assessment and into personalized management options for women with PCOS.

Material and methods

Study design/subjects

A case-control research was conducted at Bent Al-Huda Teaching Hospital in the Thi-Qar governorate of Iraq from September 2024 to December 2024. In this paper, two study groups were employed: The first group included 45 women diagnosed with PCOS according to the revised 2003 diagnostic criteria, which require the incidence of 2 out of 3: oligo- or anovulation ,presence of polycystic ovaries, clinical/biochemical manifestations of excess androgen, and the exclusion of other causes, include androgen-secreting tumors, congenital adrenal hyperplasia, and Cushing's syndrome.[8] The second group involved 45 individuals as healthy controls (HCs). All participants in the study were between 18 and 40 years old.

Exclusion and inclusion criteria

The study excluded women who had recently used hormonal contraceptives, anti-androgens, or insulin sensitizers (past three months), pregnant or breastfeeding women, those with chronic diseases (diabetes, hypertension, cardiovascular diseases), a history of ovarian surgery, or inadequate sample collection. Inclusion criteria required participants to meet clinical and laboratory PCOS criteria (menstrual irregularities, hyperandrogenism, and ultrasound-confirmed polycystic ovaries), be in the 18–40 age range, possess a body mass index (BMI) ranging from 18.5 to 35, which includes a range of normal to moderately obese individuals, and do not have a history of other endocrine diseases, such as androgens secreted tumors, Cushing's syndrome, or congenital adrenal hyperplasia.

Healthy controls were age-matched (18-40 years) with the PCOS group, had regular menstrual cycles, no history of PCOS, and a BMI within the same range to minimize confounding effects. They did not have any endocrine abnormalities (thyroid disorder, Cushing's syndrome, or congenital adrenal hyperplasia), no history of hormonal medication use, normal fertility status, and a healthy lifestyle.

Sample collection

Five milliliters of peripheral blood were obtained from each participant after fasting for 8–12 hours through venipuncture. After collection, the samples were left in gel vacuum tubes and allowed to coagulate at room tem-

perature, and then centrifuged at 3,600 × g for 10 minutes for serum separation. Each sample of serum was divided into two aliquot, one of which was stored at -80°C until it was time for subsequent analysis, and the other mixed with Trizol (500 µL of serum + 500 µL of Trizol) for miRNA extraction.

Biochemical assays

Serum AMH was measured in nanograms (ng) per mL using an enzyme-linked immunosorbent assay (ELISA) method using an AMH ELISA kit (BT LAB, China). Serum concentrations of FSH and LH were measured using the AFIAS-10 instrument, which is an automated immunoassay analyzer designed to perform up to 10 parallel tests for various parameters using the AFAIS LH kit and the AFAIS FSH kit, respectively (Boditech, Korea). The test protocol was conducted according to the manufacturer's guidelines. The findings are recorded in milli-international units (mIU)/mL. Serum insulin was measured in micro-international units (µIU)/mL using an ELISA method with a human insulin ELISA kit (BT LAB, China). The serum levels of FBG were measured using the Mindray BS-230 instrument, a fully automated biochemical analyzer. It operates based on spectrophotometry and biochemical enzymatic reactions using a glucose kit (Mindray, China). In accordance with the manufacturer's guidelines, the test procedure was conducted. The results are reported in milligram (mg)/deciliter (dL). The following equation was used to detect the homeostatic model assessment of IR (HOMA-IR).[9]

$$\text{HOMA} - \text{IR} = \text{fasting insulin}\,(\mu\text{IU/mL}) \times \text{FBG}\,(\text{mg/dL})/405$$

Molecular detection of microRNA-320a-3p: The molecular assay was conducted in a specialist laboratory in Baghdad, Iraq, following manual guidelines. Total serum RNA was extracted using the Trizol reagent method, and complementary deoxyribonucleic acid (cDNA) was synthesized for miRNA-320a-3p and RNU43 (housekeeping gene) using specific reverse transcriptase (RT) primers (Table 1) and a Thermal Cycler. cDNA concentration and quality (5–7 ng/µL) were measured with a Quantus Fluorometer. Quantification of miRNA-320a-3p and RNU43 was performed by RT-qPCR using specific forward and a universal reverse primers (Table 1) on a Mic-qPCR cycler (Fig. 1 and 2).

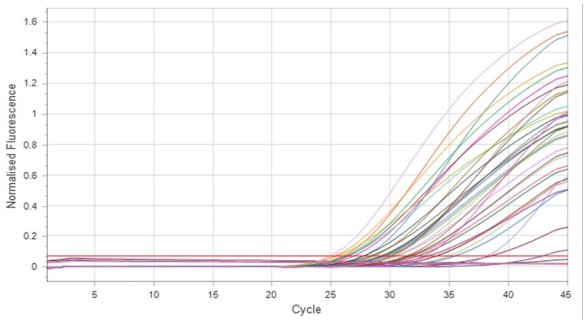

Fig. 1. The step of real-time polymerase chain reaction of microRNA-320a-3p

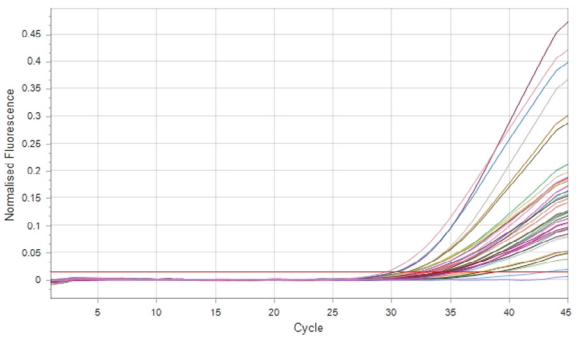

Fig. 2. The step of real-time polymerase chain reaction of RNU43

Gene expression was quantified relatively using the Pfaffl method.[10] For each sample, by using the real-time cycler software, the threshold cycle (CT) was determined. Average values were computed by running each sample twice. The expression data for the chosen genes were normalized using housekeeping genes as a reference. The ΔΔCt method was utilized for data analysis as recommended, with results presented as fold change in gene expression. The following formula was used to calculate the difference (ΔCt) between the CT values for each target gene and the housekeeping gene for each sample.[11] The normal range for microRNA-320a-3p was determined based on data from the control group.

Table 1. The study primers[a]

Primers name	Sequence ('5——————————3')	Company	Country	Temperature
microRNA-320a-3pRT*	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACTTGCCC	Macrogen	South Korea	55 °C
microRNA-320a-3pF*	GGGAAAAGCTGGGTTGAGA			
RNU43-RT*	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCG CACCAGAGCCAACAATCAG			
RNU43-F*	GTGAACTTATTGACGGGCG			
Universal reverse*	GTGCAGGGTCCGAGGT			

[a] * – from the National Center for Biotechnology Information Gen-Bank database the miRNA gene cDNA sequences were obtained. Primers for real-time qPCR were designed with a melting temperature range from 58°C to 62°C using primer Premier 3 software, the length of the primers ranging from 18 to 23 nucleotides, and the length of the PCR amplicon ranged between 75 and 150 base pairs.

ΔCT = CTgene – CT House Keeping gene
ΔΔCT = ΔCT Treated or Control – Average ΔCT Control
Folding = 2^{-}ΔΔCT

Statistical analysis

Data analysis and visualization were employed using the statistical package for social science (SPSS) (version 22, IBM, Armonk, NY, USA). The descriptive methods involved means, frequencies, and standard deviations (SD). Categorical variables were compared using the chi-square test. The statistical differences of variations in the means of continuous variables with a normal distribution was calculated using an independent t-test, which was a parametric test. For variables that were not normally distributed, the Mann–Whitney U test, a non-parametric test, was employed. The Spearman correlation coefficient, which is a non-parametric test, was calculated to determine the strength and direction of the correlations between variables. A p-value <0.05 was determined to be statistically valuable.

Results

In the current research, the differences in insulin levels between the PCOS and HC cohorts were statistically significant (p<0.001), where the mean insulin concentration was depleted in the PCOS women (6.15±2.87 μIU/mL) than HCs (7.56±2.45 μIU/mL). Regarding HOMA-IR, the mean HOMA-IR was statistically lower in the PCOS patients (1.57±0.66) than the HCs (1.75±0.58) (p=0.0227). While IR is typically more pronounced in PCOS, the current results may reflect a heterogeneous PCOS cohort or the influence of confounding factors such as BMI, physical activity levels, or dietary habits, which were not under the control of this analysis (Table 2).

Fasting blood sugar levels demonstrated a statistically significant elevation in the PCOS group (105.46±14.7 mg/dL) than the HCs group (94.3±13.51 mg/dL), with a p-value of 0.0003, supporting the presence of impaired glucose metabolism in PCOS. Luteinizing hormone concentrations were statistically elevated in the PCOS women (9.45±6.0 mIU/mL) than the controls (5.09±2.2 mIU/mL), having a difference that is statistically significant (p<0.001), consistent with the well-established association between elevated LH and the pathophysiology of PCOS. In contrast, concentrations of FSH were lower in the PCOS patients (5.8±2.13 mIU/mL) than in controls (6.89±2.02 mIU/mL), and the mean difference reached statistical significance (p=0.0145) (Table 2).

Significantly, the LH/FSH ratio was elevated in the women with PCOS (1.64±0.87) than in the HCs (0.76±0.3), with p-values <0.001, reinforcing the LH/FSH ratio diagnostic value in distinguishing PCOS. For AMH, unexpectedly, the mean concentration was lower in the PCOS patients (2.27±1 ng/mL) than in HCs (3.34±1.12 ng/mL) with a statistical difference (p<0.001). This finding is opposite to the traditionally accepted view of increased AMH levels associated with PCOS and may indicate that there are phenotypic differences within the PCOS population, or it may be the result of methodological differences, so the findings require further research (Table 2).

There was also a large difference in microRNA-320a-3p expression levels between groups. The mean expression for microRNA-320a-3p in the PCOS group was markedly lower (0.61±1.27 folds) when compared to HCs (2.81±5.03 folds) (p=0.0009). This suggests that microRNA-320a-3p could contribute to the pathophysiology of PCOS and may be used as a genetic biomarker to detect diseases or monitor progression (Table 2).

Table 2. Biochemical and molecular parameters comparison between PCOS patients and healthy controls groups (Mann-Whitney U test and independent t-test)

Parameters	PCOS Group	Healthy Control	p
	Mean±SD	Mean±SD	
Insulin (μIU/mL)	6.15±2.87	7.56±2.45	<0.001
HOMA-IR	1.57±0.66	1.75±0.58	0.0227
FBG (mg/dL)	105.46±14.7	94.3±13.51	0.0003
LH (mIU/mL)	9.45±6.0	5.09±2.2	<0.001
FSH (mIU/mL)	5.8±2.13	6.89±2.02	0.0145
LH/FSH Ratio	1.64±0.87	0.76±0.3	<0.001
AMH (ng/mL)	2.27±1	3.34±1.12	<0.001
microRNA-320a-3p Folds	0.61±1.27	2.81±5.03	0.0009

Table 3 presents the distribution of IR (measured by HOMA-IR) in relation to microRNA-320a-3p expression levels among PCOS women and HCs. The correlation between HOMA-IR and expression folds of microRNA-320a-3p was evaluated in both the PCOS and HCs groups. Among individuals with PCOS, a statistically valuable positive relationship was noted between HOMA-IR values and microRNA-320a-3p expression levels (r=0.51, p<0.001). Specifically, participants with high microRNA-320a-3p expression demonstrated a markedly higher mean HOMA-IR (1.59) compared to those with low microRNA-320a-3p expression, who exhibited a mean HOMA-IR of 1.44. Notably, 2/2 (100%) of PCOS participants with high microRNA-320a-3p expression exhibited normal HOMA-IR compared to participants with low microRNA-320a-3p expression, 13/15 (86%), yet the subgroup with high microRNA-320a-3p expression still showed a disproportionately elevated HOMA-IR level, pointing to a possible role of this microRNA in IR among patients with PCOS. On the other hand, even though there appears to be an increasing trend toward higher HOMA-IR levels in HCs with high expression levels of microRNA-320a-3p (mean=1.68) relative to those with low microRNA-320a-3p expres-

sion (mean=1.63), the relationship was not statistically valuable (r=0.23, p=0.10). This may indicate potential physiologic variability in insulin sensitivity between healthy individuals, and that this variability is not necessarily a result of underlying endocrine/metabolic disturbances, as is commonly seen in PCOS.

Table 3. Correlation of microRNA-320a-3p expression and HOMA-IR concentrations in PCOS and HCs (Spearman's correlation analysis, Mann-Whitney U test, independent t-test, and Chi-square test)

	Biomarkers		HOMA-IR						r	p
			Normal (<2)		High (≥2)		Total			
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean		
microRNA-320a-3p Folds	PCOS	Low (n=15)	13 (86.7)	1.27	2 (13.3)	2.56	15 (100)	1.44	0.51	0.00
		Normal (n=3)	3 (100)	1.55	0 (0)	0	3 (100)	1.55		
		High (n=2)	2 (100)	1.59	0 (0)	0	2 (100)	1.59		
		Total (n=20)	18 (90)	1.35	2 (10)	2.56	20 (100)	1.47		
	HCs	Low (n=7)	7 (100)	1.63	0 (0)	0	7 (100)	1.63	0.24	0.10
		Normal (n=7)	4 (57.1)	1.71	3 (42.9)	2.87	7 (100)	2.21		
		High (n=6)	4 (66.7)	1.45	2 (33.3)	2.14	6 (100)	1.68		
		Total (n=20)	15 (75)	1.6	5 (25)	2.58	20 (100)	1.85		

Table 4 displays the association between HOMA-IR values and AMH levels for both groups (PCOS and HCs). For females diagnosed with PCOS, there was a positive relationship between AMH concentrations and HOMA-IR concentrations, with a statistically significant correlation coefficient of 0.38 (P = 0.01). Women with high AMH had much higher average HOMA-IR concentrations (3.44) than women with normal AMH (1.47). Although all PCOS individuals with high AMH levels (100%) fell within the high HOMA-IR range than those with normal AMH levels (11.9%), this difference indicates that an increase in AMH concentrations correlates with an elevation in IR in patients diagnosed with PCOS and suggests the possibility of a relationship between ovarian reserve and metabolic dysfunction in patients with PCOS. For the HCs women, there was also a positive relationship between AMH and HOMA-IR, with a correlation coefficient of 0.36 (P = 0.02). The mean HOMA-IR for women in the HCs group with high AMH concentrations was 2.28, whereas women in the HCs group with normal AMH levels had a mean HOMA-IR value of 1.6. Notably, nearly half (45.5%) of the HC participants in the high AMH subgroup demonstrated elevated HOMA-IR, suggesting that even in the absence of clinical PCOS, higher AMH levels may be linked to underlying IR.

Table 5 explores the relationship between microRNA-320a-3p folds levels and several metabolic and reproductive parameters in both PCOS (G1) and HC (G2) groups. Among patients with PCOS, a statistical relationship was reported between the expression of microRNA-320a-3p and the LH/FSH ratio (p=0.007), as well as serum LH concentrations (p=0.029). Specifically, those with high miRNA expression had a notably elevated LH/FSH ratio (3.16±2.31) and LH concentration (18.80±13.73 mIU/mL), suggesting a potential role of this miRNA in modulating gonadotropin imbalance characteristic of PCOS. Conversely, there were no statistically valuable correlations found between the levels of microRNA-320a-3p and FBG, insulin, FSH, or AMH, indicating that the miRNA may not be primarily involved in glucose metabolism. No significant relationships were observed between level of microRNA-320a-3p and any of the tested parameters in the control group. These results support a disease-specific regulatory function of microRNA-320a-3p in reproductive hormone imbalance in PCOS but not in healthy individuals.

Table 4. Correlation of HOMA-IR and AMH levels in PCOS and HCs (Spearman's correlation analysis, Mann-Whitney U test, independent t-test, and Chi-square test)

	Biomarkers		HOMA-IR						r	p
			Normal (<2)		High (≥2)		Total			
			FR (%)	Mean	FR (%)	Mean	FR (%)	Mean		
AMH (ng/mL)	PCOS Group	Low (n=1)	0 (0)	0	1 (100)	2.24	1 (100)	2.24	0.38	0.01
		Normal (n=42)	37 (88.1)	1.33	5 (11.9)	2.52	42 (100)	1.47		
		High (n=2)	0 (0)	0	2 (100)	3.44	2 (100)	3.44		
		Total (n=45)	37 (82.2)	1.33	8 (17.8)	2.71	45 (100)	1.57		
	HCs	Low (n=0)	0 (0)	0	0 (0)	0	0 (0)	0	0.36	0.02
		Normal (n=34)	30 (88.2)	1.52	4 (11.8)	2.19	34 (100)	1.6		
		High (n=11)	6 (54.5)	1.57	5 (45.5)	2.98	11 (100)	2.28		
		Total (n=45)	36 (80)	1.53	9 (20)	2.63	45 (100)	1.75		

Table 5. Association between microRNA-320a-3p expression, hormonal and metabolic parameters in patients with PCOS and healthy control groups (Mann-Whitney U test, independent t-test)*

Variables/Groups (Mean±SD)		microRNA-320a-3p Folds			p
		Low (<1)	Normal (1-2)	High (>2)	
FBS (mg/dL)	G1	100.46±13.62	126.20±27.50	106.50±1.70	0.523
	G2	98.43±8.07	98.49±13.60	92.87±22	0.713
LH/FSH ratio	G1	2.23±0.53	2.20±0.49	3.16±2.31	0.007
	G2	0.70±0.13	0.54±0.18	0.65±0.19	0.728
Insulin (µIU/mL)	G1	5.89±2.61	5.23±1.99	6.06±1.56	0.928
	G2	6.78±1.08	9.20±3.47	7.27±2.09	0.742
LH (mIU/mL)	G1	13.50±4.84	14.89±3.36	18.80±13.73	0.029
	G2	5.25±1.06	3.88±1.46	4.53±1.51	0.540
FSH (mIU/mL)	G1	6.16±1.87	6.95±2.05	5.97±0.02	0.877
	G2	7.69±1.86	7.28±2.03	7.01±1.00	0.615
AMH (ng/mL)	G1	2.16±1.15	2.39±0.38	2.18±0.88	0.982
	G2	3.12±0.68	3.35±1.96	3.28±0.70	0.791

* G1 – polycystic ovary syndrome patient group, G2 – healthy control group

Table 6 evaluates the association between various biochemical and hormonal parameters and serum levels of AMH in both study groups. Among the patients

with PCOS, levels of AMH were significantly associated with FBG (p=0.006), where individuals with high AMH levels exhibited the lowest FBG (88.8±3.82 mg/dL) compared to those with normal (105.98±14.65 mg/dL) and low AMH levels (117 mg/dL). This negative correlation between FBG and AMH in PCOS-afflicted women suggests a possible protective role of AMH against hyperglycemia. Additionally, the relationship between insulin and AMH concentrations was statistically valuable (p=0.010), with the highest insulin values noted in those with high AMH levels (15.7±1.4 μIU/mL), indicating potential compensatory hyperinsulinemia or a complex interaction between ovarian reserve markers and insulin signaling pathways. There were no statistically significant relationships between AMH and LH, the LH/FSH ratio, or FSH (P > 0.05). Therefore, as there were no statistically valuable associations between the parameters studied and AMH in HCs females, this suggests that the metabolic and endocrine environments in HCs females are generally more stable than those of females with PCOS. Combining these results emphasizes the dual endocrine and metabolic relevance of AMH in PCOS pathophysiology and supports its candidacy as a multidimensional biomarker.

Table 6. Correlation between levels of AMH, metabolic and hormonal parameters in PCOS and healthy control groups (Mann-Whitney U test, independent t-test)

Variables/Groups (Mean±SD)		AMH (ng/mL)			p
		Low (<1)	Normal (1-4)	High (>4)	
FBS (mg/dL)	G1	117±0.0	105.98±14.65	88.8±3.82	0.006
	G2	0	95.7±14.8	89.94±7.47	0.590
LH/FSH ratio	G1	0.92±0	1.65±0.89	1.84±0.130	0.561
	G2	0	0.74±0.296	0.85±0.31	0.680
Insulin (μIU/mL)	G1	7.76±0	5.66±2.01	15.7±1.4	0.010
	G2	0	6.81±1.43	9.88±3.42	0.067
LH (mIU/mL)	G1	1.69±0	9.71±6.07	7.95±1.90	0.581
	G2	0	5.22±2.28	4.69±1.96	0.776
FSH (mIU/mL)	G1	1.84±0	5.97±2.08	4.37±1.34	0.082
	G2	0	7.24±1.87	5.83±2.18	0.444

Discussion

The aims of this study was to assess and compare significant molecular, biochemical, and hormonal variables of PCOS patients versus matched control groups. In studying these two populations, we identified a number of statistically valuable differences between both groups, which are indicative of the heterogeneous and multifactorial nature of PCOS. Many features of PCOS include metabolic derangements (IR), hormonal disturbances, and emerging novel molecular patterns (microRNAs). At this time, IR has been established as a primary pathogenic feature associated with PCOS with respect to hyperinsulinemia and additional concurrent metabolic changes associated with hyperinsulinemia and additional metabolic adaptations. In our study population, we also found that there were statistically depleted mean serum insulin concentrations in the PCOS patient population as than the HCs. Our findings contrast with the majority of previous research that indicates elevated concentrations of serum insulin in PCOS as a consequence of systemic IR.[12] A potential explanation for this difference may be due to the inclusion of non-obese and lean PCOS phenotypes that do not demonstrate high levels of IR.[13] Furthermore, confounding variables such as BMI, physical activity, and nutrition were not controlled in this research, and all have been shown to considerably impact insulin dynamics.[14]

Although the frequency of IR (as identified by elevation of HOMA-IR) showed no statistical significant difference between groups, the mean of HOMA-IR was surprisingly lower in PCOS participants than in controls. This contradicts previous studies indicating higher HOMA-IR in PCOS women.[15] However, as Daan et al.[16] and others have observed, the expression of IR in PCOS can vary based on age, BMI, and ethnic background. It is also possible that HOMA-IR underrepresents tissue-specific IR (e.g., ovarian or hepatic), which could still be present in PCOS despite lower systemic levels.[17]

Our findings showed significantly elevated FBG levels in the PCOS group, consistent with prior research documenting impaired glucose tolerance and increased risk for T2D in PCOS women.[18] Moran et al.[19] demonstrated that PCOS elevates the risk of metabolic syndrome and glucose dysregulation even in normal-weight individuals. These results underscore the importance of metabolic monitoring in all PCOS phenotypes, regardless of body habitus.

The elevations in serum LH and ratios of LH to FSH seen in the PCOS population studied are consistent with multiple previously reported features of PCOS. In PCOS, the elevated frequency of the LH pulsatile release of gonadotropin-releasing hormone (GnRH) leads to a predominant secretion of LH and a subsequent increase in androgen production, which are contributing factors to the ovulatory dysfunction seen in PCOS.[20] Our finding that over 50% of PCOS patients exhibited high LH levels and a significantly higher LH/FSH ratio than controls aligns with studies by Azziz et al.[21] and Rosenfield and Ehrmann,[22] further validating these parameters as useful diagnostic indicators. In contrast, FSH concentrations were modestly but significantly depleted in PCOS patients. Although the frequency distribution across FSH categories was not significant, the mean value difference may reflect disrupted granulosa cell function and impaired follicular development, as previously described.[23]

Unexpectedly, PCOS patients had lower mean AMH levels than controls, contradicting the conventional understanding of AMH elevation in PCOS due to a small

antral follicles increase.[24] Discrepancy of this could be due to heterogeneity in PCOS phenotypes, assay differences, or diminished ovarian reserve in a subset of patients.[25] Ahmad et al.[26] reported similar variability in AMH among different PCOS subtypes, suggesting it should not be used in isolation for diagnosis. This result aligns with Hmood et al.,[27] who discovered that anovulatory women with polycystic ovaries had a relative lack of AMH in their transitional and primordial follicles in the ovaries. This could be an influencing factor that leads to the abnormal early follicle development seen in PCOS.

Recent research has emphasized the role of non-coding RNAs, including miRNAs, in regulating PCOS-related pathways. Our study found a markedly reduced level of microRNA-320a-3p folds in the PCOS patient group than the controls. This is parallel with findings by Chen et al.,[28] who found that microRNA-320a modulates insulin signaling and granulosa cell proliferation. Downregulation of this miRNA may therefore contribute to follicular arrest in PCOS.[29] These findings support the growing interest in microRNA-320a-3p as a potential biomarker for disease diagnosis or progression monitoring.

The present research provides novel insights into the relationship between microRNA-320a-3p folds and IR, measured using HOMA-IR, in PCOS women than the HCs. In our PCOS group, we found a statistically valuable positive relationship between microRNA-320a-3p folds and the HOMA-IR score. This was not the case for participants in the HC group. Thus, microRNA-320a-3p may have some type of role in the pathogenesis of IR for people diagnosed with PCOS (Table 3). Our results agree with previous research that has implicated microRNA-320a in metabolic disorders, particularly in regulating signaling pathways for insulin. Luo et al.[30] showed that microRNA-320a expression was positively associated with markers of IR, including HOMA-IR and fasting insulin concentration and Vogt et al.[31] reported that microRNA-320a targets several key genes involved in insulin signaling, such as insulin receptor substrate 1 (IRS1) and phosphoinositide 3-kinase (PI3K), and its overexpression was linked to impaired insulin sensitivity in ovarian granulosa cells, further supporting its mechanistic involvement in PCOS-associated IR. In contrast, other studies have recorded conflicting findings regarding the role of microRNA-320a in metabolic regulation. For instance, Du et al.[32] discovered that the levels of circulating microRNA-320 were downregulated in patients with T2D and speculated that its loss may contribute to IR via the dysregulation of insulin-responsive genes. This discrepancy could reflect tissue-specific or context-dependent roles of microRNA-320a, whereby its expression may differ between insulin-sensitive tissues (e.g., muscle, adipose) and the systemic circulation or ovaries in PCOS. Notably, in our HCs group, although individuals with high microRNA-320a-3p expression had slightly higher mean HOMA-IR levels, the association failed to achieve statistical significance (Table 3). This may highlight the absence of underlying endocrine or inflammatory disturbances in healthy individuals that are known to amplify miRNA dysregulation in PCOS. It is possible that microRNA-320a-3p exerts a more pronounced effect on insulin metabolism in the presence of hormonal imbalances and chronic low-grade inflammation typical of PCOS, as previously suggested by Cirillo et al.[33] Taken together, our findings reinforce the growing body of evidence that microRNA-320a-3p could potentially act as a promising marker for IR in PCOS. Its expression appears to be more tightly linked to metabolic dysfunction in pathological contexts than in physiological conditions. Further study is needed with larger sample sizes and appropriate in *vitro* models to better clarify the direct relationship between microRNA-320a-3p and IR, as well as to investigate the potential for therapeutics. In current study, we also reported a positive relationship between AMH concentrations and HOMA-IR scores for both the PCOS and HCs groups (Table 4). This finding matches with other studies suggesting that AMH is also involved in metabolic problems aside from being an indicator of ovarian reserve. Additionally, we found there to be a statistically significant positive relationship between higher AMH concentrations and higher HOMA-IR scores in the PCOS cohort, which confirms previous observations made by Pigny et al.[34] results from those researchers indicated that women diagnosed with PCOS have high concentration of AMH and often have hyperandrogenism and IR, which provides evidence that AMH may help link ovarian dysfunction with metabolic abnormalities. In addition, it is interesting to note that 100% of PCOS individuals with high AMH awere found to belong to the category of high HOMA-IR, which suggests that AMH may be used as an indicator of metabolic risk stratification in patients with PCOS. This finding corroborates the findings of Jun et al.,[35] who postulated that AMH might be used as a predictor of reproductive and metabolic profiles in women with PCOS. In this same vein, high HOMA-IR was also significantly correlated with high AMH in the HC sample, with 45.5% of high AMH subjects showing high HOMA-IR levels. This observation provides preliminary evidence that the relationship between IR and AMH may not be restricted to women with PCOS, and while still limited, other studies suggest this possibility as well. Capuzzo et al.[36] noted that among normoovulatory women, persons with high AMH may exhibit subtle endocrine or metabolic abnormalities. Teede et al.[37] reported that IR could appear before symptoms of PCOS show in women with higher levels of AMH, which indicates the possibility of a preclinical metabolic phenotype. However, there are some

studies that do not support a clear relationship between AMH and IR. For instance, Simoes-Pereira et al.[38] did not report a linear correlation between AMH and IR among non-PCOS groups based on the individuals' ages, BMI, and differences in how AMH was measured. So the differences in the sample population's characteristics, how participants were chosen to be part of a study, and the methodology used by each study to analyze data might account for some of the differences found when reading these studies. Thus, our findings support the idea that AMH has an even bigger role than just being a biological marker of reproduction by suggesting that AMH could be linked as an indicator for metabolic health. This finding also has far-reaching clinical applications concerning how to recognize and prevent the development of PCOS and other metabolic diseases that are related to PCOS. To better understand the significance of our current findings, further long-term studies will need to examine the causal pathways and mechanisms linking AMH to IR in many different populations.

In this investigation, microRNA 320a-3p expression is statistically correlated with both the LH/FSH ratio and LH serum concentrations in patient's diagnosed with PCOS but not with HC (Tables 5). This implies that microRNA 320a-3p is likely to play an important disease-specific regulatory role in the imbalance of gonadotropins which is characteristic of the development of PCOS. These results align with new evidence suggesting that microRNA-320 family members are involved in regulating reproductive hormones and ovarian function. For instance, Cirillo et al.[33] reported that microRNA-320a levels were dysregulated in the granulosa cells in PCOS women, and this dysregulation was linked to impaired folliculogenesis and altered LH signaling pathways. Similarly, Zhang et al.[39] demonstrated that microRNA-320a targets the pathways of runt-related transcription factor 2 and mitogen-activated protein kinase, both of which contribute to gonadotropin responsiveness and ovarian function steroidogenesis. The current study's observation of elevated LH/FSH ratios and serum LH levels in individuals with high microRNA-320a-3p expression further supports a mechanistic link between this miRNA and hypothalamic-pituitary-gonadal axis dysregulation. Notably, the LH/FSH ratio is widely recognized as an important diagnostic and pathophysiological marker in PCOS, contributing to anovulation and hyperandrogenism.[22] The miRNA's selective association with reproductive parameters, but not metabolic indices (e.g., FBG, insulin, AMH), suggests a functional specificity of microRNA-320a-3p in neuroendocrine signaling rather than in metabolic homeostasis. Moreover, the lack of association with metabolic indicators in our cohort aligns with previous studies that questioned the role of microRNA-320a in IR or glucose metabolism among PCOS patients.[40] Overall, this study contributes novel evidence to the field by highlighting a distinct regulatory relationship between microRNA-320a-3p and gonadotropin imbalance in PCOS. The findings of this study speculate that microRNA-320a-3p could be used as a biomarker for neuroendocrine dysregulation associated with PCOS; however, further studies will need to be conducted to establish causality and elaborate on the mechanisms of action.

The current work has also demonstrated an inverse correlation between FBG and AMH concentrations in women diagnosed with PCOS. This result is in line with other investigations indicating a role for both endocrine and metabolic pathways through which AMH influences the development of PCOS. Women having the highest amount of AMH available had significantly decreased concentrations of FBG than did women having lower amounts of AMH, indicating a potential protective mechanism by AMH from the effects of hyperglycemia (Table 6). Results from our study were consistent with those reported in the cohort of González et al.,[41] who found a negative relationship between elevated AMH concentrations in PCOS patients of reproductive age and FBS. However, our findings differed from the study by Md Muslim et al.,[42] which recorded no statistically significant associations between AMH and either FBG or IR in PCOS subjects. The differences observed may be due to different BMI distributions, AMH cut-offs used by the studies which classified study participants into their respective levels of AMH, or assay variability within different research settings. The observation of elevated levels of insulin concentration among women who have high levels of AMH raises the possibility of compensatory hyperinsulinemia. Similar observations were also found by Guo et al.,[43] who proposed a paradoxical relationship between AMH and FBG, stating that while AMH may be associated with low FBG, AMH may also be positively correlated with hyperinsulinemia due to impaired action of insulin at the cellular level. This evidence supports the concept that the markers of ovarian reserve and metabolic markers may have a complex and potentially bidirectional interaction.

The lack of association between AMH and gonadotropins (LH and FSH) found in our study was inconsistent with other studies, including Lie Fong et al.,[44] who found some evidence of a correlation between LH and AMH in a smaller and more homogenous population of women diagnosed with PCOS. It is likely that differences in sample sizes and to what extent the sample was representative of the larger population explain these discrepancies. The lack of association between AMH and gonadotropins in our analysis could be attributed to the insulin-dependent disruption of the hypothalamic-pituitary-ovarian axis, which may separate the typical interactions of gonadotropins and ovarian follicular activity.

In addition to supporting the concept that AMH has many influences (e.g., metabolic and hormonal) not yet captured by standard laboratory measurements, the lack of correlation between AMH and metabolic or hormonal markers in the control group indicates that AMH has a greater range of influences (e.g., metabolic and hormonal) in the dysregulated metabolic-endocrine environment of women with PCOS than in women without menstrual irregularities.

There are various limitations within this research study. One limitation is that the sample size was very low (n=90), restricting its applicability to larger populations and decreasing the overall power of the outcome variables. Additionally, the research was conducted at only one location (Iraq), which could introduce geographic/ethnic bias that is not present in other countries. Finally, because of the case/control design of this study, there is no way to determine how levels of biomarkers evolve over time, or due to treatment/intervention. Therefore, it would be beneficial to include a larger number of microRNAs in addition to microRNA-320a-3p; this might provide additional or more accurate information for diagnostic purposes.

The unexpectedly low HOMA-IR measurement values that were recorded in the PCOS group need to be interpreted with caution. These results may be indicative of the predominant presence of a lean or non-obese phenotype within the population of patients with PCOS along with other uncontrolled confounding factors, including BMI, dietary consumption and level of exercise. In addition, the HOMA-IR calculation provides an estimation of systemic insulin IR but does not assess tissue-specific IR (eg, ovarian IR or hepatic IR), and therefore it is possible that tissue-specific IR continues to exist despite the measurement of lower systemic IR.

Conclusion

This research demonstrates that microRNA-320a-3p and AMH have diagnostic value for women with PCOS, adding to understanding of both the physiological and clinical implications of these factors. Expression of microRNA-320a-3p is statistically decreased in PCOS women, and there are changes in the amount of AMH produced by the body. The correlation between microRNA-320a-3p and IR (HOMA-IR), LH, and the LH/FSH ratio indicates that these biomarkers represent disturbances in both metabolism and the neuroendocrine system, which are inherent to PCOS.

The different patterns of expression observed between patients with PCOS and HC suggest that microRNA-320a-3p and AMH may be useful as additional diagnostic markers alongside traditional biochemical and hormonal parameters. Furthermore, the relationship between these markers supports that there is a multi-dimensional role for them in defining PCOS phenotype heterogeneity. In addition to serving as a diagnostic biomarker for PCOS, microRNA-320a-3p has the potential to be used as a therapeutic target in the future, due to its function in regulating metabolic and reproductive pathways involved in the pathophysiology of PCOS. Therefore, large-scale, multi-center, longitudinal studies are needed to confirm the observations of this study; however, this work highlights the potential value of using molecular biomarkers as part of the diagnostic framework for PCOS, thereby facilitating earlier diagnosis, improving risk stratification, and eventually providing a foundation for developing individualized management approaches.

Declarations

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Author contributions

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

Conflicts of interest

The authors affirm there is no conflict of interest.

Data availability

The data used in the research and/or the methodology are available from the corresponding author by request.

Ethics approval

Conducting this study project was approved by the ethics committee of the training and human development unit of the Ministry of Health in Iraq via the Helsinki Declaration on september 3, 2024, as it was part of my master's thesis (Approval No: 197/2024).

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ORIGINAL PAPER

Sotagliflozin prevents acute kidney injury by suppressing oxidative stress, inflammation, and apoptosis in renal ischemia/reperfusion rat model

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ABSTRACT

Introduction and aim. Acute kidney injury (AKI) is a life-threatening condition with limited effective pharmacological options. Although sodium-glucose cotransporter 2 (SGLT2) inhibitors have shown renal protective effects, the potential role of the dual SGLT1/2 inhibitor sotagliflozin in ischemia/reperfusion injury (IRI) has not been previously investigated. We aimed to evaluate its nephroprotective properties in a rat model of renal IRI.

Material and methods. Twenty-four male Sprague-Dawley rats were randomized into four groups (sham, control, dimethyl sulfoxide [DMSO], sotagliflozin). Renal IRI was induced by 40 min ischemia followed by 2 h reperfusion. Rats received either DMSO or sotagliflozin (10 mg/kg, intraperitoneally) 24 h and 1 h before surgery. Kidney function (urea, creatinine, neutrophil gelatinase-associated lipocalin [NGAL]), oxidative stress (8-iso-prostaglandin F2α [8-iso-PGF2α]), inflammation (tumor necrosis factor-alpha [TNF-α]), apoptosis (caspase-3), and histopathology were assessed.

Results. In the control group, serum urea (106.5±2.9 mg/dL), creatinine (1.52±0.09 mg/dL), and NGAL (64.5±3.6 ng/mL) were significantly higher than in the sham group (32.6±5.3, 0.89±0.06, 49.5±3.8, respectively; $p<0.0001$). Tissue 8-iso-PGF2α (63.8±5.9 pg/mL), TNF-α (186±7 pg/mL), and caspase 3 (120.3±6.5 pmol/L) were also elevated vs. sham (35.6±3.6, 137±7, 92.3±4.9; $p<0.0001$). Sotagliflozin pretreatment reduced urea (53.8±2.8 mg/dL), creatinine (1.04±0.07 mg/dL), NGAL (49.6±6.4 ng/mL), 8-iso-PGF2α (41.3±3.9 pg/mL), TNF-α (140±6.6 pg/mL), and caspase 3 (89.7±2.4 pmol/L; all $p<0.0001$ vs. control). Histological injury scores improved from 4.0 in control to 1.0 in the sotagliflozin group ($p<0.05$).

Conclusion. Sotagliflozin significantly improved renal function and histopathological damage in rats with renal IRI by attenuating oxidative stress, inflammation, and apoptosis. These findings support its potential as a candidate for further investigation in the prevention of AKI.

Keywords. acute kidney injury, apoptosis, oxidative stress, sotagliflozin

Introduction

Acute kidney injury (AKI) is common in emergency department patients, raising the risk of chronic kidney disease, and is responsible for an estimated 2 million deaths annually worldwide.[1] It can be defined as a rapid decline in kidney function typically occurring over a short period, leading to acute damage to the renal parenchyma, disturbances in electrolyte balance, fluid retention, electrolyte imbalances, and nitrogenous metabolic waste product accumulation.[2-4] Several factors can impair blood flow, including organ transplantation, systemic infections, trauma or crush injuries, and atherosclerotic plaque buildup in coronary arteries that restricts oxygen delivery to the heart.[5-6]

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Renal ischemia is especially harmful as the kidneys are highly metabolic organs that depend mostly on a continuous blood flow to operate. Particularly in the proximal tubule, the thick ascending limb, and tubular epithelial cells, whose high energy needs make them most vulnerable when blood flow is decreased.[7] By starting the ischemia phase, ATP levels decreased, and the energy-dependent processes like ion transport, protein synthesis, and maintenance of membrane integrity began to fail. The loss of cell polarity and the inability of Na^+/K^+-ATPase pump to maintain ionic gradients result from cytoskeletal disruption. This, in turn, causes intracellular sodium and calcium excess, which leads to cell swelling and activates harmful protease enzymes.[8]

When blood flow is restored during the reperfusion phase, a high level of reactive oxygen species (ROS) is created. Along with the reduction in the body's antioxidant enzyme activity, these harmful compounds damage DNA, peroxidize lipid membranes, and cause protein oxidation.[9] 8-iso-prostaglandin F2α (8-iso-PGF2α) is a part of a group of chemical compounds called f2-isoprostanes, formed in kidney tissue by non-enzymatic oxidation of arachidonic acid in membranes and released into the circulation and urine.[10] Aside from acting as indicators of oxidative stress, 8-iso-PGF2α actively worsens renal ischemia and reperfusion injury (IRI) by causing vasoconstriction and inflammation. It also impairs glomerular filtration, disturbs tubular function, and increases endothelial dysfunction and fibrosis, all of which contribute directly to the progression of renal impairment.[11] Inflammation is an early response to IRI. A pro-inflammatory cytokine such as tumor necrosis factor-alpha (TNF-α) is rapidly elevated in renal tissue following ischemia/reperfusion. It primarily communicates through TNF receptor 1 (TNFR1), which features a death domain that triggers apoptotic pathways. TNFR1 activation attracts adaptor proteins that convert procaspase 8 into its active form, caspase 8. The active caspase 8 then cleaves and enhances caspase 3 activation, leading to apoptotic cell death.[12] Now, treatments for renal ischemia are limited to supportive measures, including hydration and renal replacement therapy; no effective therapeutic alternatives are available. Many drugs have been studied for their possible kidney-protecting effects, but the dual inhibition of sodium-glucose cotransporter (SGLT1/2) has not been studied enough in this context. Sotagliflozin is a new oral dual SGLT1/2 inhibitor that uniquely acts on both glycemic and non-glycemic pathways, including modulation of inflammation, oxidative stress, and sodium handling, which are central in IRI pathogenesis.[13] It lowers blood glucose levels in people with type 2 diabetes mellitus by inhibiting SGLT2 in the proximal tubule and blocking SGLT1 in the small intestine and kidney, leading to decreased postprandial glucose absorption and enhanced incretin release, which supports glycemic control, blood pressure reduction, and weight loss alongside favorable renal outcomes.[14] Furthermore, emerging evidence indicates that sotagliflozin shows unique cardiovascular advantages that are not uniformly seen with selective SGLT2 inhibitors.[15,16] Apart from its strong blood glucose-lowering action, new data indicate that sotagliflozin also lowers inflammation, slows down cell death, and provides antioxidative effects.[17] While those outcomes are encouraging, more research is needed to better understand their long-term safety and establish the appropriate therapeutic application across a wide range of patient conditions. While renal protective effects of selective SGLT2 inhibitors have been reported, the potential of the dual SGLT1/2 inhibitor sotagliflozin in ischemia/reperfusion injury has not yet been systematically evaluated. This study explores this novel application in a rat model.

Aim

This study aims to evaluate the nephroprotective potential of sotagliflozin in an experimental model of renal ischemia/reperfusion by assessing histological changes and key biochemical markers, including serum urea, creatinine, NGAL, as well as renal tissue 8-iso-PGF2α, TNF-α, and caspase 3, employing spectrophotometric and enzyme linked immunosorbent assay (ELISA) methodologies.

Material and methods

Animal preparation

The University of Kufa, Faculty of Science, provided 24 male Sprague Dawley adult rats, weighing approximately 180–250 g and aged 12 to 18 weeks. The rats were housed in the animal facility. Equipped with a 12-hour light-dark cycle, they were maintained at room temperature (approximately 24±2°C) and humidity (approximately 60±5%). Housed separately using a group-caging approach, the animals had free access to water and food. Female rats and those with other abnormalities were excluded from consideration. All experimental procedures and animal handling were carried out after receiving approval from the Institutional Animal Care and Use Committee (IACUC) at Kufa University, following the submission of the required documents (NO.2126) on January 23, 2025.

Study design

After 7 days of adaptation, a computer-generated sequence was used to assign twenty-four Sprague-Dawley rats into four experimental groups (n = 6 each group) to ensure that the groupings were random. The sham group underwent identical procedures to the control group, excluding the clamping of the renal pedicles. However, the control group experienced bilateral re-

nal ischemia for 40 min, followed by 2 h of reperfusion, based on previous preclinical studies.[10,18] While in the dimethyl sulfoxide (DMSO) group, the rats underwent the same procedures as the control group but were given two doses of DMSO intraperitoneally (IP), the first 24 h before surgery and the second one hour before, which was considered pharmacologically inert and often used in preclinical studies.[19] The sotagliflozin group was injected with two doses of sotagliflozin (10 mg/kg), the first 24 h and the second, one hour before surgery. A dose of 10 mg/kg was selected based on previous in vivo studies.[19-20]

Model of induction ischemia/reperfusion injury

Male rats were anesthetized using IP injections of ketamine hydrochloride (100 mg/kg) from (Sigma-Aldrich, USA, Cat. No K2753) combined with xylazine hydrochloride (10 mg/kg) from (Sigma-Aldrich, USA, Cat. No X1251).[21] The level of anesthesia was monitored throughout the surgery by looking at the pedal withdrawal response, corneal reaction, and breathing rate. Body temperature was maintained between 36.8°C and 37.3°C using a feedback-controlled heating pad, with the animal positioned prone. Renal ischemia was induced surgically by making flank incisions to expose both kidneys. Bilateral renal pedicles were occluded for 40 min using non-traumatic microvascular clamps to minimize tissue damage. The successful start of ischemia was proven by the appearance of uniform kidney pallor, consistent with previously documented models.[22-23] Once the ischemic period ended, the clamps were removed to initiate reperfusion, which was maintained for 2 h. Reperfusion was visually confirmed by the return of a reddish coloration to the kidneys. Surgical incisions were then closed using standard procedures.

Drug preparation

Sotagliflozin (TargetMol, Cat. No. T3547) was administered IP as two doses. It exhibits a solubility of 50 mg/mL in DMSO, the standard solvent, according to the manufacturer's instructions, and we prepared it immediately before use. The dosage was dissolved in DMSO (10% v/v) and administered based on body weight. The time and dose were based on previous investigations that looked at how sotagliflozin works in the body and how well it gets into tissues.[20,24]

Blood sample collection

After surgery, while the rat was still anesthetized, we obtained around 4 milliliters of blood from the heart of the rat by direct cardiac puncture.[25] To separate the serum from the blood, it was placed in a gel tube from (Medic-Home, China) that does not include any anticoagulant. Then the rat was immediately euthanized under ethical guidelines, and kidney tissue was harvested promptly to assess additional experimental parameters. The blood samples were kept for half an hour at ambient temperature, then centrifuged at 5000 rpm for about 10 min using a centrifuge (Hettich, Germany). We then used the resulting serum to identify urea, creatinine by using a spectrophotometer (Emclab/ Germany), as well as to identify NGAL by using an accessible rat-specific enzyme-linked immunosorbent assay (ELISA) kit (Ideal Medical Technology, Shanghai, Cat No. ADL-EL-RT00241).

Tissue sample preparation

After euthanizing the rats and taking the kidneys, the excised left kidneys were cut sagittally: one portion was frozen at −80°C for homogenization and utilized for further biochemical investigations, while the other portion was fixed by immersion in 10% formaldehyde for further histological examination. The frozen portion was homogenized in a 1:10 weight/volume phosphate-buffered saline containing 1% Triton X-100 and a protease inhibitor cocktail.[26] The homogenate was centrifuged for 10 minutes at 4°C and 6000 rpm. The supernatants were utilized for the assessment of the following parameters using rat-specific ELISA kits from (Ideal Medical Technology, Shanghai): 8-iso-PGF2α (Cat No: ADL-EL-RT00985), TNF-α (Cat No: ADL-EL-RT00160), and caspase 3 (Cat No: ADL-EL-RT00268). The results were obtained by following the instructions provided by the kit's manufacturer. To reduce bias, the assessors responsible for assessment of outcomes of biochemical tests were blinded to the groups of therapy.

Preparation of tissue for histopathological examination

After fixation in formaldehyde, the kidney tissues were rinsed in phosphate-buffered saline (PBS) and dehydrated through a graded ethanol series followed by xylene. The tissues were then embedded in paraffin. Sections of 5 μm thickness were cut using a microtome and stained with hematoxylin and eosin (H&E) for examination in a blinded method by a pathologist using a light microscope to assess kidney histological injury. The level of renal tubular injury was determined according to the severity of alterations in tubular cells, such as necrosis, hypertrophy, glomerular atrophy, and inflammatory cell infiltrate. Scores were given to these alterations as follows: score 0 (no pathological alterations), score 1 (<25%), score 2 (25–50%), score 3 (50–75%), score 4 (>75%).[27]

Statistical analysis

GraphPad Prism, version 10 (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis. Mean with standard deviation (SD) was the method of presentation of the findings. A one-way analysis of vari-

ance (ANOVA) was utilized to verify statistical significance across various treatment groups, with Tukey's multiple comparisons serving as a post-analytical test. A p-value of less than 0.05 was statistically significant. The histopathological variations were also compared among groups using Kruskal-Wallis and Dunn's test.

Results

Effect of sotagliflozin on renal function test

The control and DMSO groups exhibited a substantial increase in urea and creatinine levels in their serum, when compared with the sham group (p<0.0001). contributing to impaired renal function, as illustrated in Figure 1. Sotagliflozin substantially decreased serum levels of creatinine and urea in contrast to each of the control and DMSO groups (p<0.0001). Compared to the control group, the DMSO group did not demonstrate any evident differences in levels of urea (p=0.276) and creatinine (p=0.828).

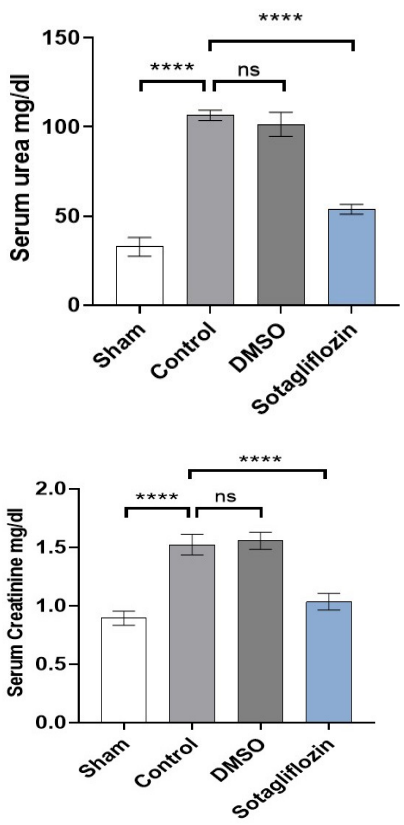

Fig. 1. Mean serum levels of urea and creatinine among experimental groups, ns – no significant difference, **** – p<0.0001, results are presented as the mean±standard deviation (n=6 per group)

Effect of sotagliflozin on neutrophil gelatinase-associated lipocalin (NGAL)

The study demonstrated that the control and DMSO groups exhibited substantially elevated serum NGAL levels compared to the sham (p<0.001), which is an earlier biomarker of tubular injury as highlighted in Figure 2. Pretreatment with sotagliflozin significantly reduced NGAL relative to the control group (p<0.001). The DMSO group did not exhibit any significant alterations concerning the control group (p=0.318).

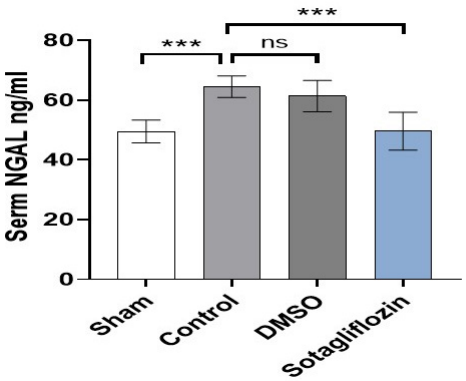

Fig. 2. Mean serum levels of NGAL among experimental groups ns. – no significant difference, *** – p<0.001, results are presented as the mean±standard deviation (n=6 per group)

Effect of sotagliflozin on 8-iso-PGF2α

Our results showed that there was an elevation in the level of 8-iso-PGF2α in the renal tissue homogenate of the control and DMSO groups compared to the sham group (p<0.0001), which is considered an indicative biomarker of lipid peroxidation and oxidative damage to renal tissues due to ischemia/reperfusion, as seen in Figure 3. Sotagliflozin significantly reduced these levels (p<0.0001) as opposed to the control. However, no observable variations in renal tissue 8-iso-PGF2α in the DMSO group compared to the control group (p=0.999).

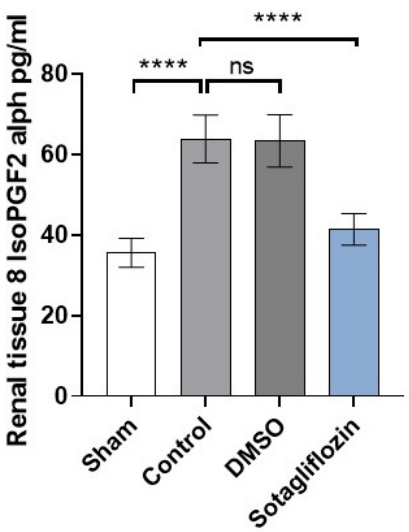

Fig. 3. Mean tissue levels of 8-Iso-PGF2α among experimental groups, ns – no significant difference, **** – p<0.0001, results are presented as the mean±standard deviation (n=6 per group)

Effect of sotagliflozin on TNF-α

Following ischemia/reperfusion, tissue proinflammatory cytokine (TNF-α) was significantly raised in the control and vehicle groups versus the sham group (p<0.0001), as shown in Figure 4. Sotagliflozin significantly reduced tissue (TNF-α) compared with a control group (p<0.0001). The DMSO and control groups did not vary significantly (p =0.705).

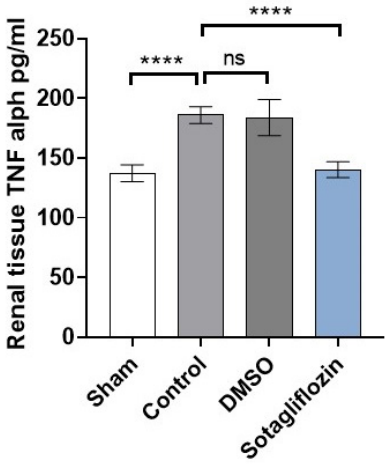

Fig. 4. Mean tissue levels of TNF-α among experimental groups, ns – no significant difference, **** – p<0.0001, results are presented as the mean±standard deviation (n=6 per group)

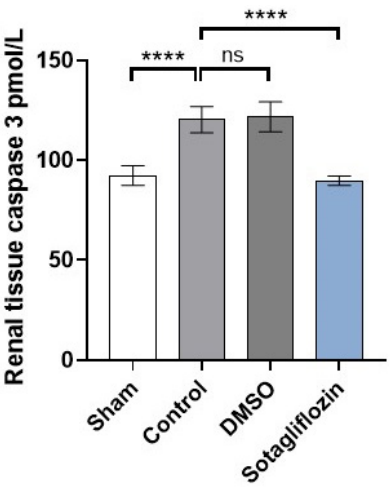

Fig. 5. Mean tissue levels of caspase 3 among experimental groups, ns – no significant difference, **** – p<0.0001, results are presented as the mean±standard deviation (n=6 per group)

Effect of sotagliflozin on caspase-3

Rats that underwent ischemia showed a significant upregulation of apoptotic marker caspase 3 in their kidney homogenate compared to sham rats (p<0.0001). This upregulation suggests that tubular cell death and kidney damage progression were accelerated in the DMSO and control groups, as shown in Figure 5. Sotagliflozin considerably decreased caspase 3 expression compared to the control (p<0.0001). There was no noticeable variance between the control and vehicle groups (p=0.902).

Histopathological examination

As seen in Figures 6 and 7A, 7B, 7C, and 7D, histological studies showed that the sham rats had normal glomeruli, tufts, and capsules, indicating a normal renal tissue microstructure a score of 0 (p<0.001). However, the control group's renal tubules developed inflammation, loss of brush border of proximal tubules, epithelial hypertrophy of lining cells, and the lumen of the proximal renal tubules became narrowed, with coagulative necrosis, and a vascular congestion with a score of 4. Similar histopathological abnormalities were seen in the DMSO group versus the control group. In contrast, the sotagliflozin group showed improvement in renal tubular architecture with preservation of the typical glomerular capsule and tuft. Moreover, mild cytoplasmic vacuolation and slight hypertrophy were observed in the tubular epithelial cells. These changes corresponded with a statistically significant reduction in injury scores compared to the control group, a score of 1, and p<0.05.

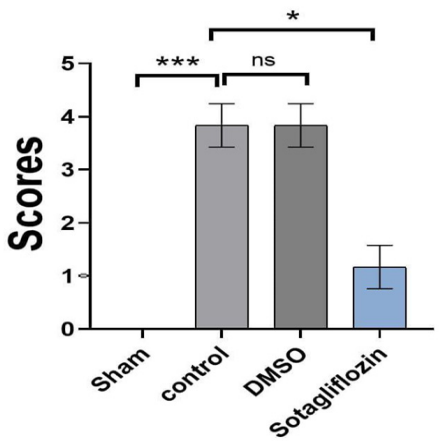

Fig. 6. Scores of renal tubular injuries in the studied groups, ns – no significant difference, *** – p <0.001 vs. sham group, * – p<0.05 vs. control group, the Kruskal-Wallis and Dunn's tests were used for analyzing the data, the data are presented as the mean±standard deviation (n=6 per group)

Discussion

To our knowledge, this is the first experimental evidence that sotagliflozin attenuates biochemical and histological features of renal IRI, expanding current understanding of SGLT inhibition beyond selective SGLT2 blockers. A major challenge in the proficiency of organ transplantation is the ischemia/reperfusion storm. So, it is essential to improve transplant techniques while developing an innovative treatment to avoid IRI.[28] There is a surge in local production of proinflammatory cytokines after injury from ischemia/reperfusion. These cytokines

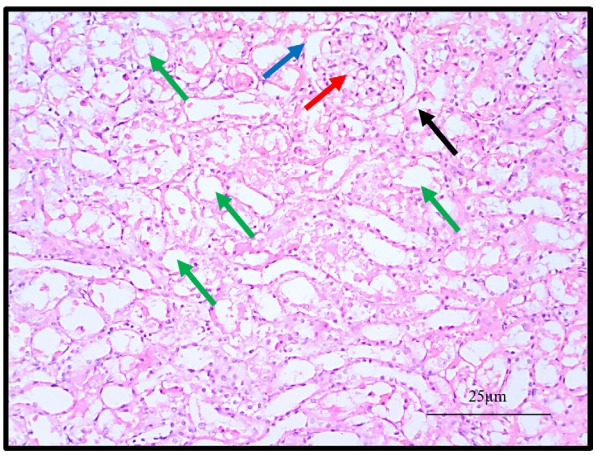

Fig. 7A. The histological section of kidney of a rat in sham group, the section shows normal renal tissue texture including normal glomeruli (black arrow), normal glomerular tuft (red arrow), and normal glomerular capsule (blue arrow), the proximal renal tubules show normal epithelial cells that line the tubules with a normal lumen (green arrows), the tissue was stained with H&E, and a digital camera and light microscope were used to observe the slice at a magnification of 100×

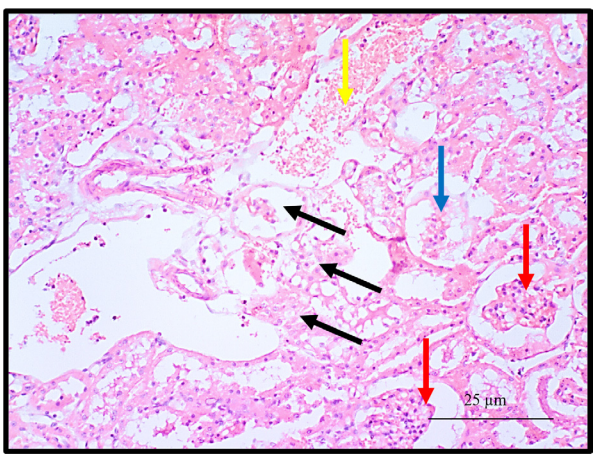

Fig. 7B. The histopathological section of kidney of a rat in control group, the section shows clear necrotic lesions in the renal tissue as coagulative necrosis (black arrows) with glomerular atrophy can be seen in the section of renal tissue (red arrows), the glomerular tuft shows severe necrotic change in epithelial cells of tuft (blue arrow), blood vessel congestion, as severe pathological changes can be seen in this section (yellow arrow), the tissue was stained with H&E, and a digital camera and light microscope were used to observe the slice at a magnification of 100×

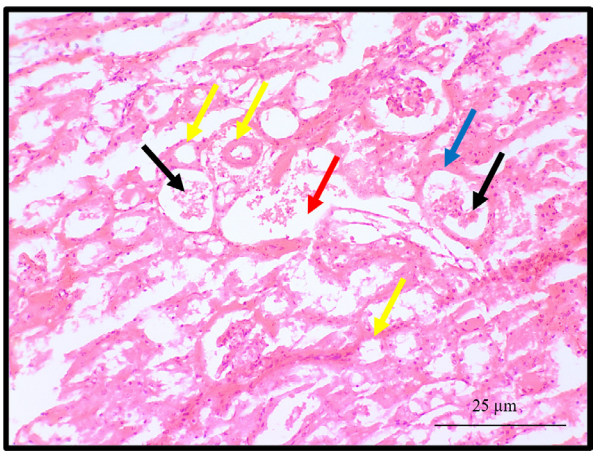

Fig. 7C. The histopathological section of kidney of a rat in DMSO group, this section of renal tissue shows severe atrophic lesion of renal glomeruli and glomerular tufts (black arrows) with severe hypertrophic changes in the glomerular capsule (blue arrow), the blood vessels show clear wall damage and congestion (red arrow), the proximal renal tubules show narrowing in the lumen with epithelial hypertrophy of lining cells (yellow arrows), the tissue was stained with H&E, and a digital camera and light microscope were used to observe the slice at a magnification of 100×

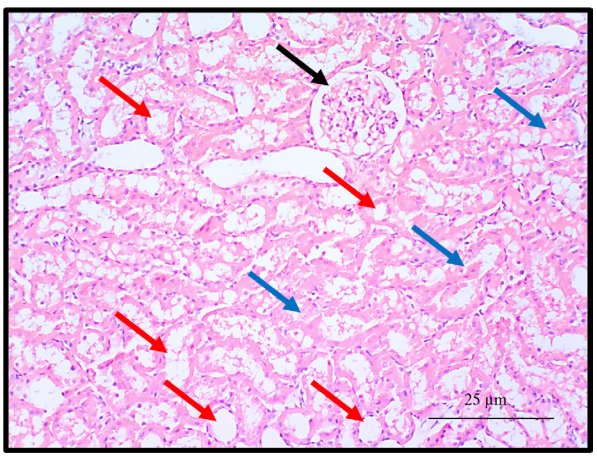

Fig. 7D. The histopathological section of kidney of a rat in sotagliflozin-treated group, this section shows a normal glomerular capsule and tuft (black arrow) with normal proximal renal tubule lumen (red arrows), the epithelial lining cells of renal tubules show moderate hypertrophy and vacuolation of cytoplasm (blue arrows), the tissue was stained with H&E, and a digital camera and light microscope were used to observe the slice at a magnification of 100×

can either intensify organ damage and dysfunction via the promotion of ROS production and the recruitment of inflammatory cells, or they may initiate protective physiological responses that limit and mitigate tissue damage.[21] The current study demonstrated that functional and structural kidney biomarkers like urea, creatinine, and NGAL levels were considerably higher in the groups undergoing renal ischemia/reperfusion surgery, indicating deterioration in renal function and tubular damage. A previous experimental study, harmonious with our findings, found that rats in control and vehicle groups demonstrated significant elevation in urea, Cr, and NGAL levels, as a consequence of renal ischemia/reperfusion.[29] However, sotagliflozin greatly lowered these renal biomarkers and improved filtration function. To the best of our knowledge, no previous research has focused on investigating the impact of sotagliflozin on urea, creatinine, and NGAL, in the context of renal IRI. Nevertheless, an earlier study demonstrated that mice administered SGLT inhibitors, such as empagliflozin, following renal ischemia and reperfusion exhibited a significant decrease in serum urea, creatinine, and NGAL.[30] When blood flow is restored in reperfusion period, the quick oxygen supply inversely causes a surge in the generation of ROS, mostly from mitochondrial complexes I and III, as well as from xanthine oxidase and NADPH oxidase pathways. These ROS start a chain reaction of cell damage, the most important of which is lipid peroxidation.[31] 8-iso-PGF2α is produced when the excessive ROS generation attacks arachidonic acid in cell membranes, leading to oxidative damage to renal tissues that weakens these membranes, making them more permeable, causing ionic equilibrium to be lost, and eventually causing necrotic and apoptotic cell death.[9,32] The current study revealed a significant elevation in 8-iso-PGF2α levels in the homogenate of renal tissue for the control and DMSO groups. This result is in line with findings of Basu and colleagues, who showed 8-iso-PGF2α levels were substantially raised upon reperfusion of the transplanted graft after human renal transplantation.[33] Interestingly, sotagliflozin reduced 8-iso-PGF2α, therefore displaying antioxidative properties. As far as we are aware, no previous projects have detailed how sotagliflozin affects the 8-iso-PGF2α in a rat model of renal IRI. However, several studies provided evidence that SGLT inhibitors such as dapagliflozin and canagliflozin have antioxidative impacts by lowering the production of ROS and these isoprostane compounds in various experimental models and settings.[34-35] At the same time, ROS is considered a powerful signaling molecules that turn on redox-sensitive transcription factors that include nuclear factor-kappa B (NF-κB), thereby stimulating pro-inflammatory cytokines, such as TNF-α, which makes the inflammatory response stronger, contributing to leukocyte recruitment, endothelial dysfunction, and tubular cell damage.[12,36] Our study demonstrated a significant increase in TNF-α in the rats after undergoing ischemia. This finding aligns with Woods and coworkers, who observed that TNF-α was significantly elevated in mice following renal IRI.[35] By dramatically reducing TNF-α, our findings exhibited that sotagliflozin has potential nephroprotective effects by mitigating inflammation and improving kidney function after renal IRI. To our knowledge, no prior research has examined the impact of sotagliflozin on TNF-α levels in renal IRI. Nonetheless, previous studies indicated that SGLT inhibitors like empagliflozin and dapagliflozin decrease the mRNA expression of TNF-α and IL-1β and demonstrate anti-inflammatory effects in diabetic renal proximal tubular cells.[30,34] Activation of caspases is a crucial component in cell death following renal IRI. In this study, levels of caspase 3 in the control and DMSO groups were significantly elevated compared with the sham group, indicating enhanced apoptosis in tubular cells. This finding aligns with the research conducted by Shan and colleagues in a rat model of renal IRI.[37] The rats that received sotagliflozin showed a notable reduction in caspase 3 levels. This indicates that sotagliflozin suppressed apoptosis in renal tubular cells. Currently, there are no published studies directly investigating the effect of sotagliflozin on caspase 3 expression in the context of renal IRI. However, earlier studies have shown that SGLT inhibitors significantly lower caspase 3 levels and reduce renal damage, suggesting that they have a protective effect on the kidneys.[38-39] Several studies revealed the protective effects of SGLT2 inhibitors like dapagliflozin and canagliflozin in diabetic and chronic kidney impairment models, and their main impact was only on the proximal tubule, where SGLT2 is found.[34-35] However, sotagliflozin works by blocking both SGLT1 in the small intestine as well as SGLT2 in the kidney. These mechanisms slow down the absorption of glucose in the intestines and change the levels of hormones like incretins that come from the gut. This dual beneficial effect may provide additional anti-inflammatory, anti-oxidative, and metabolic impacts than SGLT2 inhibitors, especially in renal IRI, where inflammation and oxidative stress make the damage worse. The histological examination displayed no aberrant pathological alterations in the sham group; the glomeruli and tubules were healthy. However, in the control group, there was evidence of loss of brush border in proximal tubules, renal interstitial hemorrhage, inflammatory cell infiltration, blood vessel congestion, cellular edema, and different intensities of coagulative necrosis. Our findings were in agreement with the findings of Zeid and coworkers.[18] Interestingly, the rats pre-treated with sotagliflozin exhibited intact tissue integrity compared to the control and DMSO groups. Our findings are in agreement with several studies that demonstrated the

SGLT2 inhibitor mitigated kidney injury, fibrosis, macrophage infiltration, and coagulative necrosis.[35,40] Taken together, as explained in Figure 8, our findings indicated that the big drops in urea, Cr, NGAL, 8-iso-PGF2α, TNF-α, and caspase-3 all point to the fact that sotagliflozin protects the kidney by not only improving metabolic balance through SGLT1/2 inhibition, but also by lowering oxidative stress, inflammation, and apoptotic pathways. These findings provide a rationale for further studies to determine whether sotagliflozin's dual mechanism may confer additional benefits compared to SGLT2 inhibitors in renal injury.

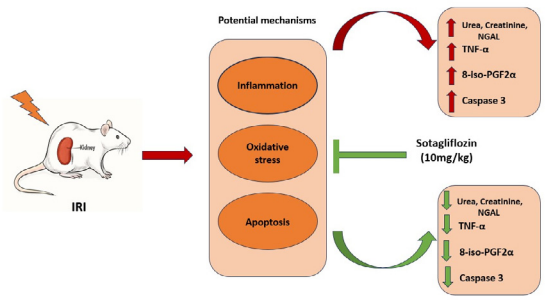

Fig. 8. Schematic diagram representing the mechanisms involved in the renoprotective effects of sotagliflozin following renal ischemia/reperfusion injury

Study limitation

This research has some notable limitations that must be highlighted. The reperfusion period was restricted to 2 h, permitting the study of acute damage signs but precluding the measurement of long-term outcomes such as fibrosis, delayed cell death, or kidney function repair. The collection of tissue and serum samples at just one time point limits understanding of the temporal evolution of sotagliflozin's impacts. Future research should include serial measurements. Another limitation was using one dosage of sotagliflozin (10 mg/kg), and this may not show all of the drug's dose-dependent effects. We selected this dosage because previous research showed that it worked in animal models of metabolic and cardiovascular disorders. Furthermore, all studies were performed only on a male Sprague Dawley rat. We didn't include female animals since hormonal differences might make things even more complicated, especially in the healing and repairing of kidney damage. Our results showed that sotagliflozin lowers oxidative stress, inflammation, and apoptotic indicators in renal I/R damage, but we still do not know precisely how it does this at the molecular level. We did not directly study the expression or activity of renal SGLT1/2 proteins and the signaling pathways that could be implicated, such as NF-κB, Nrf2/ARE, and Bcl-2/Bax that may be very important in causing the effects that were seen. The relevance of these results to human clinical scenarios is theoretical and requires additional confirmation in larger animal models and via extended investigations.

Future directions

The future studies should apply longer reperfusion intervals, such as 24 to 72 hours or even a few weeks, to see how chronic alterations happen. Moreover, further studies are needed to find out whether sotagliflozin has different impacts on male and female animals, especially when it uses to protect the kidneys. further research applying different doses of sotagliflozin are needed to find the best dose and better understand the dose-response relationship in renal IRI. Another future research using Western blotting or PCR to study NF-κB, Nrf2, and Bcl-2/Bax pathways to better understand how sotagliflozin protects the kidneys. Additionally, future studies should investigate the combination of sotagliflozin with anti-inflammatory, or antioxidant medications as well as studies comparing sotagliflozin effects with other SGLT inhibitors that might help find out whether it gives better protection.

Conclusion

This study provided evidence that sotagliflozin has renoprotective effects by improving renal function and histopathological abnormalities, as well as regulating oxidative stress, inflammation, and apoptotic cell death. These findings suggest its potential therapeutic application in preventing and treating acute kidney injury.

Declarations

Funding

No particular funds were allotted to this study.

Author contributions

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

Conflict of interest

No conflict of interest has been indicated by the researchers.

Ethical approval

The research adhered to ethical principles and received approval from the Institutional Animal Care and Use Committee (IACUC) at the University of Kufa, Iraq, after the submission of the required requests (NO. 2126 on January 23, 2025).

Data availability

Data is supplied in response to reasonable requests.

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ORIGINAL PAPER

The relationship between dry eye syndrome and serum fat-soluble vitamin levels

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ABSTRACT

Introduction and aim. Dry eye syndrome (DES) is a multifactorial ocular surface disease in which nutritional deficiencies, including fat-soluble vitamins, may play a role. This study aimed to investigate the relationship between serum levels of vitamins A, D, E, and K and DES.

Material and methods. A total of 70 eyes from 35 patients with DES and 70 eyes from 35 healthy controls were examined. Dry eye evaluation included tear osmolarity (TO), tear break-up time (TBUT), Schirmer I test, and the Ocular Surface Disease Index (OSDI). Serum vitamin levels (A, D, E, K) were measured using HPLC, LC-MS/MS, and chemiluminescence immunoassay.

Results. Patients with DES had significantly higher TO (312.7±4.9 vs. 295.2±6.5 mOsm/L, p<0.001) and OSDI scores (39.8±19.4 vs. 17.2±11.0, p<0.001), and significantly lower Schirmer I (7.0±1.8 vs. 17.3±1.3 mm, p<0.001) and TBUT values (7.3±0.8 vs. 16.1±0.7 s, p<0.001) compared with controls. Serum vitamin A (331.8±87.2 vs. 523.6±109.1 ng/mL, p<0.001) and vitamin D (14.8±6.9 vs. 34.6±14.9 ng/mL, p<0.001) levels were significantly lower in DES patients, whereas vitamin E and K did not differ between groups.

Conclusion. Deficiencies in vitamins A and D are associated with impaired tear film parameters and symptoms of DES. These findings suggest that assessing and correcting vitamin A and D deficiency may have clinical relevance in managing DES.

Keywords. dry eye, fat-soluble vitamin, tear osmolari

Introduction

Hyperosmolarity, resulting from an increase in the concentration of particles in tears, contributes to the development of dry eye disease by causing tissue damage and inflammation of the ocular surface. Dry eye syndrome (DES) is a common and complex multifactorial condition that can lead to irritation, damage to the ocular surface, vision loss, and a decline in overall quality of life.[1,2]

The global prevalence of DES ranges from 5% to 87.5%.[3] Reduced tear production and volume, increased tear evaporation, and significant changes in the lacrimal glands, including histological, functional, and structural changes, define this condition. Atrophy of the meibomian glands and metaplasia of the ocular surface are observed. These factors contribute to local ocular changes that precede the onset of DES.[3]

Numerous studies have indicated that vitamin D plays a crucial role in tear production and cell differentiation, and that DES can develop as a result of vitamin D deficiency.[4-6] A recent study has shown that megalin and cubilin, components of tear fluid that transport vitamin D, are produced by the lacrimal and accessory glands.[7] Several other eye disorders, including age-related macular degeneration, diabetic retinopathy, myo-

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pia, and uveitis, as well as systemic conditions such as cardiovascular diseases and connective tissue disorders, are influenced by vitamin D levels.[8-13] Other fat-soluble vitamins, such as vitamins A, E, and K, also have various effects on human metabolism.[14] For example, vitamin A is essential for growth, reproduction, gene transcription, bone metabolism, and immune health, and it is important for both photopic and scotopic vision. Its deficiency, particularly in malnourished children, is associated with eye diseases such as corneal ulceration and keratomalacia.[15,16] Alpha-tocopherol, one of the eight isoforms of vitamin E, is among nature's most potent fat-soluble antioxidants, and its deficiency may contribute to the development of several autoimmune diseases.[17,18]

Additionally, vitamin K plays a crucial role in regulating bone and cartilage mineralization.[19,20]

While several studies have linked vitamin D deficiency with DES, only a few have simultaneously investigated other fat-soluble vitamins. Our study, therefore, provides a broader perspective by assessing vitamins A, D, E, and K together in relation to DES.

Aim

This study aims to determine the relationship between fat-soluble vitamin levels and dry eye syndrome and, in the presence of a relationship, to support the development of new treatment methods for DES with vitamin replacement.

Material and methods

Seventy eyes of 35 patients with DES (group 1) and 70 eyes of 35 healthy volunteers without symptoms of DES and without ocular or systemic conditions that could affect the tear film (group 2) were included in this study. The study was approved to the Institutional Ethics Committee (approval no: 2016-493), by the principles of the Declaration of Helsinki, and written informed consent was obtained from all patients. Patients with a history of primary Sjögren's syndrome (pSS) or another systemic rheumatic disease, previous ocular surgery, current or recent systemic or topical drug use that may affect the lacrimal functional unit, active ocular infection, ocular allergy, current contact lens wear, and chronic tobacco or alcohol use were excluded. Visual acuity, slit-lamp, and fundus examinations were all performed on each patient. Tear osmolarity, Schirmer test, and tear breakup time (TBUT) were performed in sequence. Each subject was tested by the same ophthalmologist at the same time of day under the same fixed conditions, including the temperature and humidity. Five-minute (min) interval was determined between each measurement of the ocular surface. Three consecutive measurements were obtained for each test, and the mean values were used in the analysis. The mean data of both eyes for each patient were evaluated separately, and because the results between the two eyes were very similar, the results of the right eye were used for evaluation. Initially, all participants completed the ocular surface disease index (OSDI) survey.[21]

Tear osmolarity measurements were evaluated using a TearLab osmometer (TearLab Corp., San Diego, CA). Tears were collected from the inferior lateral tear meniscus; 3 sequential measurements were made, and their average was used in the evaluation.[22] Tear osmolarity values ≤308 mOsm/L are within the expected physiological range, whereas values >308 mOsm/L indicate tear film instability.

As previously mentioned, the Schirmer I test was performed without topical anesthesia using a 5×35 mm filter strip (Schirmer Plus; GECIS, Czech Republic). The paper was removed from the eye after a 5-minute interval, and the length of the wet portion was measured using the millimeter lines on the paper as reference points. A Schirmer test value of less than 10 mm per 5 minutes was regarded as abnormal.

As previously mentioned, tear breakup time was assessed by examining the fluorescein-stained tear film (Pricon, Iscon, India) using cobalt blue light under a slit lamp and measuring the time between blinking and the appearance of the first dry spot. TBUT values less than 10 seconds were considered abnormal. Fluorescein was used only for the TBUT test.

The OSDI is a 12-item disease-specific questionnaire developed by Schiffmann et al. that allows the evaluation of symptoms related to ocular irritation as well as quality of life (QoL).[21] There are three subgroups in the scale: five items assess ocular discomfort, four assess visual functions, and three assess environmental triggers. Each item was scored 0 to 4. OSDI scores ranged from 0 to 100, with lower scores indicating a lower quality of life in terms of vision. A dry eye score greater than 15 was used to define dry eye.

After the eye examinations were completed, all participants were directed to the laboratory for plasma vitamin level assessment. Blood samples were collected from the antecubital vein between 08:00 and 11:30 in the morning after fasting overnight in Becton Dickinson's biochemistry tube with a gel separator. Serum was obtained by centrifuging the blood sample at 4000 g for 10 minutes after coagulation, as it was taken into the biochemistry tube. The serum was portioned into sample separation tubes and stored at –80°C until the study was conducted. The pre-study portioned serum sample was analyzed on the same day after complete defrosting.

Vitamin K1 by LC-MS/MS method in Waters Corp. MS (USA), Vitamin A and Vitamin E by HPLC method in Shimadzu, SPD-M20A by HPLC analyzer (Japan), 25 dihydroxy vitamin D in Coulter UniCel DxI 800 (Germany) immunoassay analyzer using chemiluminescence method were analyzed.[23]

The deficiency was determined as <300 nanograms per milliliter(ng/mL) for vitamin A, <20 ng/mL for vitamin D, <5.5 micrograms per milliliter(μg/mL) for vitamin E, and, <1.1 ng/mL for vitamin K.[14]

Statistical analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 20; SPSS Inc., Chicago, IL). The normality of the distribution of each parameter was checked using the non-parametric two-tailed Mann-Whitney U test. An independent study t-test was used to compare tear osmolarity, Schirmer I test, TBUT values, and OSDI scores between the groups. Statistical significance was set at $p<0.05$. This statistic was obtained using the sample size estimation software package, G*Power. Based on this calculation, the required sample size was 65 patients, considering tear osmolarity (TO), tear break-up time (TBUT), and Schirmer's test as the primary outcomes. The results obtained according to the statistical program were as follows: effect size d=0.60, α=0.05, power=0.80. The sample size was increased by 10% to account for potential dropouts. Consequently, 35 patients were required for each group.

Table 1. The comparisons of the tear film parameters and OSDI scores between two groups (right eyes)

Parameter	Group 1	Group 2	p
Tear osmolarity (mOsm/L)	312.68±4.91 (290–320)	295.2±6.5 (272–306)	0.001
TBUT (sec)	7.3±0.8 (4–13)	16.1±0.7 (12–21)	0.001
Schirmer test (mm)	7.0±1.82 (4–14)	17.28±1.3 (13–22)	0.001
OSDI	34.82±19.36 (22–81)	17.19±14.97 (4–65)	0.001

Table 2. The comparison of the vitamin levels between two groups

Vitamin	Group 1	Group 2	p
A (ng/mL)	331.84±42.9 (208–733)	523.6±60.9 (309–701)	0.001
D (ng/mL)	14.79±6.86 (3–33.45)	34.56±14.87 (13.37–62.92)	0.001
E (μg/mL)	14.06±4.35 (7–24)	14.55±5.5 (6.7–34.2)	0.733
K (ng/mL)	1.32±0.51 (0.87–3.13)	1.40±0.46 (0.86–2.33)	0.584

Results

The mean age of the participants was 52.84±10.36 years (range: 38–70 years) in group 1 (19 women, 16 men) and 54.76±13.04 years (range: 33–67 years) in group 2 (17 women, 18 men). There were no significant differences in age or sex between the groups ($p_1=0.553$ and $p_2>0.999$, respectively). No differences were observed between the eyes for any of the parameters in the patients. All tear film parameters for the two groups are listed in Table 1. The mean tear osmolarity was 312.68±4.91 mOsm/L in group 1 and 295.2±6.51 mOsm/L in group 2. The OSDI scores were 39.82±19.36 and 17.19±10.97 in groups 1 and 2, respectively. The mean tear osmolarity and OSDI scores were significantly higher in group 1 than in group 2, while the Schirmer I test and TBUT measurements were substantially lower (p=0.001 for all). Vitamin A and D levels were significantly lower in group 1 than in group 2 (p=0.001), while vitamin E and K levels were similar in both groups (p>0.05) (Table 2). The vitamin A level was 331.84±87.22 ng/mL in group 1 and 523.6±109.08 ng/mL in group 2. The vitamin D level was 14.79±6.86 ng/mL in group 1 and 34.56±14.87 ng/mL in group 2. Group 1 patients exhibited statistically significant dry eye test results and significantly lower levels of vitamin A and D.

Discussion

In this study, we investigated the relationship between the serum levels of fat-soluble vitamins and dry eye syndrome, which has not been previously studied.

There are human and animal studies in the literature showing the relationship between systemic diseases and vitamins A, D, and E.[12,23,24] In recent years, many studies have been conducted showing the factors associated with dry eye syndrome, and studies showing the connection between vitamin D deficiency and DES have also been intensively studied in the literature.[2-5,8,25-29] Some studies have shown that vitamin A deficiency can also cause DES and that DES is improved with oral omega-3 treatment.[30-32]

Numerous studies have discussed the role of vitamin D in tear secretion and the corneal epithelium. Lu et al. reported that the lacrimal and accessory glands are responsible for producing tear fluid, which contains megalin and cubilin proteins, that facilitate the transport of vitamin D.[6] Yin et al. found that corneal epithelial cells possess vitamin D receptors and 1-hydroxylase mRNA, as well as high concentrations of vitamin D metabolites in both the aqueous and vitreous humor.[33] A meta-analysis conducted by Liu et al. revealed that patients with DES had lower serum vitamin D levels compared to healthy controls, suggesting that vitamin D deficiency may be a potential risk factor for DES.[25] According to this meta-analysis, vitamin D-deficient patients exhibited lower Schirmer test scores, which indicates reduced baseline and reflex tear production. This decrease in tear production may be attributed to vitamin D deficiency, which correlates with higher OSDI scores and reflects greater discomfort in affected individuals. However, TBUT scores, which assess the stability of the tear film, were similar between patients with vitamin D deficiency and the control groups. This indicates that vitamin D influences Schirmer test results and

TBUT scores differently; vitamin D deficiency appears to be associated with significantly reduced tear production (as evidenced by lower Schirmer test scores) but does not seem to affect tear film stability (as indicated by comparable TBUT scores).

The novelty of our study lies in the simultaneous evaluation of all major fat-soluble vitamins in patients with DES, which extends beyond previous research primarily focused on vitamin D. In particular, our findings highlight that vitamin A deficiency, although less frequently studied, may also contribute to ocular surface impairment.

In the present study, the Schirmer test and TBUT values were significantly lower, TO was significantly higher, and serum vitamin D was significantly lower in the DES group. The fact that we only evaluated dry eye tests and did not perform an analysis at the receptor level may be considered a limitation of our study.

The role of vitamin D supplementation in treating patients with DES has been investigated since vitamin D deficiency has been shown to be a risk factor. One study demonstrated that vitamin D supplementation resulted in a significant decrease in TO,[2] while another study revealed a notable increase in TBUT and a reduction in OSDI scores.[28] Karaca et al. reported that Schirmer 1, TBUT, TO, and OSDI scores significantly improved at the end of an 8-week oral vitamin D supplementation treatment in patients with DES who had vitamin D deficiency.[34]

Szodoray et al. found that plasma levels of vitamin A and D in patients with primary Sjögren's syndrome (pSS) were comparable to those in the control group; however, vitamin E levels were significantly elevated. They observed that vitamin A levels were notably reduced in patients with pSS exhibiting extraglandular manifestations, suggesting a more systemic and severe disease course. This observation led them to hypothesize that decreased vitamin A levels, along with immunomodulatory activity, may play a critical role in the progression of the disease, particularly in cases with a generalized and more pronounced course of the disease.[24]

Alanazi et al. reported that in their study, they administered 1500 mg of oral vitamin A for three consecutive days to male patients in both the DES and control groups. The results showed a significant increase in TO after treatment in the DES group, while there was no change in TBUT and no significant changes in any parameters in the control group. Consequently, the authors concluded that vitamin A enhances the quality of tears rather than their quantity.[30]

In this study, we measured the plasma concentrations of only the biologically active forms of vitamins A, E, 25(OH)2D3, and K. Our findings indicated that the DES group had significantly lower plasma vitamin D levels and lower, although not statistically significant, vitamin A levels. Furthermore, the DES group demonstrated significantly lower Schirmer test scores and TBUT than the control group. No vitamin treatment was evaluated in this study. Further research is needed on this topic.

Study limitations

This study has several limitations. First, the sample size was small, which makes it challenging to generalize the findings. Second, only the vitamin levels in the blood were examined, while the vitamin levels in the tears were not assessed. Future studies involving larger patient groups that examine vitamin levels in both tear secretion and serum will be essential for advancing the literature.

Conclusion

In conclusion, this study demonstrates that deficiencies in vitamins A and D – two vitamins essential for corneal epithelial function – may be associated with dry eye syndrome and vision-related quality of life. Furthermore, tear osmolarity may serve as a potential marker for vitamin deficiencies linked to DES. Additional research is needed to elucidate the pathophysiological mechanisms underlying the relationship between low vitamin levels and DES, as well as to investigate whether replenishing these deficient vitamins can result in improvements in the condition.

Declarations

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Author contributions

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

Conflicts of interest

The authors have no conflict of interest to declare.

Data availability

The study data are held by the authors and can be made available upon request.

Ethics approval

The study was reviewed and approved by the Bagcilar Training and Research Hospital Ethics Committee with the 2016-493 registration number.

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ORIGINAL PAPER

Postural, behavioural and cognitive effects of sit-stand desk use in primary school children – a crossover intervention pilot study

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ABSTRACT

Introduction and aim. This study explores the effects of implementing stand-biased desks in a classroom setting on physical posture, cognitive performance, and attention in elementary school students aged 11 to 12 years old.

Material and methods. The study group consisted of 51 boys from 5th and 6th grades. A crossover intervention design was employed, where students alternated between using traditional and sit-stand desks over the course of the study lasting three months. Anthropometric measurements, posture assessments, and cognitive tests (the d2 Test of Attention and Stroop), were conducted at three intervals (T1/T2/T3): initial, mid-intervention, and post-intervention.

Results. During the intervention, significant changes were observed in anthropometric parameters, except for a decrease in body fat percentage. Cognitive testing revealed significant improvements in attentiveness and cognitive control when using the sit-stand desks. Specifically, the d2 test indicated enhanced concentration performance and test effectiveness, particularly when conducted in a standing position. The Stroop test also showed improvements in both time and corrected errors between the second and third assessments.

Conclusion. The findings suggest that sit-stand desks may associate with better weight distribution and improved posture, with positive effects on attentiveness and cognitive performance of schoolchildren.

Keywords. attention, cognitive function, elementary school pupils, posture, sedentary behavior, sit-stand desks

Introduction

In the context of primary education, the increasing prevalence of sedentary behavior among schoolchildren has raised significant concerns about its negative impact on physical health, cognitive development, associated with various health risks, including obesity, cardiovascular diseases, and metabolic disorders, and impaired cognitive function,[1–3] academic achievement, and overall well-being. Traditional classroom settings, characterized by extended periods of sitting, have been identified as a contributing factor to sedentary behaviors. The use of sit-stand desks in educational settings has gained increasing attention as an intervention to reduce sedentary behavior among schoolchildren and enhance various aspects of their physical and cognitive development. Numerous studies have suggested that the use of sit-stand desks, as an alternative to traditional seated desks, is seen as a feasible and effective method to integrate movement into the classroom, fostering both physical health and cognitive engagement:[4,5] by allowing children to alternate between sitting and standing positions, these desks are designed to increase energy

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expenditure and reduce sedentary time, enhance classroom engagement, improve executive functions (attention, working memory) and behavioral outcomes without disrupting academic activities.

However, while the theoretical benefits are compelling, empirical evidence remains mixed, highlighting the need for further investigation to clarify the specific conditions under which standing desks might prove effective.[6,7] The current research landscape reveals significant gaps in understanding the long-term cognitive effects of standing desks on schoolchildren. Many studies are short-term, lack randomized controlled designs, or are limited by small sample sizes, thereby reducing the generalizability of their findings.

This paper builds on previous research by providing a comprehensive review of the literature on standing desks and identifying some of the key gaps by conducting a crossover intervention study to evaluate the cognitive benefits of standing desks in a primary school setting. The study will focus on their impact on physical activity levels and cognitive outcomes in children, providing a comprehensive understanding of how standing desks can influence their attention. By employing a robust experimental design, this research seeks to contribute valuable insights into the efficacy of standing desks as a tool for enhancing educational and health benefits in schools and produce implications for educational policy and practice, providing a nuanced understanding of how environmental modifications in classrooms can support holistic primary schoolchildren development. The theoretical foundation of the sit-stand desk intervention is based on the idea that physical activity can improve the overall well-being of schoolchildren, as well as some of significant health-related parameters and cognitive functions.

Based on the analysis presented above, it is possible to conclude that despite the growing body of reporting promising benefits of sit-stand desks, several gaps remain in the literature. First, while the physical activity benefits of sit-stand desks are well-documented, their direct impact on cognitive functions such as attention and executive function needs further exploration. Second, most studies focus on short-term interventions, and long-term effects on physical activity, cognitive function, and academic performance are less explored.[4] Many studies suffer from methodological weaknesses,[6] such as small sample sizes, which require further investigation to prove the reliability and generalizability of findings. Additionally, the practicality of implementing sit-stand desks in classrooms poses challenges. In a similar fashion, Sherry et al. reported logistical issues such as classroom space constraints and the need for ergonomic training for both pupils and teachers.[8–10] There is also a need for more detailed qualitative research to understand the experiences and perspectives of pupils, teachers, and parents regarding the use of sit-stand desks.[11] Furthermore, the heterogeneity in study designs, outcomes measured, and intervention implementations makes it difficult to draw firm conclusions. For instance, variations in desk types (e.g., fixed height vs. adjustable height), the duration of standing, and the specific cognitive and academic measures used complicate comparisons across studies.[12] Recent advances in computer vision-based activity recognition, extended reality–enabled learning environments, and reliable human-movement signal acquisition further support the view that classroom ergonomics and technology-mediated designs can meaningfully influence posture, engagement, and cognitive outcomes. These works provide additional methodological and contextual grounding for our sit-stand desk intervention study.[13,14] Research is needed to explore the differential impact of sit-stand desks on various pupil/student populations, including those with attention-related disorders such as ADHD. Future studies should also investigate the optimal duration and frequency of standing periods to maximize cognitive benefits without causing fatigue or discomfort. These challenges highlight the necessity for collecting more data to gain solutions to address potential barriers to the successful adoption of sit-stand desks. This is the first study to comprehensively assess the health related effects of introducing standing desks for a short period of time in a school environment on body posture, anthropometric and cognitive abilities, physical activity, and sleep quality.

Aim

The purpose of this study is to investigate the effects of sit-stand desks on cognitive function, posture, and physical activity among primary school children. The research questions guiding this study are: 1) How does the use of sit-stand desks affect the distribution of static loads and spinal curvature in children? 2) What is the impact of sit-stand desks on reducing sedentary behavior and increasing physical activity during school hours? 3) Do sit-stand desks improve attention test scores in primary school children when tasks are performed standing versus sitting?

Material and methods

Study participants

Study group included 51 boys from football sport primary school in Rzeszów (Poland), aged 11-12 years (5 and 6 grade) the particular classes were chosen because they spend most of their lessons in one room, therefore the standing desks could be used in most of school time. Present study focuses on children in this age group since significant increases in sedentary time were observed between ages 9 and 12 years of age.[15]

Research design

In this non-randomized, interventional pilot study with cross-over design, convenience sample (feasible school infrastructure and the staff of the school, parents and children interested in participation) were used. Three data collection points were performed between March and May of 2025 – first one (T1) 4 weeks before introducing of standing desks to school environment, second measurement (T2) set directly before intervention start and third one (T3) after 4 weeks of intervention.

After consultation with teachers and the school principal, two classes were selected for the study. The parents or guardians of the children were sent an information letter with a consent form and all important details about the study intervention and methods, and an online meeting with the parents was organized to answer any questions. Children with parental informed consent were additionally required to provide verbal confirmation before any measurements were taken. Children and parents/guardians could withdraw from the study at any time without giving a reason. The study was conducted in accordance with ethical standards laid down in an appropriate version of the Declaration of Helsinki and has been approved by the Rector's Committee For The Ethics Of Scientific Research Involving Humans SGGW no. 4/RKE/2025/U. Children whose parents/guardians did not agree to participate or withdrew were still using the same classroom with standing desks and had the opportunity to use them if they wished but were not included in the measurement sets. Children were excluded from the project if they had an injury or illness that prevented them from attending class or from using standing desks.

Intervention

It was assumed that the participants would use the standing desks for approximately half of the lesson (the teacher was responsible for switching between the two types of desks). Consequently, one standing desk was provided for two children of similar body height. At the commencement of the first lesson, one participant utilised a standing desk, while the other employed a standard seated desk for approximately 20 minutes. Subsequently, the teacher requested that the students exchange their respective workstations. The children who had been engaged in standing for the latter half of the preceding lesson commenced the subsequent lesson with a seated desk. The option to revert to a standard desk was permitted at any point during the lesson.

Height-adjustable sit-stand desks were installed in classrooms adjacent to standard school desks, allowing for two children to utilise one standing desk for part of the lesson. This adjustment was necessary for a number of reasons. Firstly, it was not feasible to rapidly adjust the desks to various heights (a screwdriver was required), so children using one standing desk had similar heights. Secondly, it was important to allow children whose parents or guardians had not agreed or had withdrawn from the study to participate in lessons as usual. Thirdly, it was necessary to be able to return to a sitting position at any time, either at the child's request or at the teacher's discretion, depending on the specific tasks required in the lesson.

The initial phase of the study encompassed the initial set of measurements and the installation of standing desks in classrooms. Subsequently, the children continued with their lessons as usual for a period of four consecutive weeks, during which time they were unable to utilise the standing desks. Following this, a second set of measurements was conducted in accordance with the initial procedure. Thereafter, for a period of four weeks, the children whose parents had consented to participate were permitted to use the standing desks in accordance with the aforementioned protocol. Directly after the conclusion of the four-week intervention period, a third set of measurements was undertaken using the same protocol.

Anthropometry

Body weight and height (stretched stature) were measured in accordance with the ISAK protocol by an ISAK Level 3 anthropometrist. To assess stretched stature, a SECA stadiometer (model 217, SECA, Hamburg, Germany) was utilised. The participant was instructed to stand on the device barefoot and attempt to achieve the maximum height possible without lifting the heels from the base. A deep inhalation was then performed, and the measurement was taken at the point of maximum inhalation, recorded to the nearest 1 mm. This was done with the head positioned in Frankfurt plane and the moveable arm of the stadiometer in contact with the vertex anthropometric landmark.

Body composition

Body weight and composition were evaluated through the use of a bioelectrical impedance analysis (BIA), conducted with the aid of a body composition analyser device (BC-420 MA, Tanita, Tokyo, Japan). Body weight was assessed with an accuracy of 0.1 kg. Body mass index (BMI) was calculated according to the equation proposed by Quetelet: body mass (kg) divided by height squared (m^2) (kg/m^2). The BMI percentile of each participant was calculated using Polish age- and sex-specific BMI charts, based on the BMI values obtained.[16] In accordance with the criteria set forth by the Centers for Disease Control and Prevention, the percentiles for body mass index (BMI) in all subjects can be interpreted as indicative of normal body weight.[17] It was shown that BIA method is a reliable and accurate tool for the measurement of body composition in the paediatric population.[18] The children were instructed to stand barefoot on

the device with light clothing. All measurements were taken in the morning (8:00–10:00). The children and parents/guardians of the participants were informed that they should refrain from engaging in strenuous exercise and excessive water intake for a minimum of eight hours prior to the measurement. The measurements were repeated in the following sets, always in the same hour and under the same conditions. For the purposes of the present study, the following body composition variables, estimated by the Tanita device, were analysed: body fat percentage (BFP), muscle mass (PMM) and total body water (TBW). The BFP percentage were found to yield normal values (BFP < 85th percentile and ≥ 2nd percentile) for the entire study group.[19]

Body posture analysis

The assessment of body posture was conducted using the KINEOD system (DMS Group, Gallargues-Le-Montueux, France), which enables the precise estimation of the angular values of the physiological curvatures of the spine in the sagittal plane. This is achieved through the 3D reconstruction of the posterior surface of the body, which is obtained through the use of depth cameras that acquire images using infrared. Prior to the examination, the requisite anthropometric landmarks were delineated on the child's back with the aid of a specialized hypoallergenic body marker (specifically, the C7 spinous process and multiple spinous processes in the thoracic and lumbar spine, the posterior superior iliac spines, and the inferior angles of the scapulae). Immediately following the examination, the identified points were removed using a solution of water and cleansing agent. The values of the lumbar lordosis and thoracic kyphosis angles of the examined children were estimated using dedicated software.

Foot static loads assessment

During the assessment of body posture, the subject was invited to stand freely on a tensometric platform (Free Med BASE, Sensor Medica, Guidonia Montecelio, Italy) in order to ascertain the static loads exerted on the feet during the subject's natural, unassisted stance. The device software calculated, among other variables, the maximum and mean pressure under each foot, expressed in g/cm². This was based on several seconds of data recording.

Physical activity, sedentary behavior and sleep

ActiGraph measurements were obtained over a continuous period of seven days and nights using the ActiGraph GT3X-BT monitor (ActiGraph, Pensacola, Florida, USA) and were subsequently analysed using the ActiLife 6.13 data analysis software. Actigraphy represents a valid alternative to polysomnography.[20] The device was positioned at the level of the right hip. Subjects were instructed to wear the monitor for a period of 24 hours per day, for a total of seven consecutive days and nights, during all activities, with the exception of those involving water. Parents or guardians were also provided with comprehensive instructions on how their children should utilise the activity monitor. Actigraphy data were collected at a sampling rate of 30 Hz, employing the Sadeh sleep algorithm.[21] The sleep parameters identified from the ActiGraphs were as follows: sleep efficiency (the percentage of time spent asleep during the sleep period), sleep duration (time from the child fell asleep until it woke up) and wake after sleep onset (WASO; refers to the number of minutes a child was awake between sleep onset and sleep offset).[22] After exclusion of the nocturnal sleep episode time, non-wear time was determined as 60 minutes of consecutive zeros allowing for 2 min of non-zero interruptions.[23] The time spent awake and the levels of physical activity were calculated and identified using data collected at 5-s intervals. A waking wear time of ≥ 500 minutes per day was employed as the criterion for a valid day, and ≥ 4 days were used as the criteria for a valid 7-day period of accumulated data (including ≥ 3 valid weekdays and ≥ 1 valid weekend day). For each participant, the mean minutes per day of moderate-to-vigorous physical activity (MVPA) and sedentary time were calculated. The cut-off points proposed by Evenson et al. were employed to ascertain the time spent engaged in sedentary (0-100 counts per minute) and MVPA (≥ 2296 counts per minute from all valid days). Furthermore, the number of steps taken was calculated.[24]

Cognitive test

The assessment of attention, concentration, ability to work under pressure, speed and accuracy of work was conducted and analysed by a licensed psychologist using the D2-R test. The test enables the assessment of various attention indicators, including perception, the number of errors committed, and the ability to perceive. It examines both continuous attention, which concerns the maintenance of active attention over a specified period of time, and selective attention, which pertains to the capacity to concentrate on selected stimuli. The test enables the assessment of the speed, quality and endurance of the individual being tested, as well as their ability to maintain focus over a short period of time. The test sheet comprises 14 lines, each containing 47 "d" or "p" leaders with varying combinations of lines beneath and/or above the letter. Each line comprises 21–22 letters that must be crossed out. The subject is allotted a specific time period for each line, with a maximum of 20 seconds. Once this time has elapsed, the subject must proceed to the subsequent line, regardless of whether they have completed the task for the previous line. The subject was required to identify and erase specific sym-

bols from a set of similar ones within a specified time frame (20 seconds). The results of the test provide indicators that allow for the assessment of an individual's ability to concentrate, as well as their pace and accuracy of work. The D2-R questionnaire was administered on two occasions, once at a standard desk in a seated position and once at the same time the following day at a high desk in a standing position.

Stroop test

The efficiency of executive functions, specifically the ability to inhibit a habitual response and the ability to switch to a new, previously unused response criterion, was evaluated by a licensed psychologist using the Stroop test. The initial phase of the examination comprised ten lines of five words, representing colours (blue, green, yellow, red, brown, pink, black), inscribed in black font on a white card (an achromatic colour-word reading card). The second card differs from the first in that the same words are written in coloured font, but the colour of the font in which the words are written does not correspond to their meaning (a chromatic colour-word reading). In the third part of the test, the stimuli were presented as rows of coloured squares (a pure colour card). The subject was required to complete the following task: In the initial phase, participants were required to read the names of colours presented in black font (referred to as Card A). In the subsequent phase, they were instructed to read the names of colours while disregarding the colour of the font in which they were written (referred to as Card B). Finally, in the third phase, participants were asked to name the colour of the squares presented on the card (referred to as Card C). In the fourth part of the test, the subject was required to name the colour of the font in which the word was written, ignoring its meaning (card B).

Statistical analysis

The statistical analysis was conducted using the Statistica software (StatSoft, Tulsa, OK, USA). The assumption of normality was tested using the Shapiro-Wilk test. Continuous data are expressed median and interquartile range. The Wilcoxon paired test was employed to ascertain whether statistically significant differences existed in the parameters between data collection points. The Wilcoxon signed-rank (paired) test was selected because the study compared repeated measurements of the same participants across two data collection points. Preliminary analysis indicated that the assumptions required for parametric tests, particularly normal distribution of the differences, were not met. The Wilcoxon paired test is a non-parametric alternative that does not require normality and is therefore appropriate for analyzing paired, dependent samples.

Results

At the initial assessment, 51 included boys were 11.5±0.6 years of age, with 151.4±8.6 cm height and 40.1±8.1 kg weight (BMI: 17.3±2.2 kg/m^2). In general, children exhibit a greater body weight distribution on the left foot and a more pronounced kyphosis than lordosis. The majority of children adhere to the WHO recommendations for physical activity (at least 150 minutes of MVPA per week) and report an average of more than eight hours of good-quality sleep per day (with a sleep efficiency of more than 90% and a wake after sleep onset duration of less than 25 minutes). All the anthropometric, body posture, physical activity, sleep and cognitive skills parameters are listed in Table 1.

During the intervention period, statistically significant increases were observed for all anthropometric parameters, with the exception of FATP, where a significant decrease was noted. The analysis of the static loads under the feet of the subjects revealed a significant increase in both the mean and maximum loads under the right foot. This is particularly noteworthy given that subjects exhibited a tendency to place greater weight on the left foot. The observed change therefore appeared to result in a more balanced distribution of loads under the feet of the subjects. The only significant difference in spinal curvatures in the sagittal plane was the increase in the lordosis angle between the initial and mid-intervention measurements. Nevertheless, it proved difficult to identify any discernible linear trends in this parameters (Table 2).

A comparison of the data reveals significant variations in sedentary behaviour and light physical activity (PA) across the various measurement points. Specifically, a notable increase in sedentary behaviour was observed prior to the implementation of standing desks, subsequently followed by a decrease in sedentary behaviour post-introduction of standing desks. It should be noted that there has been a constant reduction in light PA. Furthermore, there was a substantial increase in vigorous physical activity (PA) prior to the intervention, and a notable rise in step count following the intervention. The study's participants who were provided with standing desks for a portion of their lessons exhibited a notable enhancement in their sleep efficiency after a four-week period (Table 3).

The results of the d2 test revealed significant improvements in all cognitive parameters, with the exception of the Percentage of Errors and the Fluctuation Rate when the test was completed in a standing position. Moreover, when the test was performed in a sitting position, a notable decline in the Percentage of Errors was observed between the first and second measurement points. Also, the Fluctuation Rate demonstrated a significant increase between the first and second measurements and a further increase between the first and third measurements when subjects were sitting. A significant improvement was observed in the Stroop test between

Table 1. Analysed parameters at T0 – before the standing desks introduction*

	Mean	Min.	Max.	Q1	Q3	SD
Anthropometry						
Age (n)	11.5	10.0	12.0	11.0	12.0	0.6
Height (cm)	151.4	132.3	170.6	146.5	156.0	8.6
Weight (kg)	40.1	28.6	62.6	35.3	43.9	8.1
BMI (kg/m^2)	17.3	13.8	22.5	15.9	18.3	2.2
FATP (%)	12.8	5.5	25.3	9.6	14,2	4,7
PMM (kg)	33.0	23.4	50.1	29.5	35.6	6.0
TBW (kg)	25.5	18.2	38.6	22.8	27.5	4.6
Body posture						
Mean load LF (g/cm^2)	258.0	193.0	370.0	230.0	284.0	43.5
Mean load RF (g/cm^2)	203.5	154.0	263.0	179.0	230.0	31.0
Max load LF (g/cm^2)	619.0	450.0	997.0	539.0	675.0	123.8
Max load RF (g/cm^2)	537.3	358.0	776.0	470.0	590.0	102.5
Kifosis angle (°)	31.6	20.0	47.0	28.0	35.0	5.9
Lordosis angle (°)	17.3	0.0	37.0	3.0	26.0	11.9
Physical activity						
Sedentary (min)	7148.7	6430.0	8067.0	6895.5	7361.5	392.2
Light (min)	2367.3	1717.0	3091.0	2184.5	2589.0	336.0
Moderate (min)	374.3	123.0	643.0	302.5	445.0	112.6
Vigorous (min)	198.6	37.0	444.0	106.0	308.0	122.8
MVPA (min)	572.7	169.0	1069.0	433.0	717.5	204.9
Step Counts (n)	96344.7	50377.0	164193.0	80604.0	106931.5	23676.0
Sleep quality						
Average Sleep Efficiency	94.8	89.8	98.7	94.0	96.3	2.0
Average Total Sleep Time	490.0	335.6	623.8	435.5	543.3	71.4
WASO (min)	21.6	7.1	39.3	17.1	26.0	7.3
Cognitive skills						
Percentage of Errors SIT (%)	10.7	0.5	86.4	3.9	11.6	14.4
Percentage of Errors STN (%)	8.2	0.0	42.7	2.9	10.0	9.3
Concentration Performance SIT	148.6	83.0	198.0	133.5	161.5	23.2
Concentration Performance STN	179.0	115.0	256.0	156.0	198.5	31.0
The Fluctuation Rate SIT	6.4	2.0	12.0	5.0	8.0	2.3
The Fluctuation Rate STN	7.9	3.0	20.0	5.0	10.0	4.1
Total test effectiveness SIT	138.6	71.0	194.0	124.0	154.0	27.8
Total test effectiveness STN	168.9	98.0	253.0	146.0	194.0	35.3
Concentration index SIT	145.9	66.0	198.0	133.0	159.0	25.2
Concentration index STN	175.1	90.0	255.0	155.5	197.5	33.3
Stroop – time	27.8	16.0	51.0	22.0	32.5	8.3
Stroop – error corrected	0.9	0.0	4.0	0.0	1.5	1.1
Stroop – error noncorrected	0.1	0.0	2.0	0.0	0.0	0.4

* BMI – body mass index, Min. – minimum value, Max. – maximum value, Q1 – first quartile, Q3 – third quartile, SD – Standard Deviation, FATP – Fat Percentage, PMM – Muscle Mass, TBW – total body water, LF – left foot, RF – right foot, MVPA – moderate to vigorous physical activity, WASO – wake after sleep onset, SIT – sitting position, STN – standing position

Table 2. Analysed anthropometric parameters before (T1) during (T2) and after (T3) standing desks introduction*

	T1 Me (Q1–Q3)	T2 Me (Q1–Q3)	T3 Me (Q1–Q3)	p T1 vs T2	p T2 vs T3	p T1 vs T3
Anthropometry						
Height (cm)	150.2 (146.5–156)	150.3 (147–155.3)	151.0 (147–157)	0.001	<0.001	<0.001
Weight (kg)	38.0 (35.3–43.9)	38.2 (35.1–44.1)	39.5 (35.9–45.6)	<0.001	0.001	<0.001
BMI (kg/m^2)	16.9 (15.9–18.3)	16.9 (16.2–18.5)	17.0 (16–18.7)	<0.001	0.722	<0.001
FATP (%)	12.5 (9.6–14.2)	11.8 (9.5–14.1)	11.1 (8.8–13.8)	0.003	<0.001	<0.001
PMM (kg)	31.6 (29.5–35.6)	32.1 (28.8–35.6)	33.3 (29.5–37.8)	<0.001	<0.001	<0.001
TBW (kg)	24.5 (22.8–27.5)	24.8 (22.3–27.5)	25.8 (22.8–29.2)	<0.001	<0.001	<0.001
Body posture						
Mean load LF (g/cm^2)	245.0 (230–284)	255.0 (240–283)	242.0 (220.5–283.5)	0.336	0.288	0.057
Mean load RF (g/cm^2)	198.0 (179–230)	211.0 (188–231)	209.5 (189.5–247)	0.071	0.379	0.001
Max load LF (g/cm^2)	582.0 (539–675)	602.0 (543–689)	604.5 (538–717.5)	0.558	0.587	0.994
Max load RF (g/cm^2)	529.0 (470–590)	543.0 (509–628)	540.5 (477–608.5)	0.073	0.965	0.032
Kifosis angle (°)	32.0 (28–35)	33.5 (28–37)	33.0 (28–37)	0.801	0.706	0.840
Lordosis angle (°)	19 (3–26)	25 (19–31)	21 (10–28)	0.013	0.186	0.253

* T1 – measurement before intervention, T2 – measurement during intervention, T3 – measurement after intervention, Me – median, Q1 – first quartile, Q3 – third quartile, p – statistical significance, BMI – body mass index, FATP – fat percentage, PMM – muscle mass, TBW – total body water, LF – left foot, RF – right foot, SIT – sitting position, STN – standing position

crease in lumbar lordosis after four weeks of sit–stand desk use. The lordosis increase of approximately 5–6° falls within the small-to-moderate ergonomic effect range reported by Cardon et al. and Clemes et al. This supports the interpretation that alternating sitting and standing can promote healthier spinal alignment without imposing excessive lumbar extension.[4,28] While the study's non-randomized design precludes strong inference, these findings strengthen previous evidence that postural variability (rather than prolonged standing per se) can be beneficial for musculoskeletal development in schoolchildren. The observed increase in the lordosis angle between initial and mid-intervention measurements are consistent with previous research that highlights the potential of sit-stand desks to improve postural health in children.[25,29]

Cognitive improvements

The results of the d2 and Stroop tests demonstrate significant cognitive improvements. Specifically, the d2

Table 3. Analysed physical activity and sleep quality parameters before (T1) during (T2) and after (T3) standing desks introduction*

	T1	T2	T3	p		
	Me (Q1–Q3)	Me (Q1–Q3)	Me (Q1–Q3)	T1 vs T2	T2 vs T3	T1 vs T3
Sedentary (min)	7180 (6977–7357)	7956 (7675–8260)	7547 (7118–7965)	<0.001	0.003	0,031
Light (min)	2279 (2171–2433)	1568 (1321–1720)	1408 (1041–1606)	<0.001	0.015	<0,001
Moderate (min)	358 (305–415)	323 (268–393)	338 (224–434)	0.050	0.660	0,037
Vigorous (min)	175 (120–415)	222 (167–309)	292 (179–462)	0.015	0.102	0,018
MVPA (min)	550 (417–692)	580 (436–666)	638 (403–876)	0.257	0.203	0,226
Step Counts (n)	98477 (80604–106932)	92387 (70608–107565)	109752 (74378–123647)	0.334	0.038	0,379
Average Sleep Efficiency	95 (94–96)	96 (94–97)	97 (95–97)	0.410	0.068	0,038
Average Total Sleep Time	504 (436–543)	487 (449–573)	490 (456–593)	0.494	0.068	0,274
WASO (min)	21 (17–26)	17 (12–27)	16 (12–23)	0.900	0.225	0,274

* T1 – measurement before intervention, T2 – measurement during intervention, T3 – measurement after intervention, Me – median, Q1 – first quartile, Q3 – third quartile, , p – statistical significance, MVPA – Moderate to Vigorous Physical Activity, WASO – Wake After Sleep Onset, SIT – sitting position, STN – standing position

Table 4. Analysed cognitive skills before (T1) during (T2) and after (T3) standing desks introduction*

	T1	T2	T3	T1 vs T2	T2 vs T3	T1 vs T3
			D2–R test			
Percentage of Errors SIT (%)	7 (4–12)	6 (3–10)	6 (3–12)	0.028	0.992	0.373
Percentage of Errors STN (%)	5 (3–10)	6 (2–10)	6 (2–12)	0.695	0.345	0.854
Concentration Performance SIT	147 (134–162)	200 (175–231)	226 (196–256)	<0.001	<0.001	<0.001
Concentration Performance STN	176 (156–199)	219 (186–253)	231 (196–268)	<0.001	<0.001	<0.001
The Fluctuation Rate SIT	6 (5–8)	8 (6–10)	8 (5–10)	0.001	0.817	0.013
The Fluctuation Rate STN	7 (5–10)	8 (6–10)	8 (5–9)	0.809	0.169	0.498
Total test effectiveness SIT	139 (124–154)	193 (160–228)	218 (165–243)	<0.001	<0.001	<0.001
Total test effectiveness STN	170 (146–194)	202 (166–245)	220 (175–258)	<0.001	<0.001	<0.001
Concentration index SIT	144 (133–159)	199 (175–230)	224 (195–252)	<0.001	<0.001	<0.001
Concentration index STN	172 (156–198)	218 (177–243)	230 (191–266)	<0.001	<0.001	<0.001
			Stroop test			
Stroop – time	26 (22–33)	25 (22–32)	22 (20–29)	0.786	<0.001	<0.001
Stroop – error corrected	1 (0–2)	1 (0–2)	0 (0–1)	0.840	<0.001	<0.001
Stroop – error noncorrected	0 (0–0)	0 (0–0)	0 (0–0)	0.361	0.593	0.345

* T1 – measurement before intervention, T2 – measurement during intervention, T3 – Measurement after intervention, Me – median, Q1 – first quartile, Q3 – third quartile, p – statistical significance, SIT – sitting position, STN – standing position

test revealed improvements in all cognitive parameters except for the percentage of errors and fluctuation rate when completed in a standing position. The drop in the percentage of errors and the increase in the fluctuation rate observed when the test was performed in a sitting position between the first and second measurements suggest that sitting may lead to decreased cognitive stability over time. It is necessary to consider the possible learning effects which could bias the observed results. This finding aligns with studies by Swartz et al., Mehta et al. and Wallace et al., which reported improved cognitive performance and attentiveness in students using sit-stand desks.[3,30,31] Our findings are corroborated by Van der Niet et al., who found that sedentary behavior adversely impacts cognitive function, while physical fitness improves executive and cognitive function in children, highlighting the importance to include executive functioning in research on physical activity and academic achievement correlation.[32]

The significant improvement observed in the Stroop test between the second and third assessment points regarding both time and corrected error occurrence could possibly supports the cognitive benefits of the intervention. The Stroop test measures cognitive control and the ability to manage competing information, suggesting that the desks may enhance these executive functions.[5,30] However, given that cognitive testing was not blinded and standing time was self-regulated by teachers, the observed effects should be interpreted as associations rather than causal improvements. Practice effects across repeated administrations of the d2-R and Stroop tasks may have contributed to incremental performance gains; nonetheless, the greater magnitude of change understanding conditions suggests additive effect of postural activation on attentional efficiency.

Clinical implications

The significant improvements in cognitive parameters, particularly in the standing position, suggest that sit-stand desks can be a valuable tool in creating dynamic and engaging learning environments. The study's results also extend the current findings,[2,6,30] which substantiate that physical activity imposed by interchangeable sitting and standing in class improves cognitive function in children.

Table 5. Comparison of D2 test performed in sitting and in standing position*

	T1	T2	T3
Percentage of Errors SIT (%)	7 (4–12)	6 (3–10)	6 (3–12)
Percentage of Errors STN (%)	5 (3–10)	6 (2–10)	6 (2–12)
p	0.009	0.363	0.790
Concentration Performance SIT	147 (134–162)	200 (175–231)	226 (196–256)
Concentration Performance STN	176 (156–199)	219 (186–253)	231 (196–268)
p	<0.001	0.049	0.019
The Fluctuation Rate SIT	6 (5–8)	8 (6–10)	8 (5–10)
The Fluctuation Rate STN	7 (5–10)	8 (6–10)	8 (5–9)
p	0.030	0.959	0.328
Total test effectiveness SIT	139 (124–154)	193 (160–228)	218 (165–243)
Total test effectiveness STN	170 (146–194)	202 (166–245)	220 (175–258)
p	<0.001	0.041	0.008
Concentration index SIT	144 (133–159)	199 (175–230)	224 (195–252)
Concentration index STN	172 (156–198)	218 (177–243)	230 (191–266)
	<0.001	0.092	0.003

* T1 – measurement before intervention, T2 – measurement during intervention, T3 – measurement after intervention, SIT – sitting position, STN – standing position

the second and third assessment points with regard to both time and corrected error occurrence (Table 4).

A comparison of the results of the d2-R test performed in a sitting and standing position revealed that all cognitive parameters exhibited significantly better performance when the children completed the test in a standing position during the initial set of measurements. Furthermore, in the second and third measurement sets, significant improvements in the standing position were noted in both Concentration Performance and Total Test Effectiveness. Additionally, at the third measurement point, a significantly higher Concentration Index was observed (Table 5). Since the study protocol assumed multiple cognitive tests on the same population, it is necessary to bear in mind the possible learning effects that could bias the observed results.

Discussion

Anthropometric changes

The study results indicate that during the intervention period, there were statistically significant increases in all anthropometric parameters, with the exception of FATP, which showed a significant decrease. This finding aligns with previous research indicating that increased physical activity and reduced sedentary behavior can positively impact body composition.[25] The notable decrease in FATP suggests that the introduction of sit-stand desks, by encouraging more movement and standing, is more likely to be associated with sedentary behavior reduction (which resonates with the conclusions made by Benden et al., Clemens et al. and Swartz et al., and healthier body composition in children.[4,26,27]

Postural analyses demonstrated a more symmetrical weight distribution between the feet and a moderate in-

Study limitations

While the current pilot study provides insights into the possible benefits of sit-stand desks, several methodological and contextual factors constrain interpretation. First, the study included only boys from a sports-oriented primary school, which limits generalizability to mixed-sex or less active populations. Second, the non-randomized crossover and absence of blinding introduce potential expectancy, Hawthorne, and teacher-influence effects. Third, actual standing duration was not objectively logged; therefore, the fidelity and "dose" of the intervention remain uncertain. Fourth, the presence of standing desks in classrooms during the pre-intervention phase may have altered behavior ("contamination"). Fifthly, the crossover nature of the study necessitated repeated cognitive testing, which could have yielded learning effects. Therefore, any observed improvements may be partly due to familiarity with the tests. Finally, academic performance and psychosocial variables (motivation, enjoyment, fatigue) were not measured, leaving the educational implications incomplete.

Therefore, certain gaps remain that encourage further investigation. The long-term effects of sit-stand desk interventions on cognitive and academic outcomes need to be explored through longitudinal studies. Additionally, more research is needed to understand the differential impact of sit-stand desks on various student populations. Future studies should also investigate the optimal duration and frequency of standing periods to maximize cognitive benefits without causing fatigue or discomfort. Additionally, exploring the combined effects of sit-stand desks with other physical activity interventions, such as short activity breaks, could provide a more comprehensive understanding of how to best reduce sedentary behavior and enhance cognitive performance in educational settings. The combination of both quantitative and qualitative research designs (with an emphasis on the latter one, such as teachers' and students' perspective on the feasibility of sit-stand desk integration into classroom settings, as highlighted in Erwin et al. is seen as essential for the holistic approach to the studied intervention.[11]

Conclusions

This pilot study's findings demonstrate positive effects of integrating sit-stand desks into primary school classrooms. By allowing children to alternate between sitting and standing during cognitive tasks, improvements were observed in physical distribution, spinal curvature, and cognitive performance. This research contributes to a growing body of evidence supporting the adoption of dynamic workstations in educational settings to address both physical and cognitive challenges associated with prolonged sedentary behavior.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Ethics Committee (Resolution No. 4/RKE/2025/U, dated 7.03.2025) and conducted in accordance with the Declaration of Helsinki.

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REVIEW PAPER

The comparative efficacy of FDA-approved drugs for management of alcohol use disorder – a network meta-analysis

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ABSTRACT

Introduction and aim. Alcohol use disorder (AUD) represents a significant and lasting public health challenge affecting millions of people around the world. Currently, the US FDA has approved naltrexone, disulfiram, and acamprosate for AUD; however, their comparative effectiveness remains uncertain. This study aimed to evaluate the comparative efficacy of FDA-approved medications for AUD.

Material and methods. A comprehensive search of PubMed, the Cochrane Library, and Embase was performed up to January 2025. Eligible studies were randomized controlled trials of at least 12 weeks in duration, enrolling adults with AUD and investigating one or more FDA-approved medications, individually or in combination. The Cochrane Risk of Bias 2 (ROB 2) tool was used to assess study quality. A frequentist random-effects network meta-analysis (NMA) was performed. The primary and secondary outcomes were the return to any level of drinking and the return to heavy drinking, respectively.

Analysis of the literature. Fifty-two trials were included. Compared to placebo, acamprosate (risk ratio, RR, 0.87 [95% CI, 0.82-0.92]), naltrexone (0.93 [0.88-0.99]) and a combination of acamprosate and naltrexone (NAAC) (0.52 [0.35-0.76]) all statistically significantly reduced the risk of return to any type of drinking. Based on SUCRA rankings, NAAC (SUCRA = 0.99) was ranked first for efficacy. For the secondary outcome, only naltrexone (RR, 0.87 [0.80-0.95]) was found to be effective.

Conclusion. When combined with psychosocial interventions, naltrexone and acamprosate demonstrated superior efficacy compared to placebo. Furthermore, the combination of the two medications led to significantly better results.

Keywords. acamprosate, alcohol use disorder, disulfiram, meta-analysis, naltrexone, randomized controlled trial

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The list of abbreviations:

AUD alcohol use disorder, **ACAM** – acamprosate, **CI** confidence interval, **CINeMA** confidence in Network Meta Analysis, **DISL** disulfiram, **NAAC** – combination of acamprosate and naltrexone, **NALT** naltrexone, **NMA** – network meta-analysis, **PLBO** placebo, **RCTs** – randomized controlled trials RCTs, risk ratio, **SUCRA** – surface under the cumulative ranking curve

Introduction

Alcohol use disorder (AUD) is a prevalent and debilitating common behavioral health condition characterized by impaired control over alcohol consumption, leading to frequent sessions of uncontrolled drinking.[1] It poses a significant global health challenge, affecting a large proportion of the population and contributing to more than 2 million deaths annually.[2] According to estimates, individuals with AUD have a mortality risk 3-4 times higher compared to those without AUD.[3] However, the risk is reduced by half in AUD patients who receive treatment and successfully reduce their consumption of alcohol, when compared to that of those who persist with heavy drinking.[4] The principal aim in treating AUD is to enable individuals to attain and maintain abstinence, or to significantly decrease alcohol use and the accompanying behavioral harms. AUD management has several components, including a combination of behavioral interventions and pharmacotherapy. The pharmacological regimens for AUD include FDA-approved and recommended (off-label) medications. Currently, the US FDA has authorized the use of disulfiram, naltrexone, and acamprosate for this condition. Although these medications have shown promising results in clinical trials, as demonstrated by several systematic reviews and meta-analyses,[5–10] their relative efficacy is not well established.

Although the conventional meta-analysis approach is valuable, it has limitations. It can only compare two interventions at a time and is restricted to those evaluated in direct head-to-head trials. In contrast, network meta-analysis (NMA) is an advanced statistical technique that simultaneously compares several interventions utilizing evidence which is both direct and indirect. This approach offers a more comprehensive understanding of the comparative efficacy of various interventions for AUD.[11] Recent NMAs of clinical trials have assessed the comparative efficacy of various pharmacological interventions,[12,13] including treatments not yet recommended by any guidelines (ie, off-label drugs). Additionally, these NMAs included clinical trials with treatment durations shorter than 12 weeks, as many of the off-label interventions were tested over relatively brief time periods. Longitudinal research has suggested that studies with short durations of medications can produce erroneous conclusions about effectiveness due to the observed variability in alcohol consumption behavior patterns.[5] Consequently, the inclusion criteria for clinical trials in this analysis stipulate a minimum treatment period of at least twelve weeks thereby ensuring reliable findings, as was observed in a recent pairwise meta-analysis.[5]

Aim

Therefore, to address this gap, the present study conducts a systematic review and NMA of randomized controlled trials (RCTs) to determine the comparative effectiveness of FDA-approved treatments for AUD, with a focus on trials with durations of not less than 12 weeks. This study provides updated and robust evidence to help clinicians in selecting the most effective FDA-approved pharmacological interventions for people with AUD.

Material and methods

This review protocol was prospectively registered with (https://osf.io/5zfsg). The study adhered to the PRISMA guidelines, specifically the extension designed for systematic reviews that incorporate network meta-analyses.[14]

Data source and search strategy

We conducted an updated search from January 2022 to January 2025 in PubMed, the Cochrane Register of Controlled Trials (CENTRAL) and Embase to identify additional studies published since the last search date of a prior systematic review on this topic conducted by McPheeters et al.[5] Manual screening of reference lists from published systematic reviews was also performed. To ensure a comprehensive and systematic approach, search strategies were designed in accordance with the Population, Intervention, Comparator and Study Design (PICOS) framework, using a combination of indexing terms and free-text keywords.

Study selection

All RCTs with not less than 12 weeks of treatment duration that enrolled adults with AUD, regardless of comorbid conditions, and investigated an FDA approved drug (disulfiram, acamprosate, or naltrexone),[15] alone or in combination compared to control, no treatment, or another FDA-approved medication in outpatient clinics were included. The primary outcome was relapse, that is, the return to drinking, measured by the number of people who had relapsed with respect to any drinking at the end of the study.[16] The secondary outcome was the return to heavy drinking.[16] Relapse or return to any drinking was defined as the intake of any quantity of alcohol during the follow-up period. The return to heavy drinking was defined as consuming 4 or more drinks per day for women and 5 or more drinks per day for men.

Details of the search strategy are available in Table S1. Two reviewers (F.T. and A.H.) independently screened

the titles and abstracts to assess initial relevance. The full text articles were retrieved and reviewed to determine final eligibility. Any disagreements were addressed by discussion with a third reviewer (SV). We did not consider conference abstracts. Language restrictions were not applied during the study selection process.

Data extraction

Two reviewers (S.S. and F.T.) independently extracted data using a standardized extraction template. The number of participants initially randomly assigned to each study arm was recorded for all results. Data analysis was conducted regardless of the original authors' analytical approach.[17] Risk of bias was independently evaluated by two reviewers (C.J. and C.F.) using predefined criteria informed by established methodological guidance.[18,19] A third reviewer adjudicated any disagreements between the two reviewers. The risk of bias for each study was assessed and classified as low, medium, high, or unclear was assigned to each study.

Data synthesis and statistical analysis

For direct comparisons, conventional pairwise meta-analyses were conducted using a DerSimonian and Laird random-effects model to calculate pooled risk ratios (RRs) with 95% confidence intervals (CIs).[20] When more than one trial contributed to a comparison, heterogeneity was assessed using the I^2 statistics. To integrate both direct and indirect evidence, a frequentist random-effects network meta-analysis (NMA) with a consistency model was employed.[11] The network was anchored on a shared comparator, typically placebo or control. The consistency of the network was examined through a global inconsistency test using the design-by-treatment interaction model[21] and a loop-specific method.[22] Summary effects of the NMA were reported along with predictive intervals to help interpret findings in the context of observed heterogeneity.[11] To rank treatments by their relative efficacy, the surface under the cumulative ranking curve (SUCRA) were calculated.[23] Higher SUCRA scores (which can range from 0 to 1) indicated that an intervention is more likely to be better than those with lower scores. Possible small-study effects were investigated using comparison adjusted funnel plots. For the primary outcome, subgroup analyzes were performed according to the duration of follow-up (at least 12 weeks and more than 12 weeks). To assess the robustness of our results, we performed a sensitivity analysis by excluding trials of high risk of bias for the primary outcome. For statistical analysis, we used Stata version 16.0 (StataCorp, College Station, TX, USA).

Strength of evidence

We evaluated the certainty of evidence from NMA using the online software Confidence in Network Meta-Analysis (CINeMA) (https://cinema.ispm.unibe.ch/#rob [accessed January 2025]). The certainty was categorized into four levels: high, moderate, low, and very low. For the primary outcome, classification was performed across six methodological domains: reporting bias, within-study bias, , incoherence, heterogeneity, indirectness, and imprecision.[24,25] Two independent reviewers (SK, JC) performed the assessment of certainty of evidence, and in cases where there were discrepancies unresolved through discussion, a further reviewer (S.V.) was involved to reach a final consensus.

Analysis of the literature

The study search and selection process is illustrated in the PRISMA flow chart (Fig. S1). Our new search identified 169 citations following exclusion of duplicates. Following the selection of the title and abstract, 14 articles were retrieved for full text evaluation, but none met the eligibility criteria. The justifications for exclusion are reported in Table S2. Fifty-two studies[26–77] reporting primary or secondary outcomes were identified from previous reviews.[5] The characteristics of these studies are detailed in Table S3. Of the 52 studies, 4 were carried out in Asia, 2 in South America, 22 in North America, and 21 in Europe. Treatment durations varied between 12 and 52 weeks, while sample sizes ranged from 18 to 403 participants. The recruitment strategies employed in the studies involved a variety of approaches, including enrolling participants through treatment programs, using advertisements, receiving referrals, or employing a combination of these methods. Among the 52 studies, 40 (76.92%) included psychosocial cointerventions, whereas the remaining studies did not clearly report whether such cointerventions were used. Most studies (50 out of 52) used placebo as the comparator. Based on the risk of bias assessment (Table S3), 5 studies were rated as low risk, 38 as moderate and 7 as high. The risks of bias were unclear.

Primary outcome: return to any drinking

Thirty-seven RCTs (9474 patients) gave dichotomous data on the probability of returning to drinking. Five interventions have been included in the NMA (the network plot is shown in Fig. 1). The total number of comparisons of pairs with direct data was seven. Across the entire network, 7,160 events were recorded. The size of individual study results with 95% confidence intervals grouped by treatment comparison are given in Fig. S2.

NMA indicated that compared to placebo, acamprosate (RR, 0.87 [95% CI, 0.82 to 0.92]), naltrexone (RR, 0.93 [95% CI, 0.88 to 0.99]), and a combination of acamprosate and naltrexone (that is, NAAC) (RR, 0.52 [95% CI, 0.35 to 0.76]) were associated with a statistically significant reduction in the risk of relapse (return to any drinking) (Fig. 2). On the contrary, disulfiram did

not differ significantly from placebo (RR, 0.95 [95% CI, 0.82 to 1.12]). Comparative analyses between different interventions (Fig. 2) revealed that NAAC statistically significantly reduced the risk of relapse compared to disulfiram (RR, 0.54 [95% CI, 0.36 to 0.82]), acamprosate (RR, 0.59 [95% CI, 0.41 to 0.88]) and naltrexone (RR, 0.56 [95% CI, 0.38 to 0.82]). Comparison between acamprosate and naltrexone did not show statistically significant differences (RR, 1.67 [95% CI, 1.14 to 2.24]). The results of network meta-analyses resembled those obtained using standard pairwise meta-analyses (Fig. 2 and Fig. S3). Among the statistically significant comparisons in NMA illustrated in Figure 2, the 95% prediction intervals excluded the null value only in three comparisons (NAAC vs. disulfiram, NAAC vs acamprosate, and NAAC vs naltrexone) (Fig. 3). According to SUCRA rankings, NAAC (SUCRA = 0.99) had the highest probability of being the most effective, followed by acamprosate (SUCRA=0.70), naltrexone (SUCRA = 0.41) and disulfiram (SUCRA=0.31) (Fig. S4).

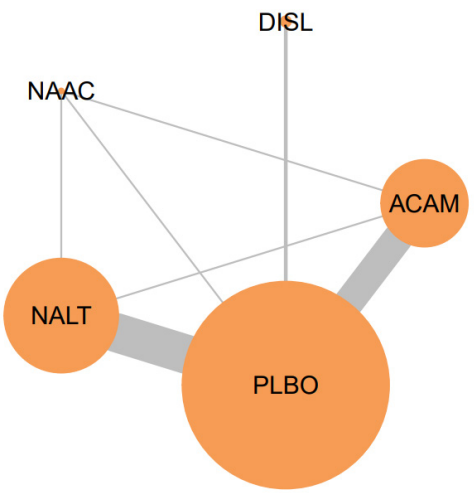

Fig. 1. Network plot for primary outcome (Notes: lines between nodes represent direct comparisons from head-to-head trials. The size reflects the number of studies for each intervention, while the thickness of the connecting lines corresponds to the number of trials that compare two strategies, ACAM acamprosate, DISL – disulfiram, NALT naltrexone, NAAC – combination of acamprosate and naltrexone, PLBO placebo)

In a subgroup analysis that included trials with follow-up of more than 12 weeks, only acamprosate demonstrated statistically significant differences compared to control (RR, 0.86 [95%CI, 0.80 to 0.92]) (Fig. S5). No trials comparing NAAC with placebo or any other interventions were included in this subgroup analysis. The subgroup analysis that included trials with a 12-week follow-up yielded findings similar to the primary analysis, except for the results regarding acamprosate. Due to the limited number of trials that evaluated acamprosate, the finding regarding its comparison to placebo was no longer statistically significant (Fig. S6). The sensitivity analysis that excluded studies with high risk of bias yielded results consistent with the primary analysis (Fig. S7).

ACAM	NA	NA	NA	0.88 (0.82, 0.93)
0.91 (0.78,1.07)	**DISL**	NA	NA	1.00 (0.93, 1.08)
1.67 (1.14,2.46)	1.84 (1.22,2.77)	**NAAC**	NA	NA
0.93 (0.86,1.01)	1.02 (0.87,1.20)	0.56 (0.38,0.82)	**NALT**	0.94 (0.90, 0.99)
0.87 (0.83,0.93)	0.96 (0.82,1.12)	0.52 (0.36,0.77)	0.94 (0.88,0.99)	**PLBO**

Fig. 2. Primary outcome: summary of findings from both pairwise and network meta-analysis (the top-right portion displays pairwise meta-analysis results, while the bottom left shows network meta-analysis results for the primary outcome, results are reported as risk ratios (RR) with 95% confidence intervals, for both pairwise and network analyses, an RR<1 suggests that treatment in the top-left position is more effective; RR>1 indicates the opposite, orange shaded results indicate statistical significance, ACAM acamprosate, DISL – disulfiram, NALT naltrexone, NAAC – combination of acamprosate and naltrexone, PLBO placebo)

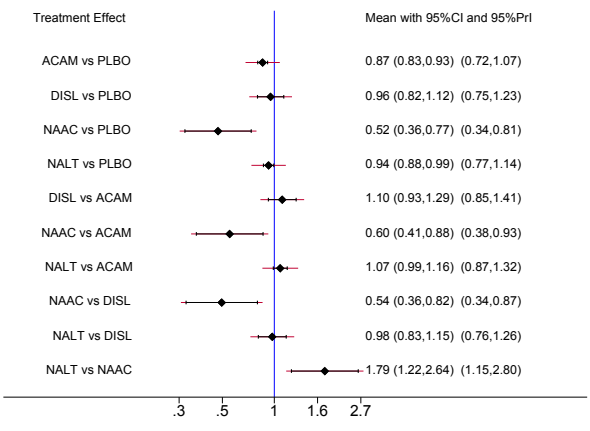

Fig. 3. Treatment effects among comparisons in NMA (reported as mean with 95% CI and 95% PrI, ACAM acamprosate, DISL – disulfiram, NALT naltrexone, NAAC – combination of acamprosate and naltrexone, PLBO placebo)

Secondary outcome: return to heavy drinking

Thirty RCTs (7012 patients) provided dichotomous data on the probability of returning to drinking. The network meta-analysis incorporated four treatment interventions, as illustrated in Fig. 4. Disulfiram was not tested in any of the trials included in the network. Six direct treatment comparisons available in the included trials, with a total of 3,992 events reported within the network.

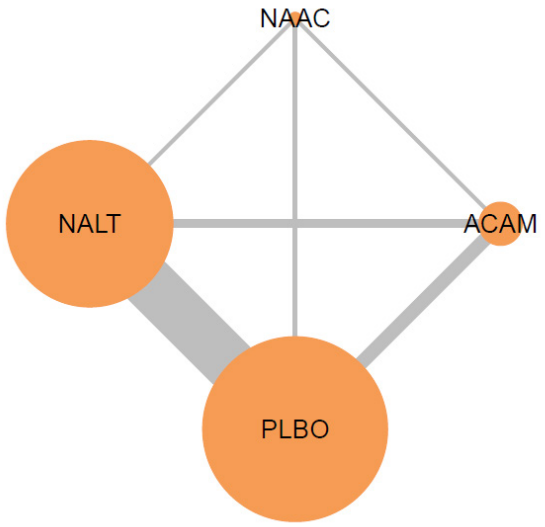

Fig. 4. Network plot for secondary outcome (Notes: lines between nodes represent direct comparisons from head-to-head trials. The size reflects the number of studies for each intervention, while the thickness of the connecting lines corresponds to the number of trials comparing ACAM – acamprosate, DISL – disulfiram, NALT naltrexone, NAAC – combination of acamprosate and naltrexone, PLBO placebo)

NMA suggested that indicated that naltrexone was associated with a statistically significant reduction in the risk of relapse compared to placebo (i.e., return to heavy drinking) (RR, 0.87 [95%CI, 0.80 to 0.95]). No statistically significant differences were observed for acamprosate (RR, 0.95 [95%CI, 0.86 to 1.04]) and NAAC (RR, 0.87 [95%CI, 0.71 to 1.06]) compared to placebo (Fig. S8). The comparative evaluation of efficacy across the different interventions did not demonstrate statistically significant superiority of one intervention over the others (Fig. S8). The SUCRA plot showed that naltrexone (SUCRA=0.79) was ranked first for efficacy followed by NAAC (SUCRA = 0.76) and acamprosate (SUCRA=0.38) (Fig. S9). The results of network meta-analyses resembled those obtained using standard paired meta-analyses (Fig. S10).

Network consistency and small study effects

For all results, the global test for inconsistency based on the design-by-treatment interaction model did not detect any significant inconsistency in the network (primary result: p = 0.22; secondary outcome: p = 0.75). Similarly, the loop-specific approach revealed no notable signs of inconsistency between the comparisons (Fig. S11). However, comparison-adjusted funnel plots (Figs. S12 and S13) suggested the possible presence of small-study effects.

Certainty of evidence

Applying CINeMA to the NMA, the certainty of evidence for all comparisons was determined to be low. Both direct and indirect evidence were also classified as low quality due to wide confidence intervals in the imprecision section. Further details on the quality of evidence can be found in Table S4.

Discussion

AUD is a significant public health problem, affecting millions of individuals and imposing a substantial economic burden on communities. The FDA has approved three medications for the treatment of AUD: naltrexone, acamprosate and disulfiram. Recent evidence from a systematic review and meta-analysis indicates that the FDA-approved medications acamprosate and naltrexone, but not disulfiram, are effective in decreasing the likelihood of returning to any alcohol consumption compared to placebo.[5] However, the previous systematic review is limited to a comparison of two interventions at a time, focusing on those evaluated in direct head-to-head trials. This study used an NMA of RCTs to comprehensively assess the comparative effectiveness of FDA-approved drugs for AUD. The findings of this review were based on trials with treatment durations of at least 12 weeks or more , as shorter follow-up periods may not adequately reflect long-term drinking patterns. The NMA results suggest that naltrexone, acamprosate, and their combination were all effective compared to placebo in reducing the likelihood of relapsing with alcohol consumption. In contrast, disulfiram did not show a statistically significant benefit in reducing the risk of relapse compared to placebo. These findings, when compared to placebo, are consistent with results from previous systematic reviews.[5] In addition, the results of NMA also suggest that combined use of acamprosate and naltrexone appeared to be the most effective intervention in reducing the risk of relapse compared to placebo and other individual FDA-approved medications for AUD.

The results of NMA suggest that acamprosate was ranked as the most effective medication compared to naltrexone in reducing the probability of relapsing to any alcohol consumption when examining the two individually. In contrast, naltrexone was the only FDA-approved intervention found to be effective in the likelihood of relapsing to reduce heavy drinking. This finding should be noted with caution, as the network had a relatively limited number of trials and sample sizes for the outcome of reducing heavy drinking for acamprosate compared to the data available for naltrexone. The available evidence suggests that acamprosate and naltrexone have shown comparable efficacy in improving outcomes such as the percentage of drinking and heavy drinking days.[5]

Previous reviews have reported that although serious side effects did not differ significantly between groups,

mild side effects such as dizziness were observed with naltrexone and gastrointestinal problems such as diarrhoea, nausea, and vomiting were reported with both naltrexone and acamprosate compared to placebo.[9,10] Oral naltrexone offers a simpler dosing regimen, requiring only a single daily dose, while acamprosate typically involves taking two tablets three times a day.[78] Acamprosate is not recommended for individuals with severe kidney dysfunction, while naltrexone is to be avoided by patients with liver failure, acute hepatitis, or those currently taking opioids. Therefore, selecting the most suitable medication should hinge on a comprehensive evaluation of the patient's clinical profile, encompassing their preference, compliance capabilities, co-existing medical conditions or contraindications, associated costs, and a thorough assessment of the potential risks and benefits.

Disulfiram has long been an FDA-approved treatment for AUD since the 1950s, underscoring its long-standing role in the management of this condition. However, the effectiveness in treating alcohol dependence has been a topic of considerable debate.[79] The efficacy of disulfiram is based on closely monitored administration and patient adherence, factors that can be difficult to sustain in practical clinical settings. Current evidence provides limited support for the effectiveness of disulfiram compared to naltrexone or acamprosate, which is consistent with the results of our NMA.

NMA evidence suggests that naltrexone in combination with acamprosate may offer potential advantages over the administration of either drug individually for the treatment of AUD, though the strength of this evidence is low because only a limited number of clinical trials were available for inclusion in the analysis. As an opioid receptor antagonist, the mechanism involves disrupting the cycle of enhanced activation of opiate receptors induced by alcohol consumption.[80] Alternatively, acamprosate is believed to work by regulating glutamatergic and GABAergic neurotransmitters systems; thus the aim is to restore the neurochemical balance disrupted by chronic alcohol exposure, which can aid in reducing alcohol relapse.[81] Studies have suggested that combining these two medications may offer synergistic effects by targeting these different aspects of alcohol dependence.[82] The existing evidence supports the tolerability of combining acamprosate and naltrexone in the management of AUD.[83] However, minor side effects, such as nausea, were observed to occur more frequently with the combined therapy compared to individual drug administration.[84] The long-term efficacy of combination therapy with naltrexone and acamprosate, with or without additional behavioral interventions, compared to monotherapy remains to be determined. Further research should be carried out to determine the optimal dosage and order of administration when using acamprosate and naltrexone together for AUD.

Treatment of AUD includes initial detoxification phase followed by rehabilitation. Relapse prevention is a key component of rehabilitation, during which these medications are used primarily alongside psychosocial interventions or with individual or group counseling. Currently, in many settings, the use of antidepressants and anxiolytics after the detoxification phase is limited, with a greater emphasis on psychosocial interventions, counseling, and ongoing support. However, acamprosate and naltrexone may have potential to reduce alcohol intake among individuals experiencing co-occurring depressive disorders. Although these medications are not specifically intended for treating depressive disorders, it is unclear how combination therapy may affect preexisting depressive conditions in these patients. To maximize the effectiveness of treatment and minimize potential risks, several important factors should be considered when selecting patients for combination therapy with naltrexone and acamprosate in the treatment of AUD. These factors include the severity of AUD, history of relapse, history of alcohol consumption, co-occurring mental and other health conditions, the potential for drug interactions and costs. A thorough evaluation of these factors allows healthcare providers to determine the suitability of combination therapy with naltrexone and acamprosate combination therapy and tailor treatment plans to the individual needs of the patient.

The limitations of this study warrant careful consideration. Firstly, this NMA encompassed studies with participants who experienced both alcohol dependence and depression, as well as those without these comorbid conditions together. Consequently, the relevance of these findings to individuals with alcohol dependence only warrants further clarification. Second, selective reporting of outcomes across studies may introduce bias. Third, the interpretation of findings should account for variations in study designs, intervention doses and dosage forms, patient populations, and outcome measured across included trials. Due to limited data, no subgroup analyzes could be performed exploring the influence of these factors on treatment outcomes. Fourth, the meta-analysis was limited in evaluating the comparative safety of the interventions due to insufficient safety data. Specifically, the small sample sizes and low number of adverse events across the included studies precluded robust NMA for safety outcomes. Future research should prioritize the collection and reporting of detailed safety data to facilitate more comprehensive risk-benefit assessments. Furthermore, the interpretation of the interactions between pharmacotherapy and psychotherapy, including cognitive interventions warrants consideration, as evidence suggests that psychotherapy reinforces the effects of medication. All study participants all received some type of concurrent psychosocial

interventions as a component of the overall treatment approach. The skills learned in therapy to avoid or manage alcohol triggers may have helped reduce drinking and self-reported cravings in treatment groups. Further research should be conducted on how psychosocial interventions and pharmacotherapy interact.

Conclusion

Our NMA offers a thorough synthesis of existing evidence, revealing the comparative efficacy of FDA-approved medications for AUD. In conjunction with psychosocial interventions, both naltrexone and acamprosate demonstrated increased efficacy compared to placebo. A combination of these two medications resulted in significantly improved therapeutic outcomes. However, more research is necessary to determine the optimal dosing strategy that maximizes therapeutic efficacy while minimizing the risk of adverse effects when these agents are used in combination. Additionally, future research should emphasize long-term results and the identification of patient characteristics that predict treatment response.

Declarations

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Author contributions

Conceptualization, S.V. and S.K..; Methodology, S.V. and S.K.; Software, S.V.; Validation, S.V., C.J. and S.K.; Formal Analysis, S.KV. and C.J.; Investigation, F.T., A.H., and C.F.; Data Curation, F.T., A.H., C.J., S.S. and C.F.; Writing – Original Draft Preparation, S.V. and S.K.; Writing – Review & Editing, F.S., S.S., P.A., and C.M.; Visualization, S.K.; Supervision, S.V. Project Administration, S.K..

Conflicts of interest

The authors have no competing interests to disclose.

Data availability

All data used in this study were obtained from existing randomized controlled trials and systematic reviews. Detailed sources are provided in the appendix. Additional data can be shared upon reasonable request.

Ethics approval

No ethical approval was required for this study, as it is a systematic review without direct involvement of human or animal subjects.

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REVIEW PAPER

Harnessing therapeutic potential of vitamins and microelements to mitigate testicular damage caused by drugs or chemical toxins – a review

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ABSTRACT

Introduction and aim. Exposure to drugs and chemical toxins has been a common cause of structural and functional impairment of the male gonad (or testis), often leading to male reproductive disorder and infertility. Health concerns due to drugs or chemical-induced testicular damage have increased the exploration of potential therapeutic agents including vitamins and microelements. This review summarizes therapeutic role of vitamins and microelements against drugs or chemical toxins in preclinical studies.

Material and methods. Relevant articles published on scientific databases like PubMed, Google Scholar, Scopus, and Web of Science were retrieved and critically reviewed for this study.

Analysis of the literature. The mitigating effect of essential vitamins (such as vitamin B2, vitamin B9, vitamin B12, vitamin B17, vitamin C, vitamin E) and microelements (such as zinc and selenium) has been demonstrated on testicular damage caused by exposure to drugs and chemical toxins in preclinical studies and associated with their antioxidant, anti-inflammatory and anti-apoptotic properties. This was further characterized with reparation of testicular histopathology, suppression of testicular oxidative damage, improved sperm parameters, elevated testicular antioxidants and testosterone level, upregulation of steroidogenic gene, inhibition of sperm DNA damage.

Conclusion. Vitamins and microelements exert therapeutic effect against drugs and chemical-induced testicular damage.

Keywords. drugs and chemical toxins, testicular damage, vitamins and microelements

Introduction

The testes are primary reproductive organs (or gonads) in males which are responsible for the gametogenic and endocrine functions.[1] Structurally, the testes are composed of seminiferous tubules and interstitial tissue (containing Leydig cells) that perform the spermatogenic and endocrine functions respectively.[2] The testes are covered by protective fibrous tissue (tunica albuginea) and suspended by the spermatic cords within a pouch (tunica vaginalis of the scrotum) outside the body, thereby enabling the surrounding temperature to be lower than the core body temperature.[3]

Essentially, the development of secondary sexual characteristics and male reproductive function are achieved under normal morphological, physiological and hormonal profile of the testicular tissue.[4] Meanwhile, the exposure of testicular tissue to toxic chemical agents, including chemotherapeutic agents such as an-

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tibiotics, anti-cancer, non-steroidal anti-inflammatory drugs (NSAIDs) and anabolic steroids, has been reported to cause damage of the testicular tissue morphology and impairment of testicular functions, leading to infertility.[5-7]

Testicular damage caused by drugs and chemical toxins is further linked with the interruption of testosterone synthesis, disruption of spermatogenesis process, testicular atrophy and when prolonged, reproductive dysfunction and infertility could happen.[8,9] The mechanisms of drugs and chemical toxins-induced testicular damage essentially involve reproductive hormonal disruption, activation of inflammatory and apoptotic signaling and induction of oxidative stress.[10] The application of potent therapeutic intervention could reverse testicular damage after acute exposure to drugs or chemical toxins while prolonged exposure leads to irreversible testicular damage, reproductive dysfunction and infertility.[11]

Furthermore, preclinical studies using experimental model of drugs and chemical toxins-induced testicular damage have been conducted to harness the efficacy of potential therapeutic agents such as vitamins and microelements. The therapeutic effects have been characterized by the modulation of associated mechanisms including the induction of oxidative stress, activation of inflammatory and apoptotic signaling, and disruption of genetic regulation of gametogenic and endocrine functions of the testes.

Aim

This review was aimed at summarizing the current information about the therapeutic potential of selected vitamins (including vitamin B_2, vitamin B_9, vitamin B_{12}, vitamin B_{17}, vitamin C, vitamin E) and microelements (including zinc and selenium) in mitigating drugs and chemical toxins-induced testicular damage in preclinical studies. The review further highlighted the mechanisms of therapeutic activity of selected vitamins and microelements.

Material and methods

The scientific publications that are relevant to the current review study were searched across databases including Google scholar, PubMed, Scopus and Web of Science. The keywords employed in the search included: 'male gonadoprotective effect of vitamins', 'male gonadoprotective effect of microelements', 'antioxidant effect of vitamins', 'anti-inflammatory effect of vitamins', 'anti-apoptotic effect of vitamins', 'antioxidant effect of microelements', 'anti-inflammatory effect of microelements', and 'anti-apoptotic effect of microelements'. Further in-depth assessment of the identified articles was conducted to extract relevant information regarding the therapeutic activity of selected vitamins and microelements against testicular damage induced by exposure to drugs or chemical toxins in experimental model. Articles containing findings that are relevant to the review study, published in peer-reviewed journals and in English language were regarded as eligible and included in the review. Conversely, duplicated articles and those containing irrelevant information were excluded from the review.

Analysis of the literature

Selected therapeutic agents possess distinct structural formula (Fig. 1) and exhibit different pharmacodynamics and pharmacokinetics which culminate into deleterious effect on the testicular tissue. Similarly, chemical toxins with diverse structural conformation (Fig. 2) exert toxic effects that lead to the morphological and functional impairment of the testes. However, some essential vitamins and microelements have demonstrated therapeutic activity against drugs and chemical toxins-induced testicular primarily based on their antioxidant, anti-inflammatory and anti-apoptotic effects.

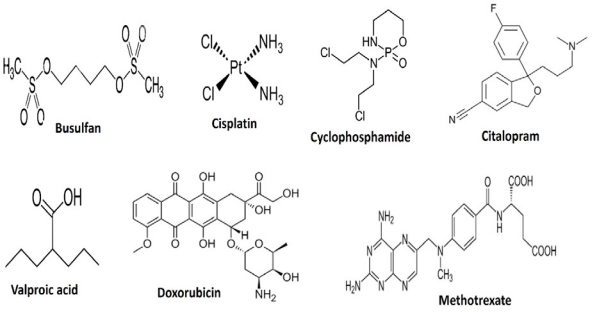

Fig. 1. Structural formula of selected drugs with testicular toxic effect

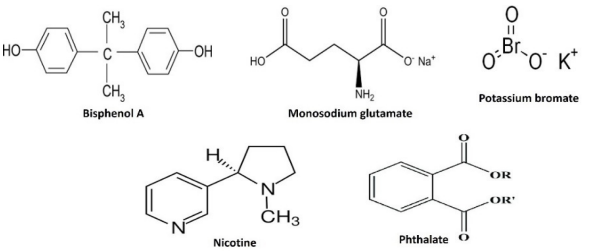

Fig. 2. Structural formula of selected chemicals with testicular toxic effect

Busulfan-induced testicular damage

Busulfan is a chemotherapeutic agent used to treat cancers with characteristic adverse effects such as testicular damage and possibly leading to reproductive dysfunction. The deleterious effect of busulfan on testicular tissue has been characterized with the induction of oxidative stress and inflammation in the testicular tissue, elevated level nitric oxide (NO) and increased myeloperoxidase activity.[12] Other features of busulfan-induced

testicular damage included decreased level of testicular glutathione (GSH), increased level of testicular malondialdehyde (MDA), reduced sperm parameters, destruction of seminiferous tubules and degenerated spermatogenic epithelium, Sertoli cells, and Leydig cells.[13-15] Busulfan-induced testicular toxicity involved reduced activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and increased level of tumor necrosis factor alpha (TNF-α).[16,17]

Cisplatin-induced testicular damage

The application of cisplatin, a common antineoplastic agent used to treat different types of cancer, often causes side effect such as testicular damage. The deleterious effect of cisplatin on the testes has been characterized with oxidative damage and upregulation of inflammatory and apoptotic signaling leading to reproductive dysfunction.[5,18] Cisplatin-induced testicular damage has been characterized with increased level of oxidative stress marker (MDA) and reduction in sperm parameters (such as count, motility, and morphology), and levels of testicular testosterone and antioxidants (SOD, GSH, CAT) as well as distortion of testicular histoarchitecture.[19,20] Other associated mechanisms of cisplatin-induced testicular damage included upregulated expressions of TNF-α, NF-κB and caspase-3 factors.[19-21]

Citalopram-induced testicular damage

Citalopram is a selective serotonin reuptake inhibitor widely prescribed to act as a serotonergic antidepressant, with characteristic toxic effects of different body tissues including the testes via induction of oxidative stress.[22,23] Citalopram-induced testicular damage is characterized with diminished sperm parameters (count, motility, viability, morphology and chromatin integrity), significantly increased NO and MDA levels and apoptotic markers.[23] Other associated mechanisms associated with citalopram-induced testicular damage included decreased GSH, increased sperm DNA damage disruption of reproductive hormones.[24,25]

Cyclophosphamide-induced testicular damage

Cyclophosphamide is an alkylating drug that function as anti-cancer agent for treatment of cancer and as immunosuppressive agent for treatment of autoimmune and immune-mediated diseases.[26] It has been reported to exhibit testicular damage mainly through the induction of oxidative damage within testicular tissue and interference with the genetic expression associated with testicular functions.[27] Cyclophosphamide-induced testicular damage has been characterized in experimental model to involve shrinkage of seminiferous tubules, decline in cellular components (Leydig and Sertoli cells) of testes, marked reduction of sperm parameters, testosterone level, tissue antioxidants while MDA level was significantly elevated.[27,28] Moreover, testicular damage due to cyclophosphamide exposure showed deleterious effect on testicular histomorphology and leads to impairment of testicular structural and functional integrity.[29]

Doxorubicin-induced testicular damage

Doxorubicin is a potent anti-neoplastic agent which constitutes the major component of anti-cancer treatment regimens.[30] It has been indicated to cause testicular damage mainly through the induction of oxidative stress, inflammation and apoptosis.[31] Doxorubicin-induced testicular damage has been characterized with prominent distortion of testicular histoarchitecture, testicular atrophy, Leydig cell degeneration, oligospermia, dysregulation of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β- hydroxysteroid dehydrogenase (17β-HSD).[32-34] Moreover, other associated mechanisms of doxorubicin-induced testicular damage included reduced testicular antioxidants (SOD, CAT, GPx), elevated level of inflammatory and apoptotic markers (IL-1β, IL-6, TNF-α, Bax, caspase-3); elevated level of MDA, reactive oxygen species, 8-Hydroxy-deoxyguanosine (8-OHdG), 4-Hydroxynonenal (4-HNE).[31,35,36]

Methotrexate-induced testicular damage

Methotrexate is a drug commonly used for treating diverse kinds of cancer and as an immunosuppressant to treat autoimmune diseases.[37] It has been reported to severe adverse including testicular damage. In preclinical studies, methotrexate-induced testicular damage has been characterized with marked decline of sperm parameters (count and motility), testicular antioxidants (SOD, GSH, CAT, GPx), and testosterone level and prominent testicular histopathological changes.[38,39] Other mechanisms of methotrexate-induced testicular damage included increased levels of MDA, protein carbonyl, and inflammo-apoptotic factors including NO, TNF-α, NF-kB, IL-6, IL-1β, caspase-3 and p53, reduced expressions of Bcl-2, IL-10, HO-1, EGF, ERK1/2 factors and steroidogenic genes in the testes.[39-41]

Valproic acid-induced testicular damage

Valproic acid is an anticonvulsant drug used to treat epilepsy. However, it has been indicated to cause tissue toxicities (including the testes) mainly on the basis of induction of oxidative stress and tissue damage. Valproic acid-induced testicular damage has been characterized with marked decline in testosterone levels, sperm parameters (count and normal morphology) and steroidogenic gene expression, as well as prominent testicular histopathological changes.[42,43] Moreover, valproic acid exposure to testicular tissue further caused abrogation of autophagy, increased inflammatory (NF-kB) and apoptotic (caspase-3) signaling and downregulation of AMPK factor.[43,44]

Table 1. Profile of selected drugs and mechanisms of testicular damaging effect

Drug	Molecular formula (molecular weight)	Therapeutic applications	Major mechanisms of testicular damage	References
Busulfan	$C_6H_{14}O_6S_2$ (246.30 g/mol)	Anti-cancer drug	- reduced sperm parameters - testicular histopathological changes - reduced SOD, CAT, GSH levels - increased levels of MDA, NO, and myeloperoxidase activity - upregulated TNF-α expression	Abarikwu et al.[12] Abarikwu et al.[13] Ezim and Abarikwu[14] Abd El-Hay et al.[15] Moghadam et al.[16] Abarikwu et al.[17]
Cisplatin	$Pt(NH_3)_2Cl_2$ (300.10 g/mol)	Anti-cancer drug	- increased level of MDA - reduced sperm parameters - reduced levels of testosterone, testicular antioxidants (SOD, GSH, CAT) - distortion of testicular histoarchitecture - upregulation of TNF-α, NF-κB, caspase-3	Abdel-Latif et al.[5] Soni et al.[18] Elsayed et al.[19] Nofal et al.[20] Othman et al.[21]
Citalopram	$C_{20}H_{21}FN_{20}$ (405.35 g/mol)	Anti-depressant drug	- diminished sperm parameters - increased NO, MDA levels - decreased testicular GSH level - increased sperm DNA damage - disruption of reproductive hormones	Kraai and Seifert[22] Moradi et al.[23] Ilgin et al.[24] Moradi et al.[25]
Cyclophosphamide	$C_7H_{15}Cl_2N_2O_2P$ (261.08 g/mol)	Anti-cancer and treatment of autoimmune diseases	- marked testicular histopathology - reduction of sperm parameters - reduced testosterone level - decreased testicular antioxidants - increased MDA level	Ahlmann and Hempel[26] Ghobadi et al.[27] Shaker Kordedeh et al.[28] Adana et al.[29]
Doxorubicin	$C_{27}H_{29}NO_{11}$ (543.52 g/mol)	Anti-cancer drug	- distortion of testicular histoarchitecture - dysregulation of testicular hormones - reduced testicular SOD, CAT, GPx - elevated levels of IL-1β, IL-6, TNF-α - upregulation of Bax, caspase-3 - elevated MDA, 8-OHdG, 4-HNE levels	Mohan et al.[32] Alafifi et al.[33] Sarman and Koca[34] Tektemur et al.[35] Ijaz et al.[36]
Methotrexate	$C_{20}H_{22}N_8O_5$ (454.40 g/mol)	Anti-cancer or anti-rheumatic drug	- decline of sperm parameters - reduced SOD, GSH, CAT, GPx, - reduced testosterone levels - testicular histopathological changes - increased levels of MDA, PC NO, TNF-α, NF-kB, IL-6, IL-1β, - upregulated caspase-3, p53 expressions - reduced expressions of Bcl-2, IL-10, HO-1, and steroidogenic genes	Akacha et al.[38] Hassanein et al.[39] Rofaeil et al.[40] Sarman et al.[41]
Valproic acid	$C_8H_{16}O_2$ (144.21 g/mol)	Anticonvulsant drug	- reduced sperm parameters - decline in testosterone level and steroidogenic gene expression - testicular histopathological changes - increased NF-kB, caspase-3 levels - downregulation of AMPK and autophagy	Conei et al.[42] Alsemeh et al.[43] Savran et al.[44]

Selected chemical toxin-induced testicular damage in preclinical studies

Bisphenol A-induced testicular damage

Bisphenol A is a synthetic compound used to manufacture polycarbonate plastics and epoxy resins. As an endocrine disruptor, it has been implicated in preclinical studies as a potent cause of chemical toxin-induced testicular damage whereby the impairment of reproductive function occurs through the characteristic anti-androgenic effect.[45] Bisphenol A-induced testicular damage was characterized with marked distortion of testicular morphology, elevated levels of oxidative stress and inflammatory markers and increased TUNEL positive cells indicating apoptosis in testis.[45-47] Moreover, bisphenol A-induced testicular damage caused significant decline of sperm parameters (viability, motility and normal morphology) and testicular antioxidants (SOD, CAT, GSH) while MDA, MMP, ROS levels were increased.[48,49]

Fluoride-induced testicular damage

Fluoride is a common environmental contaminant which causes prominent detrimental effect on body tissues such as the testes leading to male reproductive impairment and thereby constitute public health concern.[50] Fluoride-induced testicular damage and male reproductive impairment was described to involve molecular pathways including apoptosis, DNA damage, hormonal imbalance, inflammatory response, mitochondrial dysfunction.[51] Fluoride exposure caused prominent histopathological and biochemical changes characterized with poor semen quality, reduced testosterone level due

to degeneration of Leydig cells, regression of testicular tissue leading to impaired spermatogenesis and infertility.[52] Other indications of fluoride-induced testicular damage included reduced testicular weight and sperm parameters (count, motility, and viability), increased abnormal sperm morphology, induction of oxidative stress and activation of IL-17A pathway.[53,54]

Monosodium glutamate-induced testicular damage

Monosodium glutamate, chemical commonly used to enhance food flavor, has been reported to cause damaging effects on body tissue (including testes) especially after prolonged exposure via induction of oxidative stress and cell death. Monosodium glutamate-induced testicular damage has been characterized with significant reduction of testosterone level, elevated MDA level, marked testicular histopathological changes.[55] Other associated mechanisms of monosodium glutamate-induced testicular damage included decreased testicular antioxidants (SOD) and PCNA, Bcl-2 and Beclin 1 expressions; elevated levels of apoptotic and inflammatory markers (TNF-α, Bax, caspase-3) which in turn inhibited the AMPK/mTOR pathway and autophagy in the testes.[56,57]

Nicotine-induced testicular damage

Nicotine, a naturally occurring alkaloid commonly used in tobacco production and to stimulate pleasure feeling, exhibits potent adverse effect on body tissues including the testes leading to male reproductive dysfunction and infertility. In preclinical studies, nicotine-induced testicular damage was characterized with significant reduction in testosterone level, testicular sperm parameters (count, viability, normal morphology, motility), marked testicular histopathological changes.[58,59] Other mechanisms of nicotine-induced testicular damage included marked reduction in SOD and GSH, significantly increased levels of testicular MDA, NO, TBARS.[59,60] Furthermore, the exposure to nicotine caused reduction of Nrf-2 expression and destruction of blood-testis barrier (BTB) via induction of ferroptosis in testicular cells.[61]

Potassium bromate-induced testicular damage

Potassium bromate, a food additive used in production of dough, fish paste, beer, cheese or fermented beverages, has been reported to cause deleterious effects on body tissues including testes via induction of oxidative damage due to accumulation of free radicals.[62] Potassium bromate-induced testicular damage has been characterized with increased levels of MDA, ROS and RNS, reduced levels of SOD, CAT and TAC, marked distortion of testicular histomorphology.[63-65] Other mechanisms of potassium bromate-induced testicular damage included significant decline of sperm parameters (count, motility, viability, normal morphology) and reproductive hormones including testosterone, LH, FSH, 3HSD, 17HSD.[63,65,66]

Phthalate-induced testicular damage

Phthalates such as di-(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), and butyl benzyl phthalate (BBP) which are used in making consumer products and plastics, are known environmental toxicants (endocrine disruptors) that cause testicular toxicity through induction of oxidative stress, endocrine disruption, inflammation, and apoptosis.[67] Phthalates-induced testicular toxicity has been characterized with prominent testicular histopathological changes, decreased testosterone level, reduced sperm parameters, disrupted expression of steroidogenic genes (StAR, CYP11A1, CYP17A1, 17β-HSD, CYP19A1).[68,69]

Application of vitamins as mitigants of testicular damage in preclinical studies

Vitamin B2 (or riboflavin) – therapeutic effect against testicular damage

Vitamin B_2 is a member of the vitamin B family (water-soluble vitamins) which plays essential role in various biological activities and acts as a precursor to flavin mononucleotide and flavin adenine dinucleotide which perform vital roles in enzymatic reactions.[70-72] It is an essential nutrient for human health derived from dietary sources (such as egg, fish, nuts, milk, fruits, vegetables and so on) and function as antioxidant that mitigate lipid peroxidation and reperfusion oxidative injury.[71,73,74] The therapeutic effect of Vitamin B2 against fluoride-induced testicular damage has been demonstrated and characterized with reparation of testicular histopathological changes, reduction of testicular ROS and SLC3A2 levels, improved sperm parameters, and inhibition of fluoride-induced ferroptosis in testicular Leydig cells which would result into improved testosterone.[53]

Vitamin B9 (or folic acid) - therapeutic effect against testicular damage

Vitamin B_9, a water-soluble vitamin which is the synthetic form of folate that are derived from dietary sources. It has been reported to exhibit antioxidant property (comparable to vitamins C and E) and could protect tissues against some diseases caused by harmful oxidation.[75,76] In a preclinical study, vitamin B_9 had demonstrated therapeutic effect against nicotine-induced testicular damage and characterized its therapeutic activity with reparation of testicular degenerative changes, elevated testosterone level as well as suppression of inflammatory cytokines (TNF- α, IL-6) and oxidative stress markers.[77] Moreover, the therapeutic effect of vitamin B_9 against bisphenol A-induced testicular damage has been demonstrated in preclinical study and

Table 2. Profile of selected chemicals and mechanisms of testicular damaging effect

Chemicals	Molecular/Atomic formula (Molecular/atomic mass)	Common applications	Major mechanisms of testicular damage	References
Bisphenol A	$C_{15}H_{16}O_2$ (228.29 g/mol)	- used to produce polycarbonate plastics and epoxy resins	- impairment of reproductive function - marked distortion of testicular morphology - increased TUNEL positive apoptotic cells - decline of sperm parameters - reduced testicular SOD, CAT, GSH - increased MDA, MMP, ROS levels	Bordbar et al.[45] Gules et al.[46] Tekin and Celebi[47] Rezaee-Tazangi et al.[48] Asadi-Fard et al.[49]
Fluoride	F[-1] (18.99 g/mol)	- used to build healthy teeth, gums, bones	- prominent testicular histopathology - reduced sperm parameters, testosterone level - regression of testicular tissue leading to impaired spermatogenesis - activation of IL-17A pathway	Chhabra et al.[51] Patial et al.[52] Li et al.[53] Talebi et al.[54]
Monosodium glutamate	$C_5H_8NNa_2O_4$ (169.11 g/mol)	- used as food flavor enhancer	- reduction of testosterone level, - elevated MDA level - testicular histopathological changes - decreased testicular antioxidants (SOD) - downregulation of PCNA, Bcl-2, Beclin-1 - upregulation of TNF-α, Bax, caspase-3 - inhibition of AMPK/mTOR pathway	Luqman et al.[55] Gad et al.[56] Morsy et al.[57]
Nicotine	$C_{10}H_{14}N_2$ (162.23 g/mol)	- used to stimulate pleasure feeling	- reduced testosterone level, sperm parameters - testicular histopathological changes - reduced levels of SOD and GSH - increased levels of MDA, NO, TBARS and ferroptosis - downregulated Nrf-2 expression	Ikwuka et al.[58] Jalili et al.[59] Ashoub et al.[60] Zhang et al.[61]
Potassium bromate	$KBrO_3$ (167.00 g/mol)	- used as flour additive, for production of malt barley	- increased levels of MDA, ROS, RNS - reduced levels of SOD, CAT, TAC - marked testicular histopathology - decline of sperm parameters - reduced level of testosterone, LH, FSH, 3HSD, 17HSD	El-Deeb et al.[62] Mohamed et al.[63] Akinola et al.[64] Nwonuma et al.[65] Akinola et al.[66]
Phthalates	$C_8H_4O_4^{-2}$ (164.11 g/mol)	- used to increase the flexibility and softness of plastics	- testicular histopathological changes - decreased testosterone level - reduced sperm parameters - disrupted expression of steroidogenic genes	Mondal et al.[67] Tang et al.[68] Liu et al.[69]

characterized with improved sperm parameters (sperm count, motility, morphology), increased serum testosterone level, amelioration of testicular histopathological changes, as well as decreased TUNEL-positive apoptotic cells.[46]

Vitamin B12 (or cobalamin) – therapeutic effect against testicular damage

Vitamin B_{12} is a water-soluble vitamin, one of the eight vitamin B family, which participate in metabolism and function as co-factor for two critical biochemical reactions (conversion of l-methylmalonyl coenzyme A to succinyl coenzyme A, and the formation of methionine by methylation of homocysteine).[78,79] It is totally derived from dietary sources (such as fish, meat, poultry, eggs, milk) and exists in four isoforms cyanocobalamin, hydroxocobalamin, 5′-deoxyadenosylcobalamin, and methylcobalamin.[80] It has been suggested that Vitamin B_{12} exhibit antioxidant effect and may protect against induction of oxidative stress and inflammation.[81] In a preclinical study, the therapeutic effect of vitamin B12 against nicotine-induced testicular damage has been assessed and reportedly characterized with amelioration of testicular degenerative changes and increased testosterone level. Other characteristic mechanisms included suppression of inflammatory markers such as TNF- α, IL-6 and markers of oxidative stress.[77]

Vitamin B17 (or amygdalin) – therapeutic effect against testicular damage

Vitamin B_{17} is a glycoside nutrient derived from dietary sources such as apples and apricots and reported to exhibit certain therapeutic properties especially anti-tumor effect.[82] Furthermore, the therapeutic effect of vitamin B_{17} has also been demonstrated in preclinical study against methotrexate-induced testicular damage and characterized with amelioration of testicular histopathology (atrophy and lesions), improved sperm parameters (sperm count, viability, morphology index, total motility, and progressive motility), increased testosterone level, improved level of testicular antioxidants (SOD, CAT, GSH).[83]

Vitamin C (or ascorbic acid) – therapeutic effect against testicular damage

Vitamin C is a water-soluble which is abundantly derived from natural sources such as fresh fruits like oranges, lemons, grape, leafy green vegetables and exists

Table 3. Profile and therapeutic role of selected vitamins against drug or chemical-induced testicular damage

Vitamins (other name)	Molecular formula (molecular weight)	Dietary sources	Therapeutic mechanisms against drug or chemical testicular damage	References
Vitamin B_2 (riboflavin)	$C_{17}H_{20}N_4O_6$ (376.37 g/mol)	meat, eggs, almonds, nuts, spinach, apple, kidney bean, spinach, banana, potato, avocado, tomato, oatmeal	- amelioration of testicular histopathology - reduction of testicular ROS - improved sperm parameters - inhibition of ferroptosis in Leydig cells - increased testosterone level	Li et al.[53]
Vitamin B_9 (folic acid)	$C_{19}H_{19}N_7O_6$ (176.124 g/mol)	banana, papaya, orange, green peas, kidney bean, crab, tomato juice, avocado, black-eyed pea, peanuts, spinach, beef liver	- reparation of testicular degeneration - elevated testosterone level - suppression of TNF- α, IL-6 - reduced oxidative stress, apoptotic markers - improved sperm parameters - increased testosterone level	Gules et al.[46] Ray et al.[77]
Vitamin B_{12} (cobalamin)	$C_{19}H_{19}N_7O_6$ (176.124 g/mol)	liver, oyster, mussel, clam, mackerel, crab, sardines, catfish, cod, low-fat beef, skimmed milk, soy milk, salmon, cheese, eggs	- reparation of testicular histopathology - increased testosterone level - suppression of TNF- α, IL-6 - suppression oxidative stress	Ray et al.[77]
Vitamin B_{17} (amygdalin)	$C_{19}H_{19}N_7O_6$ (176.124 g/mol)	millet, apple seed oil, strawberry, raspberry, spinach, cassava, barley, almonds, yam, cashew, sweet potatoes	- amelioration of testicular histopathology - improved sperm parameters - increased testosterone level - increased level of SOD, CAT, GSH	Felemban et al.[83]
Vitamin C (ascorbic acid)	$C_6H_8O_6$ (441.4 g/mol)	oranges, tomatoes, guava, potato, tomato juice, broccoli, mango, lemon, pepper, grape fruits, strawberries	- increased testosterone level - improved sperm parameters - reparation of testicular histopathology - reduced levels of MDA, NO, - reduced TUNEL-positive apoptotic cells	Moradi et al.[25] Hajjar et al.[89] Rauf et al.[90]
Vitamin E (alpha-Tocopherol)	$C_{29}H_{50}O_2$ (430.71 g/mol)	almonds, nuts, butter, spinach, vegetable oils, olive oil, cereals, meat, egg, avocado, fruits, wheat, cottonseed oil, palm oil, potato, sunflower seeds	- improved sperm parameters - increased testosterone level - improved testicular histology - upregulation of steroidogenic gene - diminished levels of ROS, RNS - increased levels of SOD, CAT, TAC	Alsemeh et al.[43] Akinola et al.[64] Rauf et al.[90]

in the body in ascorbate form.[84,85] It is a natural antioxidant with ability to inhibit ROS production and scavenge free radicals thereby abrogating oxidative stress that follows exposure to tissue toxins.[82-84] Accordingly, preclinical studies have explored the therapeutic potential of vitamin C to combat testicular pathologies due to exposure to drugs and chemical toxins.

Therapeutic role of vitamin C, based on its potent antioxidant activity, has been demonstrated against cyclophosphamide-induced testicular damage with satisfactory outcomes. This was characterized with restoration of testosterone level, improved sperm parameters and reparation of testicular histopathological changes.[89] Similarly, preclinical studies involving experimental models of citalopram-induced testicular damage and cisplatin-induced testicular damage have demonstrated therapeutic effect of vitamin C and characterized with improvement of sperm parameters (count, motility, viability, morphology, and chromatin integrity), testosterone level, amelioration of testicular histopathology, reduced levels of MDA, NO, and TUNEL-positive apoptotic cells.[25,90]

Vitamin E (or alpha-tocopherol) – therapeutic effect against testicular damage

Vitamin E is a fat-soluble vitamin composed of eight essential compounds including four tocopherols and four tocotrienols which are all made up of a chromane ring with a hydroxyl group and side chain.[91] Alpha-tocopherol is a common component which function as an antioxidant that protects the cellular membranes and lipoproteins from peroxidation activity of free radicals.[66,92,93] As an antioxidant, the therapeutic potential of vitamin E has been explored in preclinical studies using the experimental model of tissue pathologies including the testes. The therapeutic activity of Vitamin E against cisplatin-induced testicular damage has been demonstrated in a preclinical study and characterized with amelioration of testicular histopathology (atrophy and lesions), improvement of sperm parameters (sperm count, motility, viability) testosterone level, testicular weight and lipid profile.[90]

Furthermore, vitamin E mitigated valproic acid-induced testicular damage, characterized with increased testicular weight, improved testicular histology, elevated sperm parameters and testosterone level, upregulation of steroidogenic gene and induction of autophagy through upregulation of LC3, beclin1 and downregulation of p62 factors.[43] The study by Akinola et al.[64] further demonstrated that vitamin E ameliorated potassium bromate-induced testicular damage with the ameliorative activity characterized by improved sperm parameters (count, motility, viability and normal morphology),

Table 4. Profile and therapeutic role of selected microelements against drug or chemical-induced testicular damage

Trace element (symbol)	Atomic number (mass)	Sources	Therapeutic mechanisms against drug or chemical testicular damage	References
Zinc (Zn)	30 (65.41 u)	oysters, pork, fishes, liver, meat, wheat, mollusks, dairy products, oats, dried peas, nuts, cheese	- increased testosterone level - improved sperm parameters - reparation of testicular histopathology - reduced oxidative stress markers - inhibition of sperm DNA damage - upregulation of Nrf2 factor	Hajjar et al.[89] Maremanda et al.[97]
Selenium (Se)	34 (76.96 u)	garlic, nuts, cabbage, rice, potatoes, oats, fishes, eggs, meats, lentils	- amelioration of testicular histopathology - elevated testosterone level - improved sperm parameters - reduced MDA level - increased levels of SOD, CAT, GPx, GSH, CAT	Huang et al.[100] Hamza et al.[101] Keshta et al.[102]

diminished levels of ROS, RNS, increased levels of testicular antioxidants (SOD, CAT, TAC) and hormones (testosterone, FSH and LH, 17HSD).

Application of microelements as mitigants of testicular damage in preclinical studies

Zinc – therapeutic effect against testicular damage

Zinc (Zn) is an essential microelement which participates in several body functions and physiological processes including reproduction. Aside its role in enhancing the body antioxidant system, it also acts to promote the activity of vitamin D, suppress prostaglandin synthesis and participate in immune responses and tissue homeostasis.[94,95] Zn exhibits antioxidant and anti-inflammatory properties which underscore its role in oxidative metabolism of spermatozoa. In addition, it plays vital role in maintenance of epithelial lining of reproductive tracts and exerts regulatory role during the process of capacitation and acrosome reaction.[96] Hence, Zn deficiency disrupts spermatogenesis and could result into sperm anomalies and reduced testosterone concentration.[96] The therapeutic role of Zn, based on its potent antioxidant activity, has been demonstrated against cyclophosphamide-induced testicular damage with satisfactory outcomes. This was characterized with restoration of testosterone level, improved sperm parameters, reparation of testicular histopathological changes, reduced oxidative stress markers, and inhibition of sperm DNA damage.[89,97] The mechanisms of therapeutic role Zn included the modulation of metallothionein (MT), tesmin and Nrf2 pathways.[97]

Selenium – therapeutic effect against testicular damage

Selenium (Se) is a metalloid which is essential to human health in trace amounts but could be harmful in excessive amount.[98] As an essential microelement involved in improving antioxidant defense, immune functions, and metabolic homeostasis, its deficiency increased susceptibility to various disorders and accelerate aging process leading to reduction of life expectancy.[95] Se demonstrated protective effect against testicular toxicity primarily based on its antioxidant and anti-inflammatory properties.[100] Accordingly, Se has been explored as potent therapeutic agent in mitigating testicular damage that results from exposure to drugs and chemicals in preclinical studies. The therapeutic activity of Se, through its nanoparticles, has been further demonstrated against monosodium glutamate-induced testicular damage and characterized with reparation of testicular histopathology, elevated testosterone level, reduced MDA level, increased levels of testicular antioxidants (SOD, CAT and GPx).[101] Moreover, Se nanoparticles have exhibited ameliorative effect against cisplatin-induced testicular damage. The antioxidant property of Se underscored the therapeutic role of Se which was characterized with increased levels of testicular antioxidants (SOD, GSH, GPx, CAT), decline of MDA level, improvement of sperm parameters (count, motility and abnormal morphology), amelioration of testicular degenerative changes.[102]

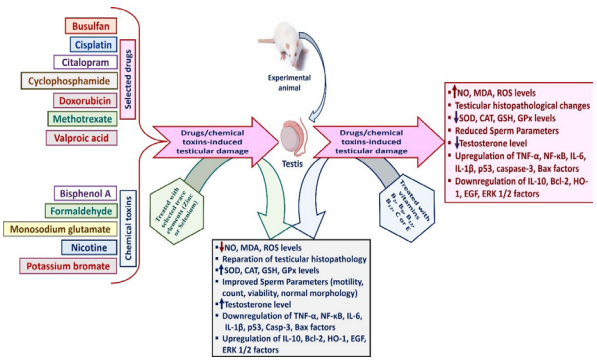

Fig. 3. Schematic summary of mechanisms of drugs and chemical toxins-induced testicular damage and therapeutic effect of selected vitamins and microelements

Conclusion

The damaging effect of drugs and chemical toxins exposure on testicular tissue has been demonstrated to result from the induction of oxidative stress, activation of inflammatory and apoptotic signaling, and characterized with disruption of genetic regulation of gametogenic and endocrine functions of the testes. Hence, potential therapeutic agents with antioxidant, anti-inflammato-

ry and anti-apoptotic properties such as essential vitamins (like Vitamin B_2, Vitamin B_9, Vitamin B_{12}, Vitamin B_{17}, Vitamin C, and Vitamin E) and microelements (like Zinc and Selenium) have demonstrated therapeutic efficacy against testicular damage caused by exposure to drugs and chemical toxins in preclinical studies.

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Conflicts of interest
The authors declare no conflicts of interest.

Data availability
The data that support the relevant findings to the review have been included in the article.

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Not applicable.

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REVIEW PAPER

Review of the therapeutic effect of alpha-tocopherol, ascorbic acid, and folic acid against ovarian toxicity induced by drugs and heavy metals

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ABSTRACT

Introduction and aim. The ovaries are almond-shaped organs that produce the female gametes and reproductive hormones. They also play a critical role of ovulation under well-coordinated hormonal regulation. However, chemotherapy involving the application of drugs to combat chronic diseases (like cancer) results in toxicity to tissues like ovaries. Similarly, exposure to heavy metals has a toxic effect on the ovaries. Hence, potential therapeutic agents including vitamin antioxidants have been explored to combat ovarian toxicity caused by drugs or heavy metals.

Material and methods. This review was based on previous articles archived on Web of Science, PubMed, Scopus and Google Scholar databases. After initial assessment, the relevant articles were selected for further critical assessment.

Analysis of the literature. Induction of oxidative stress and activation of inflammo-apoptotic signaling were indicated as the major mechanisms of ovarian toxicity due to exposure to drugs and heavy metals. Moreover, vitamins such as alpha-tocopherol, ascorbic acid and folic acid demonstrated therapeutic effects against drug and heavy metal-induced ovarian toxicity based on their modulatory effect on the downstream mechanisms of the toxicity.

Conclusion. Vitamins exert a therapeutic effect against ovarian toxicity caused by drugs or heavy metal exposure due to their antioxidant, anti-inflammatory and anti-apoptotic properties.

Keywords. experimental animal model, ovarian toxicity, vitamins

Introduction

The ovaries (or gonads) of female humans are almond-shaped organs which perform the function of production of female gametes (oocytes) and reproductive hormones.[1] The basic functional unit of the ovary, regarded as the ovarian follicle is comprised of the oocyte surrounded by granulosa and thecal cells.[2] However, the interaction of all the morphological components of the ovaries, which include the germ cells (oocytes), granulosa cells (GCs), theca cells and ovarian stroma, determines the normal physiology of the female gonads and fertility.[3] Moreover, follicular development spans through several stages including primordial primary, secondary, preantral, and graafian stages and culminate into ovulation during each monthly reproductive cycle.[2,4]

As a pivotal function of the ovaries, follicular development is highly regulated by hormones (gonadotropins) such as follicle stimulating hormone (FSH) and luteinizing hormone (LH), secreted by the anterior pituitary gland under the control of gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus.[2,3] Essentially, human ovaries achieve the critical role of oocyte release (ovulation) under well-coordinated hormonal regulation. Hence, the exposure to agents (such

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as chemotherapeutic agents or heavy metals) with ability to disrupt the highly-connected hormonal regulation of the ovarian function, could cause ovarian toxicity and dysfunction that may culminate into female infertility.[5,6]

Chemotherapy, which involves application of drugs to combat chronic diseases (like cancer), has resulted in adverse effects and toxicity of non-target tissues including the ovaries. Drug-induced ovarian toxicity has been characterized with ovarian atrophy, depletion of ovarian follicles leading to iatrogenic premature ovarian insufficiency (POI) and infertility.[7,8] The mechanisms of drug-induced ovarian toxicity included impairment of ovarian anti-oxidative capacity and oxidative damage of ovaries characterized with apoptosis of GCs due to accumulation of reactive oxygen species (ROS).[7,9] Other associated mechanisms included mitochondrial dysfunction due to excess production of superoxide leading to ROS-induced lipid peroxidation and ferroptosis.[7,10]

Furthermore, exposure to heavy metals has been reported to exhibit a significant toxic effect on body tissues including the ovaries. Heavy metal-induced ovarian toxicity primarily occurs through the induction of oxidative stress due to accumulation of ROS within the ovarian tissue. Moreover, the ROS accumulation induced DNA damage in the oocytes can activate cascades of apoptotic signaling.[11] In addition, the heavy metal-induced ovarian toxicity has been characterized with follicular atresia, reduced estrogen secretion and culminate into ovarian insufficiency and infertility.[12]

The understanding of the aforementioned mechanisms of ovarian toxicity induced by drugs or heavy metals constitute the pivotal rationale for the discovery and development of effective therapeutic intervention in order to protect the morphology and function of ovaries and preserve female fertility. In this regard, the therapeutic potential of essential vitamins has been harnessed to mitigate drug or heavy metal-induced ovarian toxicity. Accordingly, preclinical studies have demonstrated the therapeutic effect of vitamins (such as alpha-tocopherol, ascorbic acid and folic acid) via modulation of the downstream mechanisms of drug-induced or heavy metal-induced ovarian toxicity.

Aim

This narrative review was thereby aimed at presenting the current findings on therapeutic effect of the selected vitamins against ovarian toxicity induced by drugs or heavy metals in experimental animal models. The review further highlights the associated mechanisms and signaling factors that culminate into the therapeutic effect of the selected vitamins.

Material and methods

Relevant research articles were searched across the different scientific databases which include Web of Science, PubMed, Scopus and Google Scholar. The keywords used for the search included 'antioxidant activity of vitamins', 'anti-inflammatory activity of vitamins', 'anti-apoptotic activity of vitamins', 'vitamins mitigate ovarian toxicity', 'vitamins mitigate drug-induced ovarian toxicity', and 'vitamins mitigate heavy metal-induced ovarian toxicity.' The search results were critically assessed to select articles which contain relevant findings about the therapeutic role of vitamins against drug-induced and heavy metal-induced ovarian toxicity in experimental animal models. The criteria for the eligibility of articles in this review included: articles published in English language, articles which contain findings that are relevant to the scope of the review, and articles published in peer-reviewed journals. Conversely, the criteria for exclusion included duplicate articles and articles published in predatory journals.

Analysis of the literature

The induction of oxidative stress and activation of inflammatory and apoptotic signaling pathways in the ovarian tissue have been demonstrated as the major cellular mechanisms that triggers ovarian toxicity following exposure to drugs and heavy metals in experimental animal models. Hence, potential therapeutic agents such as essential vitamins like alpha-tocopherol, ascorbic acid and folic acid, with antioxidant, anti-inflammatory and antiapoptotic properties, have been explored in preclinical studies to demonstrate their therapeutic effect against drug-induced and heavy metal-induced ovarian toxicity.

Drug-induced ovarian toxicity and associated mechanisms

The selected chemotherapeutic agents in the current narrative review (including busulfan, cyclophosphamide, cisplatin and doxorubicin) possess unique structural formulas (Fig. 1) and exhibit characteristic pharmacological (anti-cancer) effects. However, they also exhibit toxic effects on the morphology and function of the ovaries (female gonads) outlined in Table 1.

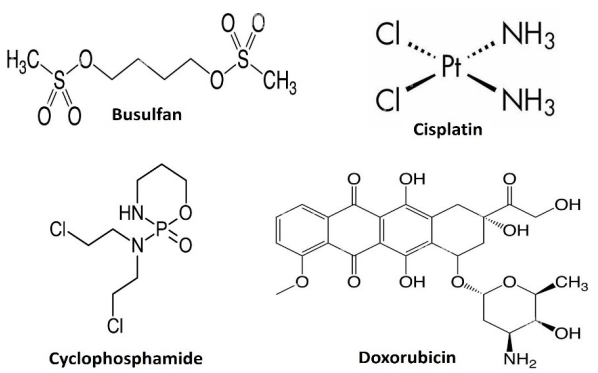

Fig. 1. Molecular structures of selected drugs with female gonadotoxic effects

Busulfan is an alkylating agent commonly used as anti-cancer drug, in combination with cyclophosphamide. Experimental animal studies have reported that the chemotherapeutic agents individually cause female gonadotoxicity and both demonstrate a synergistic toxic effect during combined exposure resulting into POI and infertility.[13] Busulfan-induced ovarian toxicity in experimental animal models is characterized with ovarian atrophy, weight loss, dysregulation of hormones, and significant depletion of primordial and primary ovarian follicles.[14,15] Other associated mechanisms of busulfan-induced ovarian toxicity included an increase in DNA-damage in oocytes and induction of apoptosis in GCs.[16] Aside from the significant loss of ovarian follicles at all developmental stages, busulfan exposure further caused a decrease in serum levels of oestradiol, progesterone, and anti-mullerian hormone (AMH) leading to toxic effect on GCs and ovarian reserve.[13,17]

Cyclophosphamide is an alkylating therapeutic agent used in the management and treatment of neoplasms which has been indicated to exhibit a female gonadotoxic effect primarily through the induction of oxidative stress and activation of inflammatory and apoptotic signaling. Cyclophosphamide-induced ovarian toxicity has been characterized with atrophy of ovarian tissue, reproductive hormone dysregulation, and marked reduction of ovarian follicles in experimental animal models.[15,18] Cyclophosphamide exposure also caused adverse morphological alterations of ovaries such as GCs derangement, ovarian stroma vacuolization, and formation of edema and fibrosis within the ovarian interstitium.[18,19] Other associated mechanisms of cyclophosphamide-induced ovarian toxicity included decreased levels of AMH, increased levels of MDA, TNF-α, IL-6 factors, downregulation of estradiol, Bcl-2, VEGF-A and SIRT1 expression while Bax, caspase-3, P13k-AKT, HIF-1, Ac-Foxo3a, and p-Foxo3a expressions were upregulated. [19,20,21]

Another chemotherapeutic agent – cisplatin, used for treatment of different forms of cancer, has been reported to cause female gonadotoxicity through induction of oxidative stress and activation of inflammatory and apoptotic signaling resulting in impairment of ovarian function. Cisplatin exposure in experimental models causes ovarian histopathological changes and decreased serum AMH levels.[22] Moreover, the cellular mechanisms associated with cisplatin-induced ovarian toxicity in experimental models included elevated levels of ROS, MDA, IL-6, TNF-α, NF-κB, and upregulation of Bax, cytochrome-c, caspase-3, PARP-1, PTEN, P-AKT, P-mTOR, and P-AMPKα expressions.[22-24] In addition, cisplatin exposure caused reduced levels of GSH, SOD, CAT, and Bcl-2 expression in ovarian tissues in experimental animal models.[22,25]

Doxorubicin, a common anti-cancer drug, is widely known for its adverse effects on multiple body tissues

Table 1. Profile of selected drugs and mechanisms of ovarian toxicity

Selected drugs	Molecular formula (Molecular weight)	Properties and uses	Mechanisms of ovarian toxicity	References
Busulfan	$C_6H_{14}O_6S_2$ (246.30 g/mol)	- white crystalline powder - slightly soluble in water or ethanol - used as chemotherapeutic drug for treating cancer	- ovarian atrophy and weight loss - dysregulation of reproductive hormones - depletion of ovarian follicles - increased DNA-damage in oocytes - induction of apoptosis in GCs - reduced levels of reproductive hormones	Thonen et al.[13] Sakurada et al.[14] Peng et al.[15] Del Castillo et al.[16] Jiang et al.[17]
Cyclophosphamide	$C_7H_{15}Cl_2N_2O_2P$ (261.08 g/mol)	- white crystalline powder - used for treating cancer and autoimmune diseases	- distortion of ovarian histoarchitecture - decreased level of AMH - increased levels of MDA, TNF-α, IL-6 factors - reduced estradiol, Bcl-2, VEGF-A, SIRT1 levels - upregulation of Bax, caspase-3, P13k-AKT, HIF-1, Ac-Foxo3a, and p-Foxo3a expressions	Peng et al.[15] Pascuali et al.[18] Nan et al.[19] Xiu et al.[20] Notghi et al.[21]
Cisplatin	$Pt(NH_3)_2Cl_2$ (300.10 g/mol)	- yellow or orange crystalline powder, - used as anti-cancer drug	- ovarian histopathological changes - elevated MDA, IL-6, TNF-α, NF-κB levels - upregulation of Bax, caspase-3, PARP-1, PTEN, P-AKT, P-mTOR, P-AMPKα - reduced levels of GSH, SOD, CAT, and Bcl-2 - reduced AMH level	Eid et al.[22] Said et al.[23] Li et al.[24] Soyman et al.[25]
Doxorubicin	$C_{27}H_{29}NO_{11}$ (543.52 g/mol)	- orange-red lyophilized, water-soluble powder - used to treat cancer	- increased ROS level - reduced MMPs level - upregulation of Bax, Bcl-2, p53, StAR expressions - impairment of folliculogenesis and oogenesis - dysfunctional ovulatory and endometrial cycle	Ben-Aharon et al.[26] Zhang et al.[27] Mohan et al.[28] Xiao et al.[29]

including ovarian tissue through induction of oxidative stress and apoptotic signaling. Previous studies in experimental animal models have reported that exposure to doxorubicin caused a reduction in ovarian size and weight which could be attributed to a significant decline in the population of different stages of ovarian follicles.[26] Doxorubicin-induced ovarian toxicity also caused induction of GCs apoptosis, increased ROS levels, and reduced MMPs levels.[27] Other mechanisms of doxorubicin-induced ovarian toxicity included upregulation of Bax, p53, StAR expressions, and oocyte mitochondrial dysfunction characterized with impaired calcium signaling and ER stress.[27,28] In essence, doxorubicin impairs folliculogenesis and oogenesis leading to dysfunctional ovarian and endometrial cycles.[28] Moreover, the toxic effect of doxorubicin on the three pivotal ovarian functions (development of ovarian follicles, hormone secretion and oocyte maturation) has been demonstrated to exhibit dose-dependency.[29]

Heavy metal-induced ovarian toxicity and associated mechanisms

Heavy metals are metallic elements which possess high densities and atomic weight, derived from different sources (both natural and industrial) and used for various applications as outlined in Table 2. Generally, exposure to different heavy metals potently exhibits a toxic effect on ovarian morphology and function (including reproductive and endocrine) by inducing oxidative stress through direct or indirect mechanisms, thereby culminating into oxidative damage, ovarian dysfunction, and infertility.[30]

The exposure to toxic heavy metals like cadmium (Cd) often leads to its accumulation in body tissues including the ovary, causing severe deleterious effects and female infertility. Cd-induced ovarian toxicity, primarily due to induction of oxidative stress, is characterized by ovarian follicular apoptosis, dysregulation of reproductive hormones and oocyte growth impairment.[31] Exposure to cadmium further caused distorted ovarian histoarchitecture, reduced ovarian follicular growth, ovarian follicular atresia, decreased serum levels of LH and FSH, and an impaired reproductive cycle.[32,33]

Copper (Cu) is a heavy metal which exhibits toxic effects on ovarian tissue. In preclinical studies, exposure to Cu has been reported to cause oxidative stress and inflammation in ovarian tissue which in turn causes impaired ovarian folliculogenesis and disrupted reproductive hormonal signaling thereby causing steroidogenesis disorder.[34,35] Cu-induced ovarian toxicity was further characterized by upregulation of Bax, Fas, Caspase8, and Caspase3 expressions thereby indicating the caspase-dependent apoptosis signaling as a pivotal mechanism.[36]

Arsenic (As) is also a heavy metal that acts as endocrine disruptor and induces oxidative stress to exert toxic effects on the ovarian tissue. As-induced ovarian toxicity has been characterized with decreased levels of

Table 2. Profile of heavy metals and mechanisms of ovarian toxicity

Heavy metals (symbol)	Molar mass	Sources	Applications	Mechanisms of ovarian toxicity	References
Cadmium (Cd)	112.41 g/mol	contaminated water, food, or beverages, earth's crust, cigarette smoke, welding, mining	production of batteries, paints, plastics, television screens, cosmetics	- ovarian follicular atresia and apoptosis - dysregulation of reproductive hormones and impairment of growth of oocytes - distorted ovarian histoarchitecture - reduced follicular growth - decreased serum levels of LH and FSH	Bhardwaj et al.[31] Oyewopo et al.[32] Massányi et al.[33] Khan et al.[44]
Copper (Cu)	63.55 g/mol	decaying vegetation, volcanic eruptions, forest fires, mining, dietary sources (liver, oysters, avocados, nuts, salmon, potatoes, mushrooms	production of electric wires, cooking utensils, heat sinks, electronics, electric motors, copper alloys, plumbing pipes, jewelry	- impaired ovarian folliculogenesis - disruption of reproductive hormonal signaling - steroidogenesis disorder - upregulation of Bax, Fas, Caspase8, and Caspase3 expressions	Wang et al.[34] Yiqin et al.[35] Chen et al.[36]
Arsenic (As)	74.92 g/mol	contaminated water or groundwater, food, earth's crust, in combined form with other metals, sulphur or oxygen	production of insecticide, alloys, lasers, bronzing, semiconductors (gallium arsenide), pyrotechnics	- mitochondrial dysfunction and autophagy - downregulation of StAR, CYP11A1, HSD3β1, mTOR and Bcl-2 expressions - decreased levels of gonadotropins - upregulation of PDK_1, PI3K, Beclin1, TSC2, AMPK, ULK1, ATG13, LC3, P62, ATG3, ATG7, and p62 factors	Chen et al.[37] Ommati et al.[38]
Mercury (Hg)	200.59 g/mol	weathering of rocks, volcanic eruptions, coal, contaminated seafood, water, air or food, industrial wastes	production of mirrors, thermometers, batteries, fluorescent lamps, cosmetics,	- overall depletion of ovarian follicles, - increased atretic, cystic ovarian follicles - elevated ovarian fat accumulation - distortion of ovarian histomorphology - decreased serum levels of LH and FSH	Massanyi et al.[33] Merlo et al.[39]
Lead (Pb)	207.2 g/mol	vehicle exhaust, burning of fossil fuels, volcanic eruptions, weathering of rocks, mining, smelting, landfills	production of pipes, gasoline, roofing sheets, paints, alloys, ammunition, weights, batteries, cosmetics, and gasoline	- distortion of ovarian cytoarchitecture - decline in ovarian follicles population - decreased AMH, MFF and MPP levels - disruption of HPG axis, steroidogenesis - increased levels of SDHA, mitofilin and MTCO2, OPA1, MFN and FIS1	Qu et al.[40] Wassem et al.[41] Fatima et al.[42] Yang et al.[43]

gonadotropins, downregulation of StAR, CYP11A1, and HSD3β1 resulting into decline of mature ovarian follicles and an increased population of atretic follicles.[37] Other mechanisms of As-induced ovarian toxicity included induction of mitochondrial dysfunction and autophagy via the upregulation of associated factors such as PDK_1, PI3K, TSC2, AMPK, ULK1, ATG13, Beclin1, LC3, P62, ATG3, ATG7, and p62 and downregulation of mTOR and Bcl-2 factors in the ovaries.[38]

Mercury (Hg) is a heavy metal which exhibits severe toxic effects during body tissue exposure including the ovaries. Hg-induced ovarian toxicity in experimental animal models is characterized by induction of oxidative stress and inflammation which further results in overall depletion of ovarian follicles, increased atretic and cystic ovarian follicles, and ovarian fat or lipid droplet accumulation.[39] Exposure to Hg has also been reported to cause distortion of ovarian histomorphology, decreased follicular growth and serum levels of LH and FSH.[33]

Exposure to lead (Pb), a common heavy toxic metal, has also been indicated as a potential cause of ovarian toxicity. Pb-induced ovarian toxicity resulted primarily from the disruption of antioxidant defense systems and excess production of ROS.[40] It was further characterized by distortion of ovarian cytoarchitecture, and a decreased population of ovarian follicles.[40,41] Pb exposure further caused disruption of the hypothalamic-pituitary-gonadal (HPG) axis and decreased reproductive hormone (AMH) level leading to dysregulation of folliculogenesis and steroidogenesis.[40,42] Other mechanisms of Pb-induced ovarian toxicity included increased levels of SDHA, mitofilin and MTCO2, OPA1, MFN and FIS1; decreased levels of MFF and MPP, and inhibition of phosphorylation in the P38 pathway.[43]

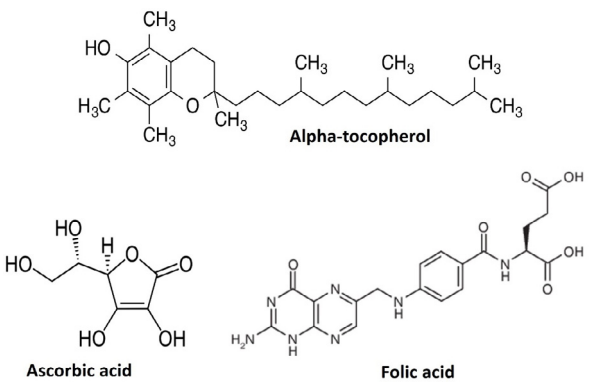

Fig. 2. Molecular structures of vitamins B_9, C and E

Therapeutic potential of selected vitamins against ovarian toxicity induced by drugs or heavy metals

Vitamins are micronutrients which play an essential role in the physiological and metabolic functions of the body, help to reduce the risk of diseases and deficiencies result in diverse tissue disorders.[45] The two classes of vitamins, namely fat-soluble and water-soluble vitamins, are comprised of different vitamins with diverse properties based on their varying structures. Vitamins such as alpha-tocopherol, ascorbic acid, and folic acid possess variable structural conformations (Fig. 2) and exhibit diverse properties. Previous preclinical studies have demonstrated their therapeutic effect against ovarian toxicity induced by drugs or heavy metals in experimental animal models as outlined in Table 3.

Alpha-tocopherol – therapeutic role against ovarian toxicity in experimental model

Alpha-tocopherol is a fat-soluble vitamin which is abundantly derived from cereals, vegetables oils, eggs, fruits, and meats, and one of the eight essential compounds (four tocopherols and four tocotrienols) of the vitamin E family.[46] The vitamin E family possesses a chromane ring with a hydroxyl group and side chain with tocopherol members possessing saturated side chain while tocotrienol members bear an unsaturated isoprenoid side chain with double bonds at third, seventh, and eleventh carbon.[47] Alpha-tocopherol acts as an antioxidant which protects tissue against oxidative damage that results from activities of free radicals.[46,48] Hence, it has demonstrated protective effect against drug-induced ovarian toxicity in preclinical studies.

In a previous study using experimental model, therapeutic activity of alpha-tocopherol against cisplatin-induced ovarian toxicity was characterized with reversal of follicle developmental disorders and ovarian fibrosis, increased level of reproductive hormones, marked inhibition of ferroptosis and abrogation of lipid peroxidation leading to the restoration of ovarian function.[49] In addition, the mitigating effect of alpha-tocopherol on cyclophosphamide-induced ovarian toxicity was characterized with elevated levels of ovarian tissue antioxidant (GSH), proliferating cell nuclear antigen (PCNA) and growth differentiation factor-9 (GDF-9).[50] Furthermore, as a natural non-enzymatic antioxidant, the ability to scavenge free radicals and inhibit lipid peroxidation highlighted its mitigating potential on heavy metal-induced toxicity of body tissues.[51,52] On this basis, alpha-tocopherol has demonstrated mitigating effect on ovarian toxicity caused by exposure to heavy metals in experimental animal models. The associated mechanisms of amelioration of heavy metal-induced ovarian toxicity by alpha-tocopherol further included the modulation of the NF-kB, Nrf2 and MAPK signaling pathways.[53]

Ascorbic acid – therapeutic role against ovarian toxicity in experimental model

Ascorbic acid (vitamin C) is a water-soluble vitamin which is abundantly derived from natural sources like

Table 3. Profile and therapeutic role of selected vitamins against ovarian toxicity

Vitamins (other names)	Molecular formula (molecular weight)	Natural sources	Possible mechanisms of therapeutic effect against ovarian toxicity	References
Alpha-tocopherol (vitamin E)	$C_{29}H_{50}O_2$ (430.71 g/mol)	almonds, nuts, butter, spinach, vegetable oils, cereals, meat, egg, avocado, fruits, wheat	- reversal of follicle disorders and ovarian fibrosis - increased levels of reproductive hormones, - inhibition of ferroptosis and lipid peroxidation - elevated levels of GSH, PCNA, GDF-9 factors - modulation of NF-kB, Nrf2 and MAPK signaling	Du et al.[49] Gürgen et al.[50] Zhai et al.[51] Fan et al.[53]
Ascorbic acid (vitamin C)	$C_6H_8O_6$ (441.4 g/mol)	orange, tomato, guava, potato, mango, lemon, pepper, strawberries, grape fruits	- stimulated antioxidant enzymes like SOD, CAT, GPx - enhanced the activity of Nrf2, Ref-1, AP-1 - preservation of ovarian morphology - elevated levels of GSH, PCNA, GDF-9 - preserve ovarian follicular growth	Gürgen et al.[50] Gęgotek et al.[56,] Gęgotek et al.[57]
Folic acid (vitamin B_9)	$C_{19}H_{19}N_7O_6$ (176.124 g/mol)	banana, papaya, orange, green peas, kidney bean, crab, tomato juice, avocado, black-eyed pea, peanuts, spinach, beef liver	- increased GSH and TAC levels - reduced MDA level - increased ovarian reserve or antral follicle count - inhibition of ovarian follicle degeneration - preservation of normal ovarian morphology - downregulation of apoptotic factors	Gliszczyńska-Świgło et al.[62] Asbaghi et al.[63] Liao et al.[64] Kadir et al.[65] Shohda et al.[66]

oranges, lemons, grape, vegetables and is composed of an almost planar five-member ring with two chiral centers that resolves into the four stereoisomers.[54,55] Essentially, ascorbic acid is a natural antioxidant which interacts with other antioxidants (including tocopherol and glutathione) and stimulates the biosynthesis and activation of antioxidant enzymes like SOD, CAT, GPx. It further enhanced the activity of Nrf2, Ref-1, AP-1 which then upregulate genes encoding antioxidant proteins.[56,57] Hence, ascorbic acid possesses ability to scavenge free radicals that follows the exposure of body tissues to potential toxicants (such as drugs or heavy metals) thereby abrogating oxidative damage of tissues due to the exposure.[57-59]

The therapeutic potential and antioxidant properties of ascorbic acid has been explored in experimental animal models to mitigate ovarian toxicity induced by drugs or heavy metals. Previous experimental studies have demonstrated the potency of ascorbic acid in mitigating cyclophosphamide-induced ovarian toxicity characterized with preservation of ovarian morphology, elevated levels of ovarian tissue antioxidant (GSH), proliferating cell nuclear antigen (PCNA) and growth differentiation factor-9 (GDF-9) thereby preserving the growth of ovarian follicles.[50]

Folic acid – therapeutic role against ovarian toxicity in experimental model

Folic acid (or folate or vitamin B_9) is a member of vitamin B family which is abundantly derived from dietary sources such as banana, papaya, orange, green peas, kidney bean, crab, tomato juice, avocado, black-eyed peas, peanuts, and spinach.[60] The chemical structure of folic acid is composed of a methylated pteridine ring attached to *p*-aminobenzoic acid (PABA), and is in turn linked to the α nitrogen of glutamic acid via carboxyl group. It is an essential vitamin that plays a key role in biosynthesis of purine and pyridine bases as well as during prenatal development.[61]

Furthermore, folic acid has been demonstrated to exhibit antioxidant properties comparable to alpha-tocopherol and ascorbic acid *in vivo,* with its supplementation contributing to the role of tissue antioxidant systems in scavenging free radicals and inhibiting lipid peroxidation.[62] The antioxidant role of folic acid was characterized with significantly increased GSH and TAC levels and reduced MDA level.[63,64] Hence, it has potential to mitigate pathologies of tissues (including ovaries) following exposure to drugs and heavy metals.

Moreover, folic acid supplementation has enhanced ovarian function and ensures high ovarian reserve or antral follicle count thereby promoting the overall female reproductive health.[65] Treatment with folic acid has further demonstrated protective effects against methotrexate-induced ovarian toxicity in experimental models characterized by inhibition of ovarian follicle degeneration, preservation of normal ovarian morphology and downregulation of apoptotic factors.[66]

Conclusion

The exposure of ovarian tissue to potential toxins, including drugs and heavy metals, triggers ovarian toxicity primarily due to the induction of oxidative stress and activation of inflammatory and apoptotic signaling. The resultant ovarian toxicity results in distortion of ovarian morphology and loss reproductive function leading to infertility. This narrative review presents the therapeutic effect of selected vitamins including alpha-tocopherol, ascorbic acid and folic acid against drug or heavy metal-induced ovarian toxicity primarily through suppres-

sion of oxidative stress and inhibition of inflammatory and apoptotic signaling pathways. The therapeutic effect of the selected vitamins thereby underscores their efficacy in therapeutic intervention against drug or heavy metal-induced ovarian toxicity.

Declarations

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Author contributions
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Conflicts of interest
Authors do not declare any conflict of interest.

Data availability
The data that support the findings of this study have been included in the article.

Ethics approval
Not applicable.

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REVIEW PAPER

Harnessing the gut-brain axis in the treatment of type 2 diabetes mellitus and obesity

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ABSTRACT

Introduction and aim. The most common metabolic disorders include type 2 diabetes (T2DM) and obesity. Their prevalence has increased in recent years. Due to their widespread prevalence and the fact that they increase the risk of cardiovascular disease, morbidity, and mortality, they pose a significant economic burden to the healthcare system. In this review, we will focus primarily on the role of gut hormone signaling produced by enteroendocrine cells (EECs), which are part of the gut-brain axis. Furthermore we will summarize applications of these mechanisms in novel therapies for T2DM and obesity.

Material and method. Literature data analysis was performed using the following databases: PubMed (MEDLINE), Scopus, Web of Science and Google Scholar. The review included articles in Polish and English published between 2000 and 2024.

Analysis of the literature. EECs are specialized transepithelial cells present throughout the intestine. The best-studied EEC subtype is the L cell, which secretes glucagon-like peptide-1 (GLP-1). GLP-1 regulates insulin secretion and contributes to satiety by increasing insulin secretion and inhibiting glucagon secretion. Significant progress in the use of intestinal hormones in the treatment of T2DM and obesity has led to the development of effective therapies for both of these conditions, such as GLP-1 analogs.

Conclusion. The growing understanding of biochemical processes, hormonal signaling, and the development of new technologies contribute to the continuation of research on new, more effective therapies that use mechanisms of action of the gut-brain axis. Despite these achievements, the need for new and more effective treatments is constantly growing, and requires innovative strategies and their potential combination with existing therapies.

Keywords. diabetes, gut-brain axis, obesity

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Introduction

Metabolic disorders include type 2 diabetes (T2DM) and obesity. These conditions frequently coexist. Their prevalence varies widely across the globe.[1,2] According to the International Diabetes Federation, 463 million people worldwide had diabetes in 2019,[3] while, according to the World Health Organization, more than 650 million adults worldwide were obese in 2016.[4] Due to their widespread prevalence and the fact that both T2DM and obesity increase the risk of developing cardiovascular disease, morbidity, and mortality, these conditions impose a significant economic burden on healthcare systems.[1]

One of the available therapies and treatment strategies for these conditions is glucagon-like peptide-1 (GLP-1) therapy. Their mechanism of action is based on the regulation of food intake, hunger and satiety, metabolism, and glucose homeostasis via the gut-brain axis.[5] The gastrointestinal tract responds to a variety of stimuli, such as mechanical factors, pathogens, nutrients contained in food, and toxins.[5] The gut-brain axis mediates bidirectional communication between the gastrointestinal tract and the central nervous system, utilizing specific signaling pathways.[5,6] These pathways include the nervous system, the immune system, the hypothalamic-pituitary-adrenal axis, and enteroendocrine cells (EECs).[6]

Aim

In this review, we will focus primarily on the role of gut hormone signaling produced by EECs, which are part of the gut-brain axis and are involved in the regulation of appetite and glucose homeostasis. Furthermore, we will summarize applications of these mechanisms in novel therapies for T2DM and obesity.

Material and methods

Literature data analysis was conducted between 2020 and 2024 using the following databases: PubMed (MEDLINE), Scopus, Web of Science, and Google Scholar. The review included articles in Polish and English published between 2000 and 2024. Key terms used to identify relevant studies included: "glucagon-like peptide-1," "enteroendocrine cells," "GLP-1 analogues," "incretin effect in type 2 diabetes," "lixisenatide," "liraglutide," "semaglutide," and "tirzepatide." The review included scientific articles, reviews, and clinical trial descriptions in pediatric and adult patients. The review addressed the role of the gut-brain axis in appetite regulation and glucose homeostasis, the role of GLP-1 analogues in the context of the incretin effect, the treatment of T2DM, the treatment of obesity, cardiovascular disease, and other beneficial and adverse effects. Articles published before 2000, articles written in languages other than English or Polish, conference proceedings, and articles with access only to the abstract were excluded. A literature review was conducted by five reviewers using a standard data extraction form. Ninety-two publications were included in the review. The analysis focused primarily on the role of gut hormonal signaling, generated by EEC, as a component of the gut-brain axis, and on the characteristics and effects of GLP-1 analogues, including tirzepatide.

Analysis of the literature

The role of the gut-brain axis in appetite regulation and glucose homeostasis

EECs are specialized transepithelial cells present throughout the intestine.[7] Several unique subtypes of EECs have been described, distinguished by their location along the intestine, and the hormones and neurotransmitters they secrete. The nomenclature used to distinguish between the subtypes is based on the detection of specific hormones.[8,9] EEC cells in the stomach produce hormones that, through feedback, regulate their own levels. These include ghrelin, histamine, and gastrin. In the distal intestine, they secrete anorexic hormones, and to support gastric motility, motilin and serotonin, as well as gastric inhibitory polypeptide (GIP).[5] The best-studied EEC subtype is the L cell, which secretes GLP-1.[10] GLP-1 regulates insulin secretion and contributes to satiety by increasing insulin secretion and inhibiting glucagon secretion, thereby suppressing endogenous glucose production and reducing postprandial glycemia. GLP-1 also delays gastric emptying[11] and increases pyloric contractions, thereby slowing the flow of food into the small intestine.[5]

EECs secrete hormones that influence neighbouring receptors, organs, and nerves (including the intestinal and vagus nerves). Therefore, their mechanism of action operates on multiple levels. The primary mechanism of action is through paracrine signaling, characterized by a short half-life.[12-15] Another mode of action is endocrine due to the presence of pancreatic β-cells, which influence the process of food regulation.[14] The third signaling pathway utilizes synaptic connections between EECs and intestinal afferent nerves; these are unable to directly detect chemical signals in the intestinal lumen. Therefore, synaptic connections with EEC cells enable the transmission of sensory stimuli from the intestinal lumen via neurotransmitters such as glutamate.[16]

Prior to food ingestion, gastric EECs produce ghrelin, which modulates orexigenic signaling to the brain. This hormone is detected at high concentrations before meals, suggesting that circulating ghrelin directly engages the hindbrain and the arcuate nucleus to promote appetite.[17] The entry of nutrients into the small intestine inhibits ghrelin secretion[18] and their absorption by the duodenal epithelium stimulates EECs to secrete cholecystokinin (CCK), GIP, and Secretin.[10] CCK

receptors act on sensory nerves located in the region of the vagus nerve and its terminals. In this way, they influence not only satiety but also pyloric contractions, inhibiting gastric emptying.[19,20] GIPs, in turn, activate insulin secretion. They regulate the activity of specific receptors on β-cells and influence the hypothalamus, which leads to fat storage in adipocytes.[21,22] EECs are most strongly activated when nutrients and bile acids pass from the duodenum into the jejunum.[19,23,24,25]

GLP-1 analogs

GLP-1 analogues, or GLP-1 receptor agonists (GLP-1RAs), are incretin-based drugs that are increasingly used in the treatment of patients with T2DM. These drugs are administered via subcutaneous injection (the exception being the oral formulation of semaglutide). Their primary role is to control and regulate blood glucose levels, which occurs through the process of insulin secretion and biosynthesis in a glucose-dependent manner. Additionally, these drugs inhibit glucagon secretion, delay gastric emptying, and promote satiety, which supports glycemic control and weight management in patients. GLP-1 analogues mimic the action of endogenous GLP-1.[26] Theoretically, pancreatic β cells should respond in the same way to glucose, regardless of whether it was administered orally or directly into the bloodstream. In practice, however, the body's response is different - after oral glucose intake, the level of insulin secreted is higher than after intravenous administration, despite similar blood glucose concentrations. This phenomenon is called the incretin effect. This effect is caused by gut hormones, the so-called incretins, which include GIP and GLP-1. GLP-1 is secreted by cells of the duodenal mucosa, while GLP-1 is produced in the cells of the small intestine. Through synthesis with appropriate receptors on the surface of pancreatic β-cells, they stimulate them to increase insulin secretion. The action of GIP and GLP-1 depends on the current glucose concentration – the higher the concentration, the greater the stimulation of insulin secretion and the simultaneous inhibition of glucagon production. When glucose levels drop to baseline, the activity of these hormones ceases. Importantly, incretins do not increase insulin secretion or inhibit glucagon when glucose levels are low – so they do not cause hypoglycemia. Additionally, GLP-1 slows down stomach emptying, which leads to slower digestion and absorption of glucose. This reduces the rapidity of the postprandial increase in blood sugar levels. Slower stomach emptying also promotes a faster feeling of satiety, which may contribute to a reduction in the amount of food consumed. In turn, GIP also affects at the cellular level – it stimulates the expression of the gene responsible for the production of proinsulin, a precursor of insulin.[27]

GLP-1 and the incretin Effect

A greater and more effective insulin response is induced by an oral glucose load than by an intravenous load.[28,29] This enhanced response is referred to as the incretin effect. GLP-1 is an incretin peptide hormone. It is secreted by L cells in the distal small intestine and colon within minutes of nutrient ingestion. It acts on pancreatic β-cells via GLP-1 receptors. It is estimated that approximately 50–70% of total insulin secretion after an oral glucose load is a consequence of incretin release from the gastrointestinal tract.[29,30,31] GLP-1 suppresses appetite, and sustained activation of the GLP-1 receptor is associated with weight loss.[29,30] The pancreas is not the only site where the GLP-1 receptor is found. It also appears in the stomach, intestines, kidneys, pituitary gland, and central nervous system. Therefore, its influence and role are extensive and multifaceted.[29,30]

Studies on the secretion and action of incretins indicate significantly reduced incretin action in individuals with T2DM.[31] The reasons for this reduced incretin action are not fully understood.[29] Secondary to the development of T2DM, pancreatic resistance to GLP-1 develops, and GLP-1 secretion by L cells decreases.[29,32] Because the attenuation of GLP-1-related incretin action occurs early in the natural history of T2DM, GLP-1 replacement therapy seems a logical choice to restore normal insulin response in patients.[29] Administration of pharmacological doses of GLP-1 has been shown to be an effective way to restore the insulin response to glucose in patients with T2DM. GLP-1 infusion not only increases insulin secretion but also normalizes its secretory pattern, restoring the first phase of the insulin response, which is characterized by a sudden and rapid increase in insulin levels.[29,33] Nauck et al. and Choe et al. demonstrated that continuous intravenous infusion of GLP-1 in patients with poorly controlled T2DM increased insulin secretion, resulting in better glycemic control, particularly of fasting glucose levels.[34,35] The antidiabetic efficacy of GLP-1 administered by continuous subcutaneous infusion was confirmed by Zander, who demonstrated normalization of daily glycemia and a reduction in glycated hemoglobin (HbA1c) levels within 6 weeks of treatment.[36,37] However, continuous administration of native GLP-1 via the intravenous or subcutaneous route is not convenient for long-term use in patients due to its very short half-life of approximately 2 minutes. Therefore, it has been important to develop GLP-1 agonists that are resistant to degradation by the dipeptidyl peptidase-4 (DPP-4) enzyme.[37]

Meta-analyses of clinical trials have shown that the use of GLP-1RAs in patients with obesity and T2DM is not associated with an increased risk of breast cancer, acute pancreatitis, pancreatic cancer, or overall cancer incidence. Furthermore, evidence suggests that GLP-1RAs therapy does not increase the risk of new thyroid nodules in patients with T2DM. Preclinical studies have

also shown potential anti-cancer activity of GLP-1RAs, including by inhibiting the growth of prostate and pancreatic cancer cells. Similarly, growth-inhibiting effects on breast and cervical cancer cells have been observed, suggesting potential future applications of GLP-1RAs in cancer therapy. In particular, liraglutide has shown antiproliferative and proapoptotic properties against pancreatic cancer cells resistant to gemcitabine and other cytotoxic drugs. Despite these promising results, some concerns have been raised about the long-term safety of GLP-1RAs. A population-based matched-case study suggested that exenatide use may increase the risk of hospitalization for acute pancreatitis. Additionally, some data suggest that incretin drugs may be associated with an approximately 1.7-fold increased risk of developing pancreatic cancer. However, due to limited statistical power, short follow-up, and uncertainties in assessing disease severity, a direct causal relationship cannot be unequivocally confirmed. In contrast, systematic reviews of studies assessing mortality and the effects of GLP-1RAs on cardiovascular and renal function in patients with T2DM have not shown an increased incidence of severe hypoglycemia, pancreatitis, or pancreatic cancer. There is currently no clear clinical evidence indicating a carcinogenic effect of GLP-1RAs. On the contrary, a growing body of research suggests that they may have anticancer effects on various types of cancer, including ovarian, breast, prostate, and pancreatic.[38,39,40]

Short-acting and long-acting GLP-1 analogs

The first GLP-1 receptor agonist approved for the treatment of T2DM was exenatide, a synthetic exendin-4 peptide isolated from the venom of the Gila monster (Heloderma suspectum).[34] Exenatide is a peptide resistant to DPP-4 and is not inactivated by it. This drug has an affinity for the GLP-1 receptor similar to native GLP-1 and stimulates pancreatic β-cells in a glucagon-like manner. After subcutaneous injection, exenatide has a half-life of about 4 hours, reaching peak concentration between 2 and 3 hours, with a total duration of action up to 5-7 hours. Two-times and three-times daily regimens have been tested. The regimen of exenatide was approved for subcutaneous injection twice daily, before breakfast and dinner.[34] The long-acting-release LAR preparation of exenatide molecule enables weekly administration while maintaining the desired therapeutic effect.[34,41] Although exenatide shares 53% sequence homology with GLP-1, it induces an immune response leading to the formation of antibodies formation, which, however, does not significantly affect its therapeutic efficacy.[34,42]

Lixisenatide is a structural analog of exendin-4, modified by adding six lysine residues at the C-terminal end, while removing a proline residue. This modification makes it resistant to DPP-4 degradation.[43,44] It is administered via subcutaneous injection once daily.[45]

Liraglutide, developed as another GLP-1 receptor agonist, shares 97% sequence homology with native GLP-1. By attaching a fatty acid chain to its structure via a linker molecule, it provides a long-acting drug. Furthermore, it promotes albumin binding in extracellular fluid and plasma.[34,46] Approximately 2% of liraglutide is unbound to albumin. However, the majority of liraglutide is a reservoir from which it can be released into specific and targeted tissues and cells with GLP-1 receptors. The estimated half-life is 13 hours.[34,47] It is approved for once-daily subcutaneous administration.[34] Dulaglutide and albiglutide represent a novel approach in which larger proteins, such as immunoglobulin fragments in the case of dulaglutide[34,48] and albumin in the case of albiglutide[34,49], are linked to two modified (DPP-4-resistant) GLP-1 molecules. Due to their slow systemic elimination, these drugs have a half-life of approximately one week, allowing for once-weekly administration.[34,50,51] The relatively rapid absorption of these drugs results in an earlier response and, consequently, a more rapid onset of clinically noticeable effects.[34,52]

Semaglutide has a molecular structure similar to liraglutide. It has been modified by replacing alanine at position 2 with α-aminoisobutyric acid, which makes it completely resistant to DPP-4 degradation. Due to the stronger binding of the fatty chain, semaglutide has an even longer elimination time, which allows it to be administered once a week.[34,53]

In summary, we distinguish short-acting and long-acting GLP-1RAs. Short-acting GLP-1RAs include exenatide and lixisenatide, administered once or twice daily via subcutaneous injection. Long-acting GLP-1RAs include liraglutide administered as subcutaneous injections once daily, and exenatide, dulaglutide, albiglutide, and semaglutide administered once weekly.[34,45]

An oral form of semaglutide has also been developed, containing the same active molecule as the injectable form, combined with an absorption enhancer, sodium N-[8-(2-hydroxybenzoyl)amino]caprylate (SNAC). SNAC locally increases pH, facilitating semaglutide absorption. However, oral semaglutide has low bioavailability. To achieve similar efficacy as subcutaneous injection (1 mg/week), significantly higher doses (up to 14 mg/day) are required.[34,54] It is recommended to take oral semaglutide once daily in the morning on an empty stomach with a small amount of water, at least 30 minutes before breakfast.[8,54]

GLP-1 analogs in T2DM treatment

The role of GLP-1RAs is to stimulate insulin secretion in a blood glucose-dependent manner – the higher the blood glucose concentration, the greater the insulin secretion.[29] Furthermore, by increasing insulin biosynthesis at the

translational level, they help maintain insulin reserves in β-cells and their secretory capacity.[29,30] Due to their direct effect on pancreatic α-cells, which become more sensitive to glucose, they secrete less glucagon, thus reducing endogenous glucose production. Glucagon stimulates hepatic glucose production, so reducing its secretion leads to reduced hepatic glucose production, which reduces insulin requirements and improves glycemic control.[29,55] Furthermore, GLP-1RAs can influence peripheral glucose metabolism via GLP-1 receptors located in the central nervous system.[29,55] GLP-1RAs reduce gastric acid secretion and slow gastric emptying, both in response to pentagastrin and after a meal. These effects are mediated by the vagus nerve and GLP-1 receptors located in the central nervous system or on afferent vagal fibers, which transmit sensory signals to the brainstem.[29,30] Slowing gastric emptying reduces the rate of glucose absorption, resulting in lower postprandial blood glucose levels. Short-acting GLP-1RAs, administered around meals, have a stronger effect on gastric motility and are more effective in reducing postprandial glycemia than long-acting forms.[29,57]

GLP-1 analogs significantly reduce the level of HbA1c. Studies show that GLP-1RAs used in monotherapy lower HbA1c level by 0.5–1.0% more than placebo. In combination with metformin, the reduction of HbA1c was 0.5–1.1%, with sulfonylureas 0.6–1.4%, and with thiazolidinediones 0.8–1.1%.[29] Direct comparisons have shown that the reduction of HbA1c achieved with GLP-1RAs is greater than in the case of DPP-4 inhibitors, due to their higher receptor binding affinity.[29,58] The reduction of HbA1c levels with the use of GLP-1RAs is comparable to that achieved with the use of insulin (including basal insulin analogs, mixed formulations, and NPH insulin), but without the risk of hypoglycemia or weight gain that are associated with insulin therapy.[29,59,60] In clinical practice, the degree of HbA1c reduction depends on the patient's initial hemoglobin level.[5]

GLP-1 analogs in obesity treatment

GLP-1 plays an important role in multiple areas of physiology. The most important in terms of obesity treatment is reducing appetite and food intake. This effect promotes long-term weight loss. Studies indicate that GLP-1 secretion from the gastrointestinal tract is impaired in obese individuals, suggesting its involvement in the pathophysiology of obesity.[61,62]

GLP-1RAs mechanism of action includes both central and peripheral processes that together promote satiety, reduce hunger, and consequently lead to decreased food intake.[61] Central effects involve the activation of GLP-1 receptors present in brain regions involved in the regulation of food intake and energy balance. Indirect mechanisms include the activation of vagal afferents originating from the intestines and portal circulation.[61,63] Slowed gastric emptying and transient nausea induced by GLP-1 analogs may contribute to weight loss, but their role is thought to be minor and short-lived.[61,64]

Weight loss was consistently observed in all pivotal clinical trials of GLP-1RAs. Studies have shown that albiglutide[45,59] and dulaglutide[32] were less effective in reducing body weight compared with liraglutide and semaglutide. The larger molecular structures of albiglutide and dulaglutide limit the ability of GLP-1RAs to penetrate the brain and affect the satiety center.[65,66,67] Food and Drug Administration -approved injectable GLP-1 analogues for the treatment of obesity include liraglutide and semaglutide.[65,68]

GLP-1 analogs and cardiovascular diseases

Cardiovascular diseases remain the most common cause of death in Poland. Cardiovascular risk is defined as the probability of developing cardiovascular disease or dying from it within a specified time period. Numerous studies confirm that people with T2DM are exposed to higher cardiovascular risk compared to the general population. The level of this risk is influenced by many factors, which can be divided into classic (e.g. hypertension, hypercholesterolemia, smoking) and non-classic (e.g. inflammation, oxidative stress, metabolic disorders). T2DM is often accompanied by hyperglycemia and other metabolic disorders, which significantly increase the risk of developing both cardiovascular diseases and microvascular complications. Randomized clinical trials have shown that maintaining proper glycemic control reduces the risk of microvascular complications. However, its effect on preventing macrovascular complications, such as atherosclerosis, is moderate. Despite this, it has been shown that more effective treatment of hyperglycemia can lead to a reduction in the number of serious cardiovascular events. GLP-1 analogues, in addition to their hypoglycemic effect, also have a beneficial effect on cardiovascular risk factors. It is suspected that drugs from this group affect them indirectly, mainly by reducing body weight. Therapy with GLP-1 analogues often also leads to an improvement in the lipid profile – a reduction in the level of total cholesterol, low-density lipoprotein (LDL) fraction and triglycerides – as well as a decrease in systolic blood pressure. Additionally, preclinical and clinical studies suggest that stimulation of GLP-1 receptors affects the functioning of endothelial cells, the immune system and platelets, which are involved in the process of atherogenesis. It has also been shown that drugs such as exenatide, liraglutide or semaglutide reduce oxidative stress and the expression of adhesion molecules in blood vessels, which may slow down the development

of atherosclerosis. In recent years, many studies have been conducted to assess the effect of GLP-1 analogues on the risk of cardiovascular events. For example, the use of liraglutide at a dose of 1.8 mg for 3.5 to 5 years in patients with T2DM led to a significant reduction in the risk of cardiovascular death, heart attack and non-fatal stroke. The effect of individual GLP-1 analogues on the cardiovascular system varies. Studies have shown that lixisenatide and exenatide do not differ in terms of efficacy from placebo in the context of cardiovascular risk. On the other hand, liraglutide, albiglutide and dulaglutide showed a beneficial effect in reducing this risk. Although most studies concerned patients with T2DM, the effect of GLP-1 analogue therapy in people without diagnosed diabetes is increasingly being analyzed. Preliminary results suggest that these drugs may improve glucose metabolism, reduce the amount of perivascular and epicardial adipose tissue, which is important in preventing atherosclerosis. In addition, they are indicated to have a potential role in reducing chronic inflammation and oxidative stress – two important factors increasing cardiovascular risk – which may be indirectly related to weight loss. [69,70]

Studies show that GLP-1 analogues can reduce the risk of cardiovascular events in overweight or obese individuals, regardless of diabetes. They also have potential protective effects on the circulatory system, helping to reduce the risk of cardiovascular complications. Table 1 shows cardiovascular and renal effects in obese individuals caused by GLP-1 drug supplementation.

Table 1. Cardiovascular and renal effects in obese individuals caused by GLP-1 drug supplementation

With diabetes	No diabetes
weight loss	weight loss
lowering systolic blood pressure	lowering systolic blood pressure
lowering the level of total cholesterol, LDL fraction and triglycerides	improving kidney function, reducing the risk of developing or worsening kidney disease
reducing oxidative stress and the expression of adhesion molecules in blood vessels, which may slow down the development of atherosclerosis	reducing oxidative stress and the expression of adhesion molecules in blood vessels, which may slow down the development of atherosclerosis
reducing the incidence of cardiovascular death, heart attack and stroke	reducing the incidence of cardiovascular death, heart attack and stroke

Other positive effects of GLP-1 analogs

GLP-1 analogs may also benefit patients with conditions other than diabetes and obesity, as indicated by subsequent studies.[71] It has been demonstrated that GLP-1 analogs have a protective and therapeutic effects in the central nervous system through the reduction of neuroinflammation, enhancement of signal transduction in cells, and stimulation of neuronal growth and differentiation.[71,72] Particularly beneficial effects have been observed in case of Alzheimer's disease, Parkinson's disease, depression, and in post-stroke conditions. In Alzheimer's disease, GLP-1 analogs improve glucose metabolism in brain, improving glucose transport across the blood-brain barrier.[71,73] They also have a protective effect on dopaminergic neurons in the substantia nigra, which may positively influence motor activity in Parkinson's disease.[71,74] GLP-1RAs have also demonstrated a significant impact on reducing stroke incidence and enhancing neuroprotection in both preclinical and clinical studies.[71,75] Furthermore, GLP-1 analogs may exhibit antidepressant effects by improving cognitive function, promoting neuroprotection, and modulating neurotransmitter release. They may be effective as adjunctive medications not only in neurodegenerative diseases and substance abuse disorders, but also in mood disorders such as depression.[71,76] In addition, GLP-1 analogs appear to be a promising therapeutic option for the treatment of chronic pain, offering analgesic effects without severe adverse effects or addiction risks.[71,77]

These medications show cardioprotective properties by exerting anti-inflammatory effects and reducing myocardial damage induced by ischemia. They also modify the processes of lipid synthesis and secretion, while improving endothelial function. Studies suggest that GLP-1 analogs may be effective in the treatment of hypertension and atherosclerosis.[71,78]

In individuals reporting typical or atypical chest pain, increased circulating GLP-1 levels were correlated with reduced atherosclerotic plaque burden as assessed by coronary computed tomography. In animal models of atherosclerosis, treatment with GLP-1RAs, particularly semaglutide, has been shown to reduce fatty changes in the aortic root, ascending aorta, and iliac bifurcation. On the other hand, one study of type 2 diabetic patients receiving liraglutide reported increased plaque volume. Despite the growing body of evidence, it remains unclear whether GLP-1RAs therapy actually contributes to plaque regression or stabilization of those more likely to rupture.[79]

Atherosclerosis is a chronic inflammatory-degenerative process in which fibrolipid changes occur in the arterial wall. It is the leading cause of death worldwide. Particularly dangerous are cases in which thrombosis overlaps – this leads to the most serious complications, such as myocardial infarction or stroke.

One of the causes of atherosclerosis is elevated plasma cholesterol levels, especially in the form of low-density lipoproteins. These are responsible for transporting cholesterol through the bloodstream. Emerging atherosclerotic plaques undergo fibrosis over time, forming fibrous caps and calcium deposits. Numerous studies have shown that semaglutide and liraglutide can positively impact lipid profiles and lower blood pressure, reducing the risk of developing atherosclerosis and car-

diovascular disease. Furthermore, animal models have shown that drugs from this class inhibit the progression of atherosclerosis. The mechanisms of action of GLP-1RAs in atherosclerosis include: reduced inflammation, decreased intima-media thickness, improved lipid profile, and normalized endothelial function. These drugs have also been shown to lower systemic inflammatory markers, which is important because chronic inflammation plays a key role in the pathogenesis of cardiovascular disease. Furthermore, there is evidence that GLP-1RAs may prevent the formation of macrophage foam cells, thereby delaying the progression of atherosclerotic lesions. Although no GLP-1 agonists have been officially approved for the treatment of atherosclerosis, the results of previous studies suggest that these drugs may represent a promising therapeutic option for the prevention and treatment of this disease.

Research results indicate that GLP-1 analogs may also play a beneficial role in the treatment of polycystic ovary syndrome, contributing not only to weight reduction but also beneficially affecting androgen levels.[71,80]

Moreover, these medications seem to be effective in the treatment of non-alcoholic fatty liver disease. GLP-1RAs have been shown to directly influence adipogenesis, lipotoxicity, fatty acid oxidation, and the release of cytokines associated with liver inflammation and fibrosis. They also reduce visceral obesity and hepatic fat deposition.[71,81]

Adverse effects of GLP-1 analogs

When presenting the characteristics of GLP-1 analogues, it is also important to mention their potential side effects. The most common adverse effects include nausea and vomiting. Their incidence is lower with long-acting preparations. These symptoms may be due to delayed gastric emptying or the analogues' effects on the central nervous system. Nausea and vomiting are particularly common at the beginning of therapy, but their severity usually decreases within the first 4–8 weeks of treatment, as observed in studies with exenatide and liraglutide.[29,82] Other adverse effects of GLP-1RAs inhibitors include diarrhea and constipation. Diarrhea occurs in approximately 10–20% of patients, and constipation in approximately 4–10% of those treated with these drugs.[29] Injection-site reactions, such as itching or soft tissue nodules, may also occur in patients taking GLP-1 analogues. These reactions are usually mild and transient. Injection site nodules are observed primarily with exenatide and are the result of an inflammatory reaction to the polymer.[29] Additionally, GLP-1RAs use may slightly increase heart rate.[29] It is worth noting that when GLP-1 analogs are used in monotherapy, the risk of hypoglycemia is low because the analogs stimulate insulin secretion and inhibit glucagon release in a glucose-dependent manner. The risk of hypoglycemia may be a concern when analogs are combined with insulin therapy.[29]

GLP-1RAs for weight loss were associated with an increased risk of pancreatitis, gastroparesis, and intestinal obstruction compared with bupropion/naltrexone therapy, but no increased risk of biliary tract disease. Although these adverse events are relatively rare, given the increasing use of GLP-1 agonists, they should be taken into account by patients considering the use of these drugs for obesity treatment.[83]

Patients taking GLP-1 analogues often experience side effects from the digestive system, such as nausea, vomiting, diarrhea or constipation. These symptoms may result from the drug's effect on the motility of the digestive tract and the mechanisms regulating the feeling of satiety.

In gastroenterology practice, you should be prepared for the following phenomena:

- Nausea, vomiting, diarrhea - these are the most common side effects, which may affect even over 50% of patients.
- Constipation - slowed intestinal peristalsis caused by the drug may lead to constipation, which can be particularly troublesome in patients with existing pelvic floor dysfunction.
- Bloating - may be a consequence of both constipation and a general slowing of intestinal transit.
- Abdominal discomfort - manifested by abdominal pain, a feeling of fullness or tension, may be related to the effect of the drug on the digestive tract.
- Intestinal obstruction – a rare but potentially serious complication that can occur in those with anatomical or functional predispositions.
- Pancreatitis – although rare, some studies suggest a possible association between the use of GLP-1 analogues and an increased risk of pancreatitis.
- Gastroparesis – slowed stomach emptying, known as gastroparesis, can also occur as a side effect of therapy.

Tirzepatide (Mounjaro) a new agent in T2DM and obesity treatment

Currently, drugs are also available that can perform dual functions. Tirzepatide is the first dual agonist of the GIP and GLP-1 receptors. Currently, tirzepatide is approved only for the treatment of type 2 diabetes.[84,85] It is a synthetic peptide composed of 39 amino acids and structurally similar to incretins. Its structure contains a fatty acid residue linked to hydrophilic linkers. Additionally, the peptide contains two non-encoded amino acid residues, which are responsible for its extended half-life and strong affinity for albumin. Similar to other drugs, tirzepatide has a mean half-life of approximately five days.[85] Tirzepatide acts through the aforementioned incretin effect. Studies indicate that

it lowers HbA1c levels more effectively than semaglutide and induces weight loss, depending on the dose administered. This effect is primarily mediated by GIP, although the combination of GIP and GLP-1 receptor agonism demonstrates synergistic effects.[86-89] The phase III SURPASS trials demonstrated that tirzepatide, both as monotherapy and as adjunctive therapy, is more effective in lowering blood glucose and insulin levels compared with GLP-1RAs. Furthermore, the risk of hypoglycemia and other adverse cardiovascular events is low with tirzepatide.[84] Currently, tirzepatide is also being evaluated for its use in the treatment of obesity in patients with T2DM. It is also anticipated that this drug could be used in the future to treat nonalcoholic steatohepatitis or obstructive sleep apnea.[90,91,92] Adverse events are rare, usually during dose escalation, and are generally mild to moderate in severity. Five SURPASS clinical trials confirmed the potent glucose-lowering and weight-reducing properties of tirzepatide, with an overall safety profile comparable to that of GLP-1RAs.[84,88] Figure 1 shows the effect of GLP-1 on selected internal organs.

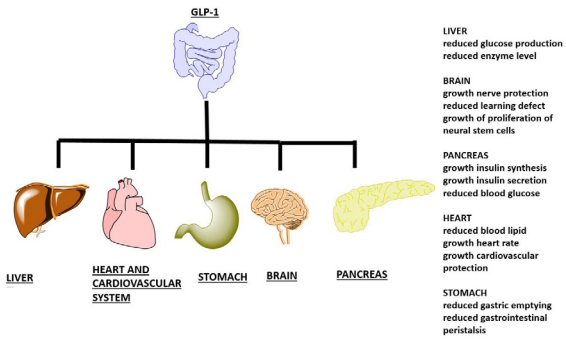

Fig. 1. Effects of GLP-1 on organs

Conclusion

Significant progress in the use of intestinal hormones in the treatment of T2DM and obesity has led to the development of effective therapies for both of these conditions, such as GLP-1 analogs. These drugs are now commonly used to treat T2DM, and recent studies have shown that they are also effective in weight loss. The growing understanding of biochemical processes, hormonal signaling, and the development of new technologies contribute to the continuation of research on new, more effective therapies that use mechanisms of action of the gut-brain axis. Despite these achievements, the need for new and more effective treatments is constantly growing, and requires innovative strategies and their potential combination with existing therapies.

Declarations

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Author contributions

Conceptualization, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Validation, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Formal Analysis, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Resources, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Writing – Original Draft Preparation, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Writing – Review & Editing, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Visualization, Ko.K.; Ka.K.; M.M.; J.F-R.; A.P.B.; K.D.; D.A.; R.W. and A.K-K.; Supervision, A.K.K.

Conflicts of interest

The author(s) declare no competing interests.

Data availability

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

Ethics approval

Not applicable.

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REVIEW PAPER

Doxorubicin-induced cell cycle arrest and apoptosis in HL-60 leukemia cells – a narrative review

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ABSTRACT

Introduction and aim. Doxorubicin is an anthracycline widely used in cancer therapy. Although the complete mechanism of action of doxorubicin is not fully understood, it is known to effectively destroy cancer cells by intercalating into DNA. This article aims to evaluate the anticancer effect of doxorubicin with particular emphasis on HL-60 leukemia cells and cell cycle/apoptosis.

Material and methods. This narrative review contains a collection and evaluation of peer-reviewed publications on the anticancer properties and therapeutic applications of doxorubicin. It focuses on doxorubicin-induced cell cycle arrest and apoptosis in HL-60 leukemia cells, with particular emphasis on toxicity and resistance. A literature review was conducted using the following databases: PubMed and Google Scholar.

Analysis of the literature. Doxorubicin exhibits potent anticancer activity against many cancer types. Unfortunately, due to serious side effects, primarily related to the cardiotoxicity of the drug, its potential cannot be fully exploited**.**

Conclusion. In recent years, many approaches have been developed to circumvent the limitations of doxorubicin. Identifying all the pathways of doxorubicin action is important for planning combination therapy, mitigating side effects, and developing new drugs. However, further clinical studies are needed to determine their long-term safety and efficacy.

Keywords. cancer, cell line, doxorubicin, HL-60 cells, leukemia

Introduction

HL-60 cells are a well-established model of human promyelocytic leukemia, widely used to study mechanisms of cell cycle control, differentiation, and apoptosis. Doxorubicin is one of the key chemotherapeutic agents used in the treatment of hematologic cancers. Together, this model allows researchers to investigate how leukemia cells respond to cytotoxic treatment, explore mechanisms of sensitivity and resistance, and identify pathways that may be targeted to improve therapeutic outcomes.

Characteristics of acute promyelocytic leukemia

The term leukemia refers to neoplastic diseases of the hematopoietic system characterized by abnormal proliferation, impaired maturation, and the release of white blood cells from the bone marrow and other tissues. These diseases are accompanied by organ infiltration and the presence of immature cell forms in the peripheral blood. Leukemia is divided into acute and chronic forms. In acute leukemia, it is mainly immature blast cells that have lost their ability to differentiate that proliferate.[1]

Acute promyelocytic leukemia is an aggressive subtype of acute myeloid leukemia. With a characteristic clinical course. It was first identified and described in 1957. Its characteristic feature is a translocation between chromosomes 15 and 17, resulting in the formation of the PML-RARA fusion protein (a fusion of the promyelocyt-

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ic leukemia (PML) gene and the retinoic acid receptor α (RARα)), which disrupts normal cell differentiation.[2]

Apart from this form of translocation (t(15;17)(q22;q21)-PML-RARA), there are other less common variants of translocation, but always involving the RARA gene. In addition to chromosomal translocation, there is an accumulation of abnormal promyelocytes in the blood and bone marrow, as well as coagulation disorders with features of disseminated intravascular coagulation and a tendency to bleed due to hypofibrinogenemia.[2–4]

Of all types of leukemia in adults, acute leukemia accounts for 40% of cases, the vast majority of which are acute myeloid leukemia. According to data from the Adult Acute Leukemia Registry, between 2004 and 2010, an average of over 600 cases per year were reported, mainly in people over 50 years of age.[5]

Treatment of leukemia

The treatment of acute promyelocytic leukemia is a complex process based on targeted therapy. It most often involves administering all-trans retinoic acid (ATRA) or arsenic trioxide (ATO) to patients, often in combination with chemotherapy.[6] High concentrations of ATRA in the cell increase the expression of typical retinoic acid receptors, which displace the PML-RARα protein. ATRA induces the differentiation of cancer cells and also limits their proliferation, as has been demonstrated, among others, in acute promyelocytic leukemia.[7,8] Arsenic trioxide, on the other hand, binds to the PML subunit, leading to degradation of the PML-RARα fusion protein and disrupting cancer cell self-renewal.[8] Therapy based on combining all-trans retinoic acid with anthracycline chemotherapy is a highly effective method of induction treatment for patients with acute promyelocytic leukemia. It allows for complete remission in up to 95% of patients and is associated with a lower recurrence rate than monotherapy.[8]

Doxorubicin as an anticancer drug

Doxorubicin is an anthracycline antibiotic. It was isolated from the bacterium *Streptomyces peucetius*, which was modified to find a substitute for another anthracycline discovered earlier – daunorubicin (also anticancer, but more cardiotoxic). It is a widely used substance in anticancer therapies, primarily for the treatment of leukemia, breast cancer, bladder cancer, lung cancer, and Hodgkin's lymphoma.[9,10] Although the complete mechanism of action of doxorubicin is not fully understood, it is known to intercalate into DNA, causing double-strand breaks and inhibiting nucleic acid biosynthesis (both DNA and RNA). In addition, it inhibits topoisomerase II and (in combination with iron) generates free radicals, which also initiate DNA damage.[10] According to Ye et al., doxorubicin may affect proliferation by proteolytically activating the transcription factor CREB3L1 at the endoplasmic reticulum membrane.[11] CREB3L1 is a protein with one end located in the lumen of the endoplasmic reticulum and the other (terminated with an NH2 group) in the cytoplasm of the cell. Through a ceramide-mediated mechanism, the drug triggers regulated intramembrane proteolysis (RIP). This results in a two-step cleavage of the molecule, allowing the N-terminal domain of the protein to enter the cell nucleus, where it drives the transcription of genes that inhibit cell proliferation.[11,12] Fig. 1. illustrates the molecular mechanisms of action of doxorubicin at the cellular level.

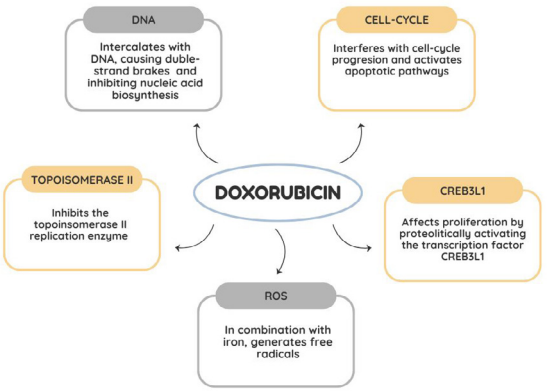

Fig. 1. Mechanisms of doxorubicin action

Aim

The aim of this review is to summarize current knowledge on the effects of doxorubicin on HL-60 leukemia cells, with a focus on its influence on cell cycle regulation, induction of apoptosis, and the key mechanisms that shape therapeutic response and limitations. By integrating findings from recent in vitro studies, the review seeks to highlight the molecular pathways involved and identify areas that may guide future research on improving the efficacy and safety of doxorubicin-based treatments.

Material and methods

This article is a narrative literature review focusing on acute promyelocytic leukemia, the HL-60 cell line, and the biological effects of doxorubicin. A literature search was conducted using the PubMed and Google Scholar databases to identify relevant publications published between 2000 and 2024. The selected time frame was chosen to include both classical studies describing leukemia biology, cell cycle regulation, and apoptosis, as well as more recent research addressing molecular mechanisms of doxorubicin action, multidrug resistance, and treatment-related toxicity. The search strategy involved the use of predefined keywords and their combinations, including "acute promyelocytic leukemia," "HL-60," "cell line," "doxorubicin," "anthracyclines," "apoptosis," "cell cycle," "DNA damage," and "multidrug resistance." Boolean operators (AND/OR) were used to broaden or narrow the search scope. The reference lists of selected articles were manually reviewed to identify additional

relevant publications. Inclusion criteria included original research articles, review articles, reports, and textbook chapters that addressed: (i) the epidemiology, pathogenesis, and molecular characteristics of acute leukemias; (ii) the mechanisms of action of doxorubicin in cancer cells; (iii) in vitro studies using the human promyelocytic leukemia cell line HL-60; and (iv) cellular processes relevant to this review, including apoptosis, cell cycle regulation, responses to DNA damage, and mechanisms of drug resistance. Exclusion criteria included studies unrelated to leukemia or anthracycline-based therapy, publications of no biological relevance to the topic, and conference abstracts. After selection, 37 publications were included in the final narrative review.

Analysis of the literature

Characteristics of the HL-60 cell line

HL-60 is a human leukemia cell line established in 1977 from lymphocytes from the blood of a patient with acute promyelocytic leukemia. It is a suspension cell line with lymphoblastic morphology and a doubling time of up to 48 hours. The cells proliferate continuously and are capable of spontaneous and induced differentiation. HL-60 cells are cultured in RPMI 1640 medium containing 2 mM glutamine and 10-20% FBS. However, the cells can also proliferate in the absence of serum, provided that insulin and transferrin are present.[13,14]

Most HL-60 cells have a variety of surface antigens characteristic of immature myeloid cells. Many karyotypic changes, such as chromosomal translocations and alterations in chromosome number, are observed in HL-60 populations. One of the distinct genetic changes in HL-60 is increased amplification of DNA sequences encoding the *c-myc* gene, which encodes a transcription factor that plays an essential role in regulating the cell cycle, proliferation, apoptosis, and metabolism. Although the amplification magnitude varies between sublines, the relative abundance of c-myc RNA in cells of each subline is proportional to the number of gene copies.[14,15]

HL-60 cells constitute a population arrested at the stage of immature myeloid precursors. However, in each culture, approximately 10% of cells exhibit the characteristics of mature myeloid cells and are therefore capable of spontaneous differentiation. However, some factors can increase the proportion of differentiating cells in the population to as much as 90%. Among the known factors inducing HL-60 differentiation are phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), vitamin D3, and retinoic acid.[13,14]

Characteristics of the processes most frequently analyzed in response to anticancer treatment

Cell cycle control systems

The cell cycle is a strictly ordered set of events throughout the life of a cell. Cell division is an integral and crucial part of this cycle. This process maintains the continuity of life in both prokaryotes and eukaryotes. The division of a single cell, during which it passes on its genetic material, allows the formation of new daughter cells that are separate organisms and the development and repair of entire tissue structures in multicellular organisms.[16] For the entire cell cycle to proceed correctly, the events of each phase must be completed before the cell enters the next phase; therefore, mechanisms that regulate the cycle are necessary. This role is performed by cycle checkpoints, the three main ones being located in the G1 phase and at the transition between the G2 and M phases.[16,17] They prevent the progression of mutated or damaged G0 cells (which are in a dormant state). When cell abnormalities are recognized by checkpoints, either the error is repaired or the cell cycle is arrested. The integrity of the cell genome is monitored by the p53 transcription factor, which halts the cell cycle in response to DNA damage. The p53 factor regulates the transcription of the cyclin-dependent kinase inhibitor (CDKI) p21, an active inhibitor of CDK4, 6, and 2, thereby preventing retinoblastoma protein phosphorylation and keeping the cell in G1.[18,19] Another highly sensitive marker of DNA damage in cells caused by ionizing radiation, oxidative stress, or genotoxic stress is histone H2A.X, whose phosphorylated form accumulates at sites of damage.[20]

Cancer proliferation

During the cell cycle, the number of cells increases over time through division, i.e., proliferation. Mammalian cells divide approximately every 24 hours, with the S phase being the longest stage. As mentioned earlier, this process is strictly regulated in healthy cells, but in cancer, it becomes uncontrolled. Although proliferation is not accelerated in every case of cancer, it is always chronic.[21]

In clinical practice, several techniques are used to assess cancer cell proliferation, including methods based on the percentage of cells in the S phase, the mitotic index, and proliferation markers (cyclins, proliferating cell nuclear antigen (PCNA), nuclear protein Ki67). Based on cyclin A expression, it is possible to distinguish DNA-synthesizing cells. Cyclin B is a regulator of the mitotic phase. An increase in the percentage of cells expressing cyclins A, B, and E indicates rapid tumor proliferation. Similarly, the expression of Ki67 and PCNA proteins provides information about the intensity of proliferation in the population.[22]

Apoptotic death

Apoptosis is a strictly controlled, orderly mechanism of programmed cell death. This process is controlled by a series of signaling pathways in the cell, which can be initiated by signals from both inside and outside the cell.

Table 1. Table summarizing key studies on HL-60 cells treated with doxorubicin

Author, year	Doxorubicin concentration	Analyzed markers	Main effect	Detailed effects
Żuryń et al., 2007	0.5–10 µmol/L	cyclin A	dose-dependent increase in apoptosis; altered cyclin A expression and subcellular localization; G2/M cell cycle arrest	1) the expression of cyclin A in HL-60 cells increases in a dose-dependent manner following exposure to doxorubicin 2) higher concentrations of doxorubicin increase the proportion of apoptotic cells and induce the cytoplasmic translocation of cyclin A 3) doxorubicin modulates cyclin A expression, inducing apoptosis and morphological changes
Suzuki et al., 2005	0.5–16 µmol/L	caspase-3, caspase-8, caspase-9	caspase activation and DNA damage	1) doxorubicin exhibits higher cytotoxicity in cancer cell lines than in healthy cells 2) activation of caspases is observed, predominantly caspase-3 3) doxorubicin induces DNA fragmentation in HL-60 cells
Al-Abbas and Shaer, 2021	100 ng/mL (with 100–1000 µg/mL coumarin)	annexin V	induction of apoptosis	1) combined treatment with coumarin and doxorubicin increases apoptotic cell death in HL-60 and HL-60/ADR cells in a coumarin dose-dependent manner

During apoptosis, DNA and organelles are destroyed, and the cell's contents are packaged into vesicles (apoptotic bodies), which are then digested. Apoptosis, as a controlled process of cell destruction, protects normal cells by preventing inflammation in their vicinity.[16] It is also involved in cell replacement in healthy, mature tissues and in the focal elimination of cells during normal embryo development. Its mechanism has been relatively well understood thanks to research on *Caenorhabditis elegans,* where three proteins key to apoptosis have been identified: Ced-3, Ced-4, and Ced-9. Ced-3 and Ced-4 are present in the cell in an inactive form, but when the cell receives a death signal, they activate, triggering a cascade that activates nucleases and other proteases. Ced-9 is a protein in the outer mitochondrial membrane and, in a state where the cell does not receive a programmed death signal, it inhibits apoptosis, while Ced-3 and Ced-4 remain inactive. In mammalian cells, this process is more complex, but proteins very similar to those mentioned above are involved.[16,23]

Apoptosis pathways and signals

There are two interconnected pathways in programmed cell death: one that uses cellular death receptors (the exogenous pathway) and the other that uses mitochondria (the endogenous pathway). The exogenous pathway begins with the binding of a ligand (most often from the tumor necrosis factor (TNF) family) to the appropriate receptor on the cell surface. This is followed by the activation of adaptor proteins and the caspase-8 mechanism, which, when activated, can directly initiate the apoptotic program.[24] The intrinsic pathway is triggered by cell-damaging factors such as oxidative stress, DNA damage, or growth factor deficiency. During this pathway, the mitochondria undergo disintegration, leading to the release of proapoptotic factors such as cytochrome C into the cytoplasm. The described pathways converge in the execution phase. Effector caspases degrade some nuclear and cytoskeletal proteins.[24] Caspase-3 plays a significant role here, inducing the disintegration of the cell into apoptotic bodies. As the cell dies, phosphatidylserine is exposed on its cell membrane, which is later recognized by phagocytes. Annexin V, a protein commonly used as a marker of apoptosis, binds strongly to phosphatidylserine.[24]

Fundamental processes such as cell cycle progression and DNA integrity are crucial for proper cell function; therefore, disturbances in these processes directly affect cell survival and proliferative potential. As summarized in Table 1, studies investigating the effects of doxorubicin on HL-60 cells demonstrate concentration-dependent alterations in apoptosis and cell cycle regulation.

In a study examining cyclin A expression in HL-60 cells under the influence of doxorubicin, Żuryń et al. showed that, with increasing doxorubicin dose (0–5 µmol/L) and the proportion of apoptotic cells in the population, the number of cells expressing cyclin A decreased.[25] The cyclin A2/Cdk1 complex initiates the transition from the G2 phase to the M phase. In contrast, the cyclin A2/Cdk2 complex initiates the transition from the G1 phase to the S phase, whereby the cyclin A level reflects proliferative activity.[22,26] As reported by Denard et al., doxorubicin inhibits cancer cell proliferation by activating proteolytic cleavage of CREB3L1.[11] It stimulates the proteolytic cleavage of CREB3L1, a membrane-associated transcription factor, allowing the NH2-terminal domain of CREB3L1 to enter the cell nucleus, where it activates transcription of genes encoding cell cycle inhibitors, thereby limiting cell proliferation.[11]

Doxorubicin has a toxic effect not only on target cancer cells but also on healthy human cells with normal physiology and morphology, albeit to a noticeably lesser extent. In a 2005 study, Suzuki et al. compared the cytotoxic activity of doxorubicin against cancer cells using the HL-60, HSC-2, HSC-3, and HSG cell lines and regular cell lines from oral tissues – HGF, HPLF, and HPC.[27] The cytotoxicity of HL-60 cells was assessed using the trypan blue exclusion method, while the MTT assay was used for the other lines. Doxorubicin, tested in a range of concentrations, showed higher cytotoxicity

in cancer cell lines than in healthy cell lines, and the tumor specificity (TS) index, determined based on the ratio of 50% cytotoxic concentration of cancer and healthy cell lines, was determined to be in the range of 54-255. In the apoptosis induction analysis, they compared the effect of the drug on HL-60 and HSC-2 cells at seven concentrations (0; 0.5; 1; 2; 4; 6; 8 and 16 μmol/L for HL-60 and 0; 0.1, 0.3, 10, and 30 μmol/L for HSC-2), showing activation by doxorubicin of caspases-3, -8, and -9 in both cell groups, with a clear predominance of caspase-3. In addition, gel electrophoresis performed to assess the level of genetic material damage showed DNA fragmentation in HL-60 cells and none in HSC-2 cells, despite confirmed caspase activation.[27]

Despite its widespread and well-established efficacy in cancer therapy, doxorubicin is associated with a range of adverse effects, including bone marrow suppression, nausea and vomiting, and alopecia. The cytotoxic impact of doxorubicin on healthy tissues represents a major dose-limiting factor in clinical practice. Among the most severe complications of doxorubicin therapy is cardiomyopathy, which may ultimately lead to congestive heart failure. Cardiac toxicity can be acute, with an incidence of approximately 11% in patients taking the drug, or chronic, with an incidence of approximately 1.7%.[10,28,29] Factors that increase the risk of doxorubicin-induced cardiotoxicity include dose (including single and cumulative lifetime anthracycline exposure), concomitant cardiovascular disease, previous chemotherapy, method and rate of drug administration, and patient age.[30] Although the details of the mechanisms responsible for the antibiotic's harmful effects on myocardial cells are not fully understood, the main factors mentioned include increased oxidative stress through excessive production of reactive oxygen species, mitochondrial damage, apoptosis induction, and inhibition of cardiomyocyte-specific gene expression.[10,31] The cardiotoxicity of doxorubicin has prompted the search for analogues and alternative methods of drug administration that would be less toxic.[32]

As studies show, cytostatics may also be associated with premature ovarian failure. Although alkylating agents are mainly responsible for reducing the reserve of primordial follicles in the ovaries, other chemotherapeutic agents also have a detrimental effect on organ function.[33] In their study, Ben-Aharon et al. confirmed that doxorubicin is toxic to the ovaries in female mice.[34] The antibiotic was administered intraperitoneally at a dose of 7.5 or 10 mg/kg of doxorubicin, depending on the test. They observed a significant decrease in ovarian weight in mice treated with doxorubicin, both 1 week after injection (85% of control weight) and 1 month after injection (only 52% of control weight). When examining ovulation rates, they found a drastic decrease in ovulation frequency after one week, followed by a partial increase after one month. In addition, they observed vascular changes in ovarian sections and a reduction in the number of both primary and secondary follicles.[34]

Over the years, many approaches have been developed to overcome the limitations of doxorubicin, particularly its cardiotoxicity. These include low-dose continuous infusion regimens, antibiotic administration with cardioprotective drugs, and liposomal encapsulation technology. The latter involves lipid-based drug carriers that alter drug distribution, thereby reducing its concentration in heart cells. This method was based on the assumption that liposomes cannot escape from vessels in areas with tight capillary connections, but can leave the circulation in tissues and organs lined with cells that are not tightly connected, such as cancerous tissues. Thus, encapsulating anthracyclines in liposomes results in higher concentrations in tumor tissue while limiting their effects on healthy cells, making liposomal encapsulation one of the effective methods for increasing the therapeutic index of doxorubicin and other anthracyclines.[9] Another factor reducing the therapeutic potential of doxorubicin in cancer treatment is the occurrence of multidrug resistance. The mechanism underlying resistance development is reduced intracellular drug concentration after long-term dosing, leading to upregulation of efflux systems in cancer cells.[35,36] Chen et al. confirmed the presence of multidrug resistance in the sensitive HL-60 line at progressively higher doxorubicin doses.[37] In a 2021 experiment, Al-Abbas and Shaer used the HL-60 and HL-60/ADR (chemotherapy-resistant) cell lines. They investigated the effect of doxorubicin therapy combined with coumarin, a naturally occurring compound with broad bioactivity, including antioxidant, anti-inflammatory, and anticancer properties.[36] In their study, they showed that the simultaneous use of coumarin (0, 100, 250, 500, and 1000 μg/mL) and doxorubicin (100 ng/mL) after 24 hours significantly induced apoptosis in drug-resistant HL-60/ADR cells, an effect not observed with monotherapy. The rate of cell death clearly increased with increasing coumarin concentration. Their results suggest that coumarin inhibits extracellular doxorubicin efflux, thereby increasing antibiotic efficacy against resistant human myeloid leukemia cells.[36] These and many other data in the literature suggest the existence of multiple pathways to enhance the therapeutic potential of doxorubicin in cancer treatment by circumventing its toxicity to healthy cells and cancer cells' resistance mechanisms.

Conclusion

To summarize the gathered information, doxorubicin clearly affects HL-60 cells by disrupting cell cycle progression and activating apoptotic pathways, leading to a marked reduction in their proliferative capacity. Although its anticancer activity is well established, the

therapeutic impact of the drug continues to be limited by toxicity toward healthy tissues and the emergence of resistance. Ongoing research exploring molecular mechanisms, protective strategies, and combination approaches offers promising directions for improving the safety and effectiveness of doxorubicin-based therapies in hematologic malignancies.

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Conceptualization, D.R. and E.K.; Methodology, D.R.; Writing – Original Draft Preparation, D.R. and E.K; Writing – Review & Editing, E.K.; Supervision, E.K.

Conflicts of interest
The authors declare no conflict of interest.

Data availability
No datasets were generated or analyzed during the current study.

Ethics approval
Not applicable

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CASE REPORT

Postoperative diagnosis of pulmonary actinomycosis in a patient with bronchiectasis – a rare case

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ABSTRACT

Introduction and aim. Pulmonary actinomycosis is a rare chronic infection caused by *Actinomyces* species, often mimicking tuberculosis, malignancy, or other chronic lung diseases both clinically and radiologically. These similarities frequently lead to diagnostic delays. We present a rare case of pulmonary actinomycosis in a patient with long-standing bronchiectasis who developed acute hemoptysis during anticoagulant therapy.

Description of the case. A 59-year-old woman with a ten-year history of bronchiectasis and recent mechanical aortic valve replacement presented with persistent hemoptysis while on warfarin. Thoracic computed tomography revealed bronchiectatic changes and opacities in the right middle lobe. Bronchoscopy showed bloody, encrusted material, but no definitive diagnosis was made. Due to ongoing symptoms and radiological suspicion of malignancy, an urgent right middle lobectomy was performed. Histopathological analysis confirmed pulmonary actinomycosis, revealing filamentous organisms via PAS, Gram, and Grocott's staining.

Conclusion. This case highlights the importance of considering pulmonary actinomycosis in patients with bronchiectasis and unexplained hemoptysis, particularly when standard treatment fails. Surgical resection may be essential for both diagnosis and treatment. The case underscores how structural lung disease and anticoagulant use can reveal rare infections otherwise masked by chronic symptoms.

Keywords. actinomycosis, bronchiectasis, hemoptysis, lobectomy, pulmonary infection

Introduction

Thoracic actinomycosis is a rare chronic pulmonary infection caused by filamentous, Gram-positive, facultatively anaerobic bacteria of the genus Actinomyces. Although the disease more commonly affects anatomic regions such as the oral cavity, pharynx, face, and abdomen, thorax infection occurs in approximately 15% of cases and can extend to the lung parenchyma, central airways, pleura, mediastinum, and chest wall.[1] The clinical presentation is usually nonspecific and may include symptoms such as chronic cough, productive sputum, hemoptysis, chest discomfort, fever, and weight loss. These symptoms in conjunction with overlapping radiographic features often resemble more common pulmonary diseases such as tuberculosis, lung cancer, or chronic bronchiectasis. As a result, timely and accurate diagnosis can be difficult. Radiographic findings often show mass-like consolidations, cavitary lesions, infiltrative shadows, morning opacities or pre-existing bronchiectatic changes.[2]

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The diagnosis of pulmonary actinomycosis is particularly difficult in individuals with preexisting structural lung abnormalities such as bronchiectasis. In such cases, clinical symptoms are often mistakenly attributed to the underlying chronic disease, leading to a delayed diagnosis. Furthermore, radiological findings are often similar to those seen in malignant neoplasms or other chronic pulmonary infections, further complicating the clinical picture. When hemoptysis occurs in patients receiving anticoagulant therapy, the differential diagnosis usually focuses on neoplastic processes or exacerbations of bronchiectasis, while rare infectious causes such as actinomycosis may be overlooked inadvertently.[3]

Histopathological examination remains the cornerstone in the diagnosis of pulmonary actinomycosis, as it allows the identification of filamentous organisms that stain positive using Schiff-, Gram-, and Grocott periodic acid techniques. Microbiological detection, on the other hand, is often limited due to the slow growth of the organism and its need for strict anaerobic conditions. Recognized risk factors include underlying structural lung abnormalities such as bronchiectasis or emphysema, poor oral hygiene, dental infections, chronic systemic disease, and immunosuppression. Of the various species, Actinomyces israelii is the most frequently isolated. Clinical symptoms often remain latent until complications such as hemoptysis, abscess formation, pleural effusion, or fistula formation occur.[4]

Due to its clinical and radiographic similarity to pulmonary malignancies, actinomycosis is often misinterpreted as cancer, leading to delays in diagnosis and potentially unnecessary invasive procedures. Surgical resection is usually reserved for cases with persistent hemoptysis, failure of drug therapy, or when malignancy cannot be definitively ruled out. Histopathological analysis of surgically removed tissue remains the most reliable diagnostic tool in these cases.[3]

The present case is particularly noteworthy because long-standing bronchiectasis and hemoptysis exacerbated by anticoagulant therapy occurred simultaneously and ultimately required emergency surgical resection. It emphasizes the importance of considering thoracic actinomycosis in the differential diagnosis of hemoptysis in patients with structural lung disease, especially when conventional diagnostic methods are inadequate to clarify the underlying cause.

This case illustrates a particular clinical context characterized by the coexistence of two unusual but clinically relevant conditions: chronic bronchiectasis and hemoptysis triggered by anticoagulant therapy, which eventually led to the diagnosis of pulmonary actinomycosis. There are few reports in the literature of thoracic actinomycosis that occurs under such circumstances. By highlighting this atypical diagnostic convergence, this case emphasizes the need to maintain a heightened clinical suspicion for rare infectious etiologies, especially in patients with pre-existing structural lung disease and unexpected clinical deterioration. Furthermore, it emphasizes the crucial role of a multidisciplinary approach in cases where standard diagnostic pathways do not provide a definitive diagnosis.

Aim

This report describes a rare case of pulmonary actinomycosis in a patient with long-standing bronchiectasis who developed clinical symptoms after starting anticoagulant therapy. The diagnosis was finally confirmed by surgical resection. This case illustrates the importance of considering infectious causes in the differential diagnosis of patients with unexplained hemoptysis, especially when structural lung abnormalities are present.

Description of the case

A 59-year-old woman was admitted with complaints of hemoptysis and cough that had persisted for about a week and had worsened significantly in the last few days. The medical history revealed a productive chronic cough that had persisted for 10 years and had been treated intermittently with antibiotics. Previous imaging studies had revealed bronchiectasis in the right middle lobe, which had remained stable but symptomatic over the years.

Her comorbidities included type 2 diabetes mellitus, hypertension, hyperlipidemia and a history of surgical treatment for aortic stenosis. She had undergone mechanical aortic valve replacement a year earlier and continued to receive long-term oral warfarin therapy. She was a lifelong non-smoker but had been exposed to textile dust due to her many years of working in the textile industry.

On physical examination, the patient was alert and in good general health with stable vital signs. Auscultation revealed diminished breath sounds in the right middle lung zone and a mechanical heart valve murmur was heard on cardiologic examination. Peripheral oxygen saturation was 96% in room air. Laboratory results showed a hemoglobin level of 8.8 g/dL, a leukocyte count of 6,610/mm^3, a platelet count of 283,000/mm3, a CRP of 16.2 mg/L, and an INR of 1.1. Liver and kidney function tests were within normal limits.

Thoracic computed tomography (CT) showed traction bronchiectasis in the right middle lobe surrounded by morning opacities, parenchymal fibrosis, and volume loss (Fig. 1). No mass lesions, cavitary changes, or lymphadenopathy were observed. Flexible bronchoscopy revealed bloody, encrusted, irregular, nonspecific material in the right middle lobe bronchi, which was aspirated and removed. No signs of massive active bleeding were noted during the procedure.

As hemoptysis persisted during anticoagulant therapy and conservative treatment did not respond, a

multidisciplinary team and emergency surgery was performed to remove the right middle lobectomy. Postoperatively, she was monitored in the intensive care unit for one day and then transferred to the general ward in stable condition. On the second postoperative day, 250 ml of chest drainage and an air leak were detected. On day 3, the drainage volume had decreased and the air leak had disappeared, so that the chest tube could be removed. The patient was mobilized, tolerated oral nutrition, and had adequate oxygen supply. She was discharged on postoperative day 4 without complications.

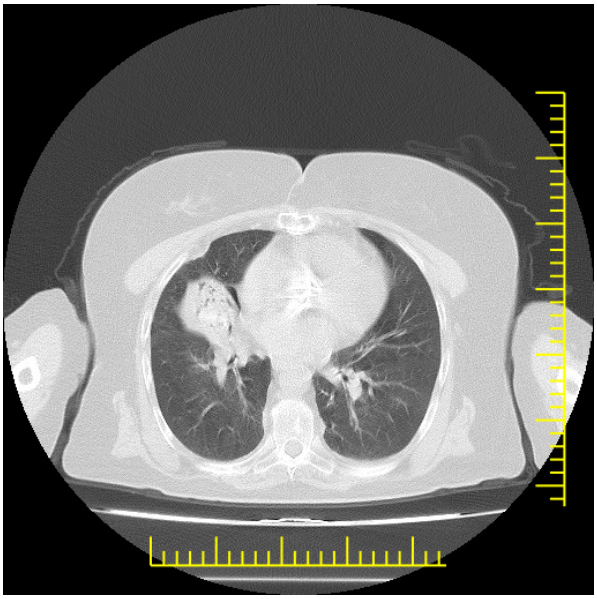

Fig. 1. Thoracic CT showing traction bronchiectasis in the right middle lobe

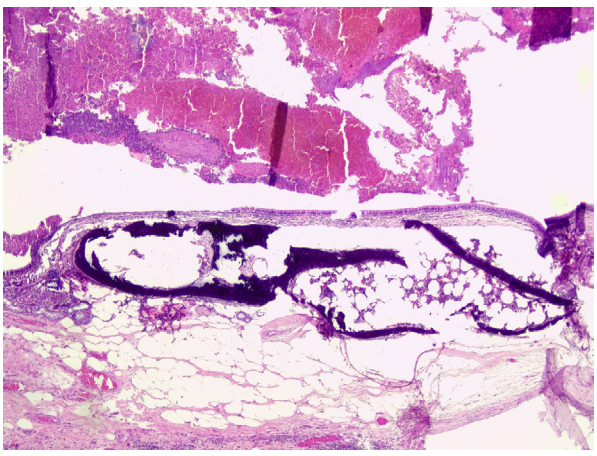

Fig. 2. Ossification and bone marrow formation in bronchial cartilage (H&E stain, x40)

Histopathologic analysis of the resected sample revealed advanced destructive bronchiectasis, parenchymal fibrosis, ossification in cartilage, and necrotic areas in the bronchial wall (Fig. 2). These necrotic areas contained filamentous organisms that stained positive with PAS, Gram, and Grocott's techniques (Figs. 3 and 4), suggesting pulmonary actinomycosis. No bacterial growth was observed in culture. Examination of the mediastinal lymph nodes showed reactive changes and anthracosis without evidence of malignant or specific pathology.

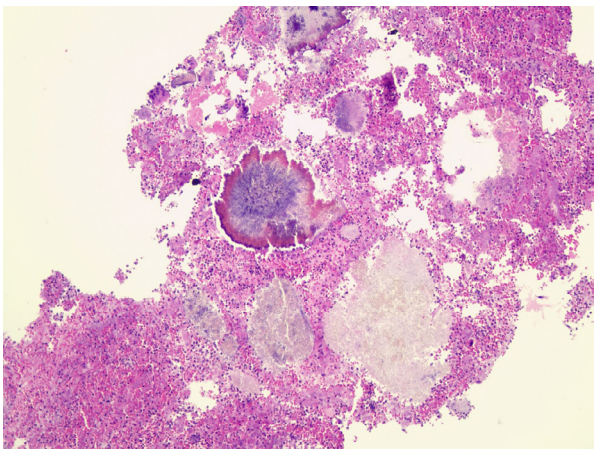

Fig. 3. Basophilic filamentous microorganism – *Actinomyces* (H&E stain, x100)

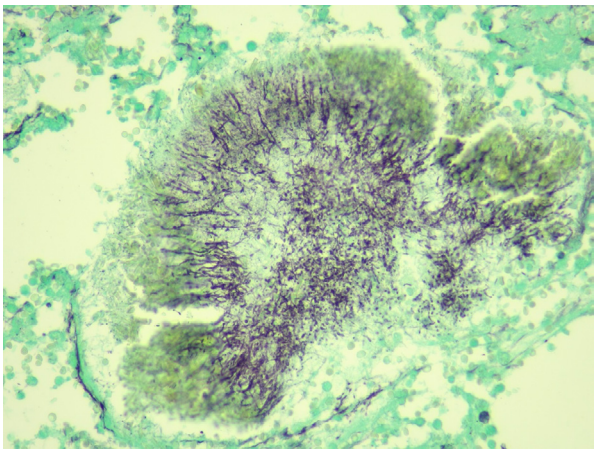

Fig. 4. Filamentous *Actinomyces* organisms highlighted by Grocott's methenamine silver stain (×400)

The infectious disease team recommended 6 months of oral amoxicillin (1 g, three times a day). Due to possible interactions between warfarin and amoxicillin, close monitoring of INR was recommended. The patient was referred to the outpatient departments of thoracic surgery and cardiology for follow-up care.

Discussion

Pulmonary actinomycosis is a rare but clinically significant infection that poses significant diagnostic challenges due to its similarity to more common pulmonary diseases such as tuberculosis or malignancies. The nonspecific clinical presentation and radiologic characteristics often contribute to delayed diagnosis.

In the present case, radiological imaging initially suggested a malignancy and the final diagnosis was made only after surgical resection and histopathologic analysis, confirming the reputation in the literature as the 'great masquerader'.[5]

A critical aspect in this case was the presence of long-standing bronchiectasis, which likely created a favorable environment for colonization and the development of low-grade, chronic infection. Cardoso et al.[6] reported that actinomycosis is often inconspicuous in patients with bronchiectasis, which can delay clinical recognition. In our case, the patient's chronic respiratory symptoms were initially attributed to her known bronchiectasis. However, the onset of hemoptysis, likely exacerbated by anticoagulant therapy, led to the clinical recognition of a previously hidden underlying infection. This demonstrates how structural lung abnormalities and anticoagulation can work together to reveal severe, previously unrecognized infections.

Bloody, crusted material was observed on bronchoscopic examination, but this finding was nonspecific and non-diagnostic according to the literature, which states that such features are not pathognomonic. Although modern diagnostic methods such as endobronchial ultrasound (EBUS) or bronchoscopic biopsy can be effective in selected cases, their utility is often limited by the accessibility of the lesion and the patient's condition. In our patient, progressive hemoptysis required urgent surgical intervention, which not only relieved symptoms, but also allowed histopathological confirmation.[7]

Microbiologic confirmation of actinomycosis is inherently difficult. In this case, culture results were negative, reflecting the well-documented difficulties in isolating Actinomyces species due to their slow growth and strict anaerobic requirements. Yuan et al.[8] successfully identified Actinomyces graevenitzii using advanced bronchoalveolar lavage and techniques, but such methods are not commonly used in routine clinical practice. Therefore, histopathology remains the most reliable diagnostic method, especially in diagnostically complex cases.

In the differential diagnosis, the possibility of coexisting malignancies should also be considered. Sugihara et al.[9] described a case in which pulmonary adenocarcinoma and actinomycosis were present simultaneously, which further complicated the clinical evaluation. Although no malignancy was detected in our patient, this possibility was carefully considered because the radiographic features suggested a neoplastic process.

Immunosuppression is another recognized risk factor for actinomycosis. Facchin et al.[10] reported a more aggressive disease course in patients with hematologic disease. Although our patient did not have immunosuppressive disease, chronic bronchiectasis may compromise local immune defenses and lead to a similar outcome.

Another consideration is the effect of long-term antibiotic use on the airway microbiota. Choo et al.[11] found that chronic macrolide therapy can alter microbial balance and possibly increase susceptibility to anaerobic infections. Although our patient did not receive such treatment, this factor remains relevant in the broader context of the treatment of patients with bronchiectasis.

Comparative case analyses illustrate the diagnostic challenges associated with pulmonary actinomycosis. Cardoso et al. pointed out that in patients with bronchiectasis, clinical overlap with other chronic infections often led to delays in diagnosis. Similarly, Sugihara et al.[9] reported a case in which actinomycosis coexisted with adenocarcinoma, increasing the suspicion of malignancy and further complicating the diagnostic process. Our case mirrors these findings, as the initial symptoms were attributed to pre-existing bronchiectasis, which delayed further investigation. These examples confirm the label of the 'great masquerader' and emphasize the need for a comprehensive and critical differential diagnosis in such cases.

Finally, several diagnostic limitations contributed to the late recognition of thoracic actinomycosis in this case. Although positron emission tomography (PET/CT) can be helpful in differentiating between infectious and malignant lesions, it was not performed due to the urgency of the clinical situation. A preoperative biopsy was also not possible due to the peripheral location of the lesion and the unspecific bronchoscopic findings. These challenges highlight the difficulty of making a diagnosis in the absence of definitive imaging or biopsy data, a problem that has also been encountered in previous case series of thoracic actinomycosis.

Study limitations

This case highlights several diagnostic challenges associated with rare pulmonary infections. First, although the histopathological findings were suggestive of actinomycosis, microbiological confirmation was lacking due to the difficulty in isolating Actinomyces species, which require strict anaerobic conditions and grow slowly. Second, no PET/CT scan was performed, which, although not standard for infections, could have provided metabolic information and helped differentiate between malignancy and infection, potentially speeding up the diagnosis. Thirdly, bronchoscopy did not provide diagnostic material, probably due to the peripheral location of the lesion or the patchy nature of the infection, emphasizing the limited sensitivity of minimally invasive methods. Therefore, a definitive diagnosis could only be made by surgical resection. As this is an isolated case, generalizability is limited. However, it emphasizes the diagnostic complexity of pulmonary actinomycosis in

patients with structural lung disease and anticoagulation and the need for increased clinical suspicion and timely use of advanced or surgical diagnostics when standard procedures are not conclusive.

Conclusion

Pulmonary actinomycosis is a rare but clinically relevant infection that frequently mimics malignancy or other chronic pulmonary disease, often leading to a delayed or inaccurate diagnosis. This report contributes to the limited literature by presenting a rare case in which pulmonary actinomycosis developed in a patient with chronic bronchiectasis and became clinically apparent after anticoagulant-induced hemoptysis. The need for surgical resection to make a definitive diagnosis despite nonspecific radiographic findings and nondiagnostic bronchoscopic evaluations highlights the diagnostic limitations of conventional noninvasive procedures in such complex cases. This case emphasizes the need to include rare infectious etiologies, such as actinomycosis, in the differential diagnosis of hemoptysis, especially in patients with preexisting structural lung abnormalities. Early recognition and a coordinated multidisciplinary approach are crucial to minimize diagnostic delays and achieve optimal clinical outcomes. To our knowledge, this case is one of the few documented cases in which pulmonary actinomycosis has been associated with bronchiectasis and hemoptysis during anticoagulation therapy.

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Declarations

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Author contributions

Conceptualization: M.K. and K.C. Methodology: M.K. Clinical data acquisition and interpretation: M.K., K.C., and G.K.G. Pathological analysis and reporting: G.K.G. and N.B. Writing – Original Draft Preparation: M.K. and T.D. Writing – Review & Editing: T.D. and N.B. Visualization: G.K.G. and M.K. Supervision: T.D. and N.B. Project Administration: M.K. and K.C.

Conflicts of interest

The authors declare no competing interests.

Data availability

Not applicable.

Ethics approval

Written informed consent was obtained from the patient for the use of clinical data, medical images, and related information presented in this case report. The patient was assured that all personally identifiable information would be treated confidentially and that all data would be anonymized to protect privacy.

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CASE REPORT

Self-injection of kerosene with chemical pneumonitis and abscess formation – a case report and literature review

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ABSTRACT

Introduction and aim. Kerosene, a widely available household fuel in Sri Lanka, is a common cause of accidental poisoning, especially in children, and is occasionally used for self-harm, which can cause complications such as chemical pneumonitis, abscesses, and thrombosis. Its unsafe storage practices increase the risk of accidental exposure.

Description of the case. A 16-year-old Sri Lankan male self-injected kerosene into the left cubital fossa. Although initially stable, he later developed chemical pneumonitis, left cephalic vein thrombosis, and a sterile abscess at the injection site.

Conclusion. Early multidisciplinary intervention and close clinical monitoring led to a favorable outcome. A review of the literature reveals that intravenous hydrocarbon injection, although rare, can lead to significant local and systemic toxicities.

Keywords. abscess, kerosene, pneumonitis, self-injection

Introduction

Kerosene, a widely used household fuel and solvent in low-resource settings such as Sri Lanka, poses significant public health risks due to its toxic properties and frequent involvement in accidental poisonings, particularly among children.[1,2] While ingestion and inhalation are well-documented routes of exposure, intravenous self-injection of kerosene remains a rare and underreported method of deliberate self-harm with severe systemic complications. Chemically, kerosene consists of a complex mixture of hydrocarbons, including paraffins, naphthenes and aromatics, as well as potentially toxic compounds such as n-hexane, naphthalene and benzene.[1] Its low viscosity, low surface tension, and high volatility facilitate rapid absorption into tissues, leading to local necrosis, systemic toxicity, and, in cases of aspiration or hematogenous spread, chemical pneumonitis, a life-threatening complication.[2,3]

Although the previous literature has described kerosene-induced pneumonitis after inhalation or ingestion, reports of intravenous injection leading to concurrent complications, such as deep vein thrombosis, sterile abscess formation, and acute lung injury, are exceedingly rare, particularly in previously healthy adolescents.

Aim

This case presents a unique clinical scenario in which a 16-year-old man developed multisystem involvement after self-injection of kerosene, highlighting the need for increased clinical awareness and prompt intervention in such atypical presentations.

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By detailing this case, we aim to emphasize the novelty of this presentation, underscore the pathophysiological mechanisms of intravenous kerosene toxicity, and improve the recognition and management of similar cases in emergency and clinical settings.

Description of the case

A 16-year-old right-handed school-going adolescent male was admitted to the Teaching Hospital Anuradhapura, Sri Lanka, on February 2, 2024, at 6:50 PM, approximately one hour and 20 minutes after self-injecting 4 ml of kerosene into his left cubital fossa. He was preparing for the Ordinary Level upcoming national exam, the General Certificate of Education, conducted throughout the island.

On admission, he was conscious and rational, with a noticeable kerosene odor on his breath and an erythematous patch over the injection site. His blood pressure was 130/80 mmHg, his heart rate was 90 beats per minute and he was tachypnoeic with a respiratory rate of 28 breaths per minute and a Glasgow Coma Scale of 15. His oxygen saturation, as measured by pulse oximetry, was 100% while breathing room air, with clear lung fields on auscultation. Empirical broad-spectrum intravenous antibiotics were started, including ceftriaxone 1 g twice a day and metronidazole 500 mg every eight hours with normal intravenous normal saline for hydration.

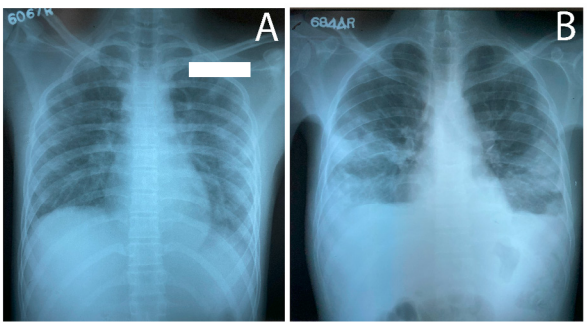

Fig. 1. Chest radiographs taken during the hospital stay, A: February 3, 2024 (day 2), normal chest X-ray, B: February 4, 2024 (day 3), evidence of pneumonitis by widespread bilateral poorly defined small opacities

On day 2, arterial blood gas analysis revealed type 1 respiratory failure (PaO_2 7.53 kPa, $PaCO_2$ 3.02 kPa, pH 7.508) with respiratory alkalosis, indicating early pulmonary involvement despite a normal initial chest radiograph. This discrepancy likely reflects the limited known sensitivity of plain radiography in early chemical pneumonitis. Intravenous hydrocortisone (100 mg six-hourly) was initiated alongside oxygen therapy (2 L/min via face mask). A repeat chest X-ray (Figure 1B) on day 3 showed widespread, multiple, poorly defined, small opacities over both lung fields consistent with pneumonitis, confirming kerosene-induced pneumonitis. During the next 24 hours, the patient developed worsening respiratory distress, fever, and cellulitis at the injection site.

Despite initial stabilization, his respiratory status deteriorated, and he was transferred to the intensive care unit (ICU) for close monitoring. In the ICU, oxygen therapy through a 60% venturi device, intravenous antibiotics, fluid, hydrocortisone, and subcutaneous enoxaparin.

On the fourth day of admission, acute cephalic vein thrombosis, along with soft tissue inflammation, was observed on ultrasound and Doppler imaging of the left forearm without any evidence of compartment syndrome. Intravenous heparin therapy was started to prevent further propagation of the thrombus.

On the sixth day, he was discharged from the ICU and moved to the ward. Before discharge, a psychiatrist and was prescribed sertraline 50 mg once a day. On admission, the patient was evaluated using the SAD PERSONS scale for suicidal risk,[4] with a score of 3/10 (indicating that the risk of suicidality is low and discharge may be safe). However, no formal suicide risk assessment tool (for example, C-SSRS) was administered before discharge.

On the seventh day, he developed pain and swelling at the injection site, and an ultrasound confirmed an abscess at the cubital fossa and the biceps muscle. The incision and drainage of the abscess was performed under general anesthesia on the ninth day. The culture did not yield bacterial growth, suggesting sterile abscess formation.

Table 1. Investigations summary

	February 3	February 6	February 8	February 10	February 12
White blood cells (4–10 x10^9/L)	5.06	12.21	12	15.6	14.65
Neutrophils (40–60%)	84	86	81	82.7	60
Lymphocytes (20–40%)	12	7	13	12.1	32
Hemoglobin (12–16 g/dL)	13	13.3	13	12.6	12
Platelets (150–450 x10^9/L)	192	238	198	308	388
Blood urea (2.5–6.5 mmol/L)	–	–	3.3	–	–
Serum creatinine (45–120 micromoles/L)	73	56	52	–	–
Serum sodium (135–148 mmol/L)	137	137	138	144	–
Serum potassium (3.5–5.5 mmol/L)	3.5	4.1	3.4	4.4	–
Aspartate transaminase (0–50 U/L)	28	21	–	–	–
Alanine transaminase (0–45 U/L)	10	11	–	–	–
Gamma-glutamyl transferase (0–40 U/L)	–	16	-	18	-
Total bilirubin (3–20 micromoles/L)	–	8.6	–	–	–
Direct bilirubin (0–14 micromoles/L)	–	4.3	–	–	–
Albumin (35–55 g/L)	–	38	–	–	–
Globulin (15–35 g/L)	–	33	–	–	–
International normalized ratio (<1.4)	–	1.01	1.28	–	–
Activated partial thromboplastin time (<25 seconds)	–	34.4	37.2	–	–
C-reactive protein (0–6 mg/L)	–	382	109	49	19

Management presented a significant therapeutic challenge, requiring concurrent administration of systemic corticosteroids for chemical pneumonitis and anticoagulation for cephalic vein thrombosis. This was further complicated by the subsequent development of a sterile abscess at the injection site, raising concerns about (1) the potential risk of bleeding from combined anticoagulant and high-dose steroid therapy and (2) the possible abscess rupture with anticoagulation. We successfully managed these competing risks through clinical follow-up and maintaining therapeutic anticoagulation goals. The patient did not experience bleeding complications, and the abscess remained contained until surgical drainage on day 9.

He was discharged from the hospital on the eleventh day with a tapering course of prednisolone. His lungs were clear two weeks later and the wound healed. Refer to Table 1 for a summary of the investigations and Table 2 for the timeline.

Table 2. Timeline*

Date and time	Day	Clinical sequence
February 2, 2024	01	5:30 PM – self-injection of kerosene oil
		6:50 PM – admitted to Teaching Hospital Anuradhapura
February 3, 2024	02	first chest X-ray performed – normal ABG – type 1 respiratory failure supplementary oxygen started
February 4, 2024	03	second chest X-ray done – evidence of acute pneumonitis
February 5, 2024	04	USS/Doppler – acute thrombosis of the left cephalic vein transferred to the ICU for close monitoring
February 6, 2024	05	sertraline started after a psychiatric evaluation
February 7, 2024	06	discharged from the ICU to the ward
February 9, 2024	08	large abscess over the injection site
February 10, 2024	09	incision and drainage of abscess
February 12, 2024	11	discharged
February 27, 2024		review healed wound, no lung signs

* ABG – arterial blood gas analysis, USS – ultrasound scan, ICU – intensive care unit

Discussion

We present a patient who attempted to inject kerosene intravenously, which caused chemical pneumonitis and the formation of sterile abscesses at the injection site, probably caused by accidental extravasation. There is no history of mental illness or drug addiction.

A review of kerosene or hydrocarbon injections based on a PubMed search and references (Table 3) analyzed 65 case reports and series involving 114 patients and 117 incidents (including three attempts by a single individual). Hydrocarbon injections predominantly affected men (67%, 10% unspecified gender), and 12 fatalities were reported (two unspecified genders, one female). The findings suggest that intentional hydrocarbon injections (mainly suicidal) are most common among young men with a history of substance abuse or mental illness. The route of administration had a significant influence on clinical outcomes. Subcutaneous or intramuscular routes (76%) caused localized tissue damage (e.g., abscesses), while intravenous or intrathoracic injections (36%) led to systemic toxicity (eg arrhythmias, acute lung injury).

However, nine patients who reported intravenous injections (including five deaths) did not show evidence of pulmonary involvement. Among the deaths, four were attributed to cardiac arrhythmia, which occurred before chemical pneumonitis could develop.[5–8] The other death was from a 39-year-old man who injected kerosene into his hemorrhoids in the mistaken belief of its therapeutic potential. He died one month later due to a myocardial infarction, a day after major surgery.[9] In particular, two of the four deaths due to arrhythmia resulted from accidental injection of kerosene by healthcare workers.[5,8] Only one patient was confirmed as a suicidal attempt,[6] while the other was a homicidal act.[7] The remaining four survivors without pulmonary involvement probably attempted intravenous self-administration but instead injected subcutaneously, as evidenced by local tissue reaction.[10–14]

Further analysis of fatalities revealed that five of the other seven deaths were reported in a case series of 10 patients with substance use disorder,[15] had multiorgan failure and acute respiratory distress,[16] and in the other death, we could not obtain the full text for further analysis.[17] Geographically, seven out of the 12 deaths are from Iran. Systemic complications, though rare, were severe in intravenous cases, with mortality associated with rapid absorption or high-volume injections.

Three studies from Iran and Iraq represent the most extensive documented case series of intentional kerosene injection.[15,18,19] All three articles contextualize the sociocultural and psychological landscape of the socioeconomically strained environment of postwar Iran and Iraq, where stigma, limited psychiatric care, and war-related trauma appear to fuel such self-destructive behaviors.

Furthermore, a case report describes the injection of mineral oil for cosmetic purposes in the USA, leading to sclerosing lipogranulomatosis.[20] Although only one case report was included in this review, numerous other case reports document similar complications with the injection of mineral oil subcutaneously for cosmetic purposes.

When kerosene is taken orally, aspiration occurs due to its low viscosity, high volatility, and low surface tension. As little as a milliliter of aspirated kerosene can cause significant lung injury.[3] In the absence of aspiration, chemical pneumonitis will not occur.[3] It is poorly absorbed through the gastrointestinal tract and, even if absorbed, does not cause pneumonitis.[3] Due to the risk of aspiration, gastric lavage is contraindicated in case of kerosene poisoning.[3]

The mechanism of pneumonitis after hydrocarbon injection was elucidated in an elegant animal experi-

Table 3. Summary of literature (references are in supplementary file S1)[a]

No	Reference	Age	Sex	Injection				Complications and outcome	Pulmonary involvement and imaging	Intention	Comorbidity, attempts, and other info
				Route	Site	Vol	Chemical				
	Green 1977 USA[1]	21	M	IV	L & R cubital fossa	3–4 mL	Naphtha	Sterile abscess. Discharged on day 6	Lung involvement was not observed	Suicidal	Substance use disorder, intoxicated
	Neeld 1978 USA[2]	31	M	IV	–	2–6 mL	Hydrocarbon insecticide	No local reaction. Left against medical advice after 3 days	Pneumonitis with patchy consolidation & atelectasis	Suicidal	Substance use disorder for 12 years
	Vaziri 1979, 1980* USA[3,4]	40	M	IV	L cubital fossa	3 mL	Naphtha	No local reaction. Discharged on day 5	Pneumonitis, bilateral ground glass shadows, L pleural effusion	Suicidal	–
	Erichsen 1979#^ Denmark[5]	–	–	SC	–	–	–	–	–	–	–
	Kósa 1981#^ Hungary[6]	NS		IV	Arm?	20 mL	Petrol	The patient died 45 minutes after repeated seizures	Probably died before pneumonitis	Accidental injection by health care worker	After surgery
	Beck 1981 UK[7]	27	M	IV & SC	L arm	4–9 mL	Hydrocarbon insecticide	Extensive necrosis, sterile abscess discharged on day 25	Pneumonitis, alveolar & interstitial infiltrates in both lungs.	Suicidal	Substance use disorder
	Goldberg 1982 USA[8] 2 cases	22	M	SC	L forearm	NS	Hydrocarbon insecticide	Two sterile abscesses. Discharged on day 9	Lung involvement was not described	Suicidal.	Substance use disorder & depression
		24	M	SC	L forearm	NS	Hydrocarbon insecticide	Sterile abscess discharged on day 9	Lung involvement was not described	Suicidal	Substance use disorder
	Layton 1983 USA[9]	38	M	SC & IM	R forearm	5 mL	Gasoline	Compartment syndrome. Ischemic contracture damage to all three major nerves. Rehabilitation for the R arm discharged on day 44	Lung involvement was not described	To get 'high'	Substance use disorder amphetamine, barbiturates narcotics
	Buseti 1984^#Italy[10]	–	–	IV	–	–	Kerosene	–	Respiratory insufficiency	–	–
	Qaryoute 1984 Jordan 2 cases[11]	18	F	SC	R inguinal area	5 mL	Kerosene	Abscess, major surgery, recurrent hospitalizations.	Lung involvement was not described	Non–suicidal, believed therapeutic	–
		22	M	SC	L arm	5 mL	Kerosene	Necrosis, major surgery. Recovered.	Lung involvement was not described	Assault. forceful injection	–
	Saulsbury 1984[12] 2 admissions	9/12	M	IV	–	NS	Naphtha	Survived, developmental delay, cerebral palsy and cortical blindness	Respiratory arrest, pneumonitis	Mother injected	Child unresponsive before injection
		11/12		IV	Injected into IV line				Respiratory arrest,	Mother injected	Child placed in foster care. Mother guilty for manslaughter
	Wedin 1984 USA 2 cases [13]	23	M	SC	R forearm	NS	Hydrocarbon insecticide	Sterile abscess. Discharged on day nine	Lung involvement was not observed	Suicidal	Paraplegic, IV drug abuse.
		16	M	SC	R forearm	1.5–2 mL	Turpentine	Sterile abscess	Lung involvement was not observed	Suicidal	The man came from a psychiatric hospital
	Case 1985^ USA[14]	Elderly	M	IV	–	25–40 mL	Naphtha	Arrhythmia and sudden death from blunt chest trauma.	Probably died before pneumonitis	Homicidal	High concentration of naphtha in cardiac blood
	Rubinstein 1985 Nixon 1985 Israel*[15,16]	22	F	SC	Multiple sites	NS	kerosene	Multiple abscesses on the skin, epidural space, knee, etc. Acute arthritis, paraparesis, & sensory loss T8 laminectomy, but paraplegic after 18 months	Lung involvement was not observed	Homicidal. (boyfriend injected after sedating with hypnotics)	He murdered his wife using the same technique
	Wason 1986 USA[17]	22	M	IV & SC	R cubital fossa	5 mL	Turpentine	Sterile abscess discharged on day 20	Pulmonary edema	Suicidal	Depressed, unable to offer sex change operation
	Bushe 1986 UK[18]	18	F	SC	R cubital fossa	2 mL	Petroleum lighter fuel	Cellulitis, no abscess, discharged on day 10	Lung involvement was not observed	DSH	–
	Bozzuto 1987 USA[19]	29	M	IV	R cubital fossa	12 mL	Hydrocarbon insecticide	Cellulitis, microscopic hemoglobinuria, and myoglobinuria. Transferred to psychiatric ward	Lung involvement was not observed	To get 'high'	Previous suicidal attempts. Injected previously 4 days ago, multiple injection marks
	Sevcik 1992 Czech[20]	30	M	IV	–	8 mL	Xylene	Ventilated, hemodialyzed, GTC seizures, referred to psychiatric facility	ARDS, ventilated	Suicidal	–

Geoffray 1992 France 2 cases[21]	24 case 1	M	SC	Both hands, the elbow folds & neck	10 mL	Naphtha	SC necrosis with abscess formation. Skin graft Diabetes insipidus.	No lung involvement was observed.	Suicidal	–
	43 case 2	M	SC	Both buttocks	0.5 mL	Petrol	Two abscesses with fistulas formed on the buttocks. Complete cure	No lung involvement was observed.	Sexual stimulation	–
Larsen 1992 USA[22]	17	F	SC	Both hands	3 mL	Hydrocarbon insecticide	Abscess, compartment syndrome	No lung involvement was observed.	Suicidal	–
Rimmer 1993 UK 2 cases[23]	30	M	SC	L index finger	2 mL	White spirits	I&D	No lung involvement was observed.	Accidental	No
	45	M	SC	L thumb	NS	White spirits	Abscess ruptured spontaneously	No lung involvement was observed.	Accidental	No
Greenberg 1993 USA[24]	38	M	IV	NS	5–15 mL	Gasoline	Atrial fibrillation. Left against medical advice	Lung involvement was not described	DSH	Substance use disorder (IV & tobacco)
Bindlish 1993 Canada[25]	23	M	SC	R forearm and L pectoral muscle	NS	Gasoline	Pectoral muscle necrosis, compartment syndrome, fasciotomy of the arm and chest wall, and psychiatric evaluation. discharged on day 12	Lung involvement was not observed	Suicidal	NS
Grimmet 1996 Australia[26]	28	F	IV	NS	1 mL	Endosulfan in xylene 30%	Status epilepticus, rhabdomyolysis, proximal myopathy, hypoxic brain injury, discharged on day 21	Ventilated. R lower lobe pneumonia	Suicidal	Substance use disorder (IV) & 2 previous suicide attempts
Khammash 1997^ Jordan 3 cases[27]	–	–	SC	–	–	–	Severe necrotizing inflammation skin grafting	–	–	–
Rush 1998 USA[28]	33	M	SC	L cubital fossa	4 mL	Naphtha	Cellulitis & large abscess. Discharged on day 20	Lung involvement was not observed	Suicidal	A previous suicide attempt, depression, substance use disorder (IV)
Shustermann 1999 USA[29]	34	M	SC	L cubital fossa	1.5 mL	Dripless oil	Cellulitis. Discharged with psychiatry review	Lung involvement was not observed	Suicidal	Psoriasis with folliculitis, HIV, depression, two suicidal attempts
Guerguerian 2000 Canada[30]	18	M	IV	Cubital fossa (Side NS)	NS	Hydrocarbon	Acute lung injury discharged on day 15	Xray increased vascular markings. pulmonary oedema	Suicidal	Self–inflicted hematuria.
Aguemon 2000# Benin[31]	18	M	IV	R upper limb	2.5 mL	Paraffin oil	Local thrombophlebitis. Necrosis and shock, Recovered	Cough & Chest pain	DSH	Anxiety neurosis
Federmann 2000^# Germany[32]	26	M	SC	L cubital fossa	–	Heating oil	Necrosis & abscess. Surgical debridement with mesh graft	Lung involvement was not observed	–	–
Mussoff 2000# Germany[33]	26	M	SC	Surgically treated site of an inguinal hernia	–	Petroleum distillates	Significant localized painful swelling of the soft tissues, large colliquation necrosis of the subcutaneous tissue with petroleum odour.	Lung involvement was not observed	Self–mutilating behaviour	Recent surgical treatment of an inguinal hernia.
Buchman 2000 USA 5 cases[34]	54	F	IM & SC	L & R forearms	–	Raid Roach Killer	Swelling in both forearms and hands, liquefaction necrosis in multiple sites.	Lung involvement was not observed	Suicidal	History of hypertension, diabetes mellitus, alcohol abuse, and suicide attempts
	38	M	SC & ? IV	Cephalic vein by another substance abuser	–	Unknown insecticide contains hydrocarbon	Incision and drainage, the thrombosed portion of the cephalic vein was resected. Referred to a drug rehabilitation centre	Lung involvement was not observed	Not suicidal	Substance use disorder
			IM	Upper extremity		Ant & roach killer	Muscle & fat necrosis, multiple debridement	Lung involvement was not observed	Suicidal	
			IM	Upper extremity		Roach killer	Muscle & fat necrosis, multiple debridement	Lung involvement was not observed	Suicidal	
			IM	Upper extremity		Roach killer	Muscle & fat necrosis, multiple debridement	Lung involvement was not observed	Suicidal	
Perings 2001# Germany[35]	20	M	IV	NS	20 mL	Lamp oil (Liquid paraffin)	R heart failure, mild DIC, psychiatric therapy	Lipoid pneumonia	Suicidal	–
Awe 2003 Saudi Arabia[36]	33	M	SC	Both side of his R leg	5 mL	Kerosene	Acute necrotizing fasciitis with compromised venous return. Skin grafts were applied. Discharged cured	Lung involvement was not observed	DSH	Injected twice with kerosene, ingested razor blades. Diagnosed with personality disorder.
Solak 2006 Turkey[37]	53	M	IT	L hemithorax through the 5th intercostal space	10 mL	Thinner	No local effects. Discharged on day 35. CT after 1 year of pleural thickening.	Atelectasis with massive effusions (chemical empyema) L lung CT L pleural effusion, atelectasis in the L lower lobe, and pleural thickening.	Suicidal	Major depression

Domej 2007 Austria[38]	26	M	IV	L cubital vein.	10 mL	Gasoline	Atrial fibrillation, multiorgan failure, BL hemorrhagic effusions ARDS, ventilated, AKI, CWH, rhabdomyolysis. Discharged for psychiatric care. 6 months after restrictive lung diseases with nodular & reticular opacities	FOB Hemorrhagic bronchopneumonia diffuse mucosal hemorrhage. X–ray interstitial & BL alveolar opacities CT severe, patchy consolidations, ARDS, BL pleural effusions.	Suicidal	–
Thaha 2007 UK 3 attempts[39]	35	F	SC	L cubital fossa (June 1998)	3–5 mL	Lighter fluid	Compartment syndrome. surgical decompression	Lung involvement was not observed	Suicidal	Psychiatric illness
			SC	L Femoral vein (October 1998)	10 mL		Local inflammation, sterile abscess, and extensive fat necrosis. Incision and drainage done. Left to heal by secondary intention	Lung involvement was not observed	Suicidal	
			SC	Volar aspect of the right arm (March 1999)	NS		Multiple stab wounds were made on the skin under local anesthesia and a thorough 'saline flush out' done	Lung involvement was not observed	Suicidal	
Hazari 2008 USA[40]	34	M	IM	Thighs and buttocks	5 L	Mineral oil	Multiple abscesses in the lower extremities, multiple surgeries, pneumonia, effusion & septicemia (sclerosing lipogranulomatosis) discharged after 6 months	Developed effusion and pneumonia, unlikely to be direct effect	Aesthetic	–
Amiri 2009 Iran 10 cases[41]	mean20.3 (16–26)	9M 1F	IV	Cubital vein	five > 5mL	kerosene	5 died & 4 of them injected more than 5 ml. 7–phlebitis 6–arrhythmia 8–loss of consciousness	All had pneumonitis. No soft tissue injuries	Suicidal	All had substance use disorder (IV), 4 had previous suicidal attempts
Farahvash 2009 Iran 21 cases[42]	16–85	15M 6 F	8 SC 10 IM 3 SC& IM	All injected into arm but one injected into arm & abdomen	1–30 mL mean 4.9 mL	Hydrocarbon	Compartment syndrome–13 (fasciotomy 5 skin graft 10) Abcess–5 No surgery3 4 had long–term deformity	Lung involvement was not observed	Suicidal	14/21 had comorbidities. 4 Major depression 2 schizophrenia 1–epilepsy 1–migraine 7 Substance use disorder opium 5 previous suicide attempt.
Fink 2010 Germany[43]	22	M	IV, SC & IM	L cubital vein	7 mL	Gasoline	Cellulitis 10 cm around the injection area. Surgical debridement. Referred to psychiatrist	Multiple opacities on X-ray in the bases of the lung with bilateral effusion CT basal ground–glass opacities	Suicidal	Asperger syndrome, suicidal attempt by benzodiazepine overdose.
Rostami 2010 Iran[44]	39	M	SC?	Haemorrhoids. Repeatedly injected for 6 days	NS	Kerosene	Necrosis in the perineal region & scrotum oedema. Laparotomy and colostomy performed. Died of MI	Lung involvement was not observed	Suicidal	–
Eskandarlou, 2010 Iran[45]	31	M	IT & SC	40 mL into the L hemithorax 10 mL into the L cubital fossa	50 mL	Petroleum	Decortication & segmentectomy recovered after 2 months of being sent to a psychiatric facility	Pyothorax, empyema, collapse, necrosis of the chest wall. Xray airfluid level in the L hemithorax. Complete CT collapse L lung with mediastinum shift and bronchopleural fistula.	Suicidal	–
Ra, 2011 Republic of Korea[46]	23	M	IV	NS	1 mL	Isoparaffin	Cellulitis and necrosis site of injection. Complete resolution of lung findings	X–ray ill–defined pulmonary nodules BL CT multiple, wedge–shaped, ill–defined ground glass opacities BL consolidations and BL pleural effusion	Suicidal thoughts	–
McGeary 2011 UK[47]	40	M	IV	L cubital vein	10 mL	White spirit	Soft tissue abscess in the arm– I&D	Bilateral X-ray opacities CTPA– air space consolidation	Suicidal thoughts	Substance use disorder (IV) Psychiatric history
Wiper 2011 UK[48]	27	M	SC	L arm and both buttocks	40 mL	Petrol	Necrosis. Excised necrotic muscle and fat. Discharged	Lung involvement was not observed	Suicidal	Schizophrenia, sectioned (detained in hospital)
Mahmoodpoor 2012 Iran[49]	58	M	IV & SC	R arm	10 mL	Gasoline	cellulitis R forearm fasciotomy, ventilated ARDS AKI multi–organ failure, died day 21	Symptoms of acute lung injury	Suicidal	Previous suicidal attempts.
Godarad 2012[50]	19	F	IT	Chest	15 mL	white spirit	R effusion consolidation, pneumothorax ARDs	Symptoms of acute lung injury	Suicidal	Substance abuse disorder & depression

Nelson, 2013 USA 2 cases[51]	23 Case–1	M	IV	Arm R injected over 12 hours (1 mL at a time)	15 mL	Lubricant (WD–40)	The necrosis and abscess R arm. Was discharged on day 9 to inpatient psychiatric care	Xray pulmonary edema with right lower lobe infiltrate	Suicidal (with his fiancée, case 2)	Substance use disorder IV heroin, depressed. Homeless
	29 Case–2	F	SC	Dorsal aspect of R hand & wrist dorsal aspect	NS	Lubricant (WD–40)	Necrotic tissue, compartment syndrome, psychiatry unit after 12 days	Lung involvement was not observed	Suicidal (with her fiancé, case 1)	Substance use disorder IV heroin. Homeless
Liu 2013 China[52]	33	M	IM	Distal region of the left arm	10 ml	Phoxim (although an OP, it is a complex hydrocarbon)	The distal L arm was swollen cellulitis I&D	Lung involvement was not observed	Suicidal	Past craniocerebral trauma
Omori 2013 Japan[53]	21	M	SC	L cubital fossa	5 mL	Spot remover fluid (hydrocarbons as solvents)	Pain, oedema, and erythema of L upper limb I&D	Lung involvement was not observed	Suicidal	Psychiatric treatment for previous suicide attempts
Jayaprasad 2013 India[54]	34	M	SC	L hand at two sites	5 ml	Kerosene	Cellulitis left against medical advice	Lung involvement was not observed	Suicidal	Chronic alcoholic
Sharquie 2014 Iraq 11 cases[55]	17–25	1 M 10 F	SC	Arm–8, thigh–1 buttock–1		Kerosene	Ulcer and cellulitis, one developed contracture	Lung involvement was not observed	DSH	Severe emotional tension due to social, economic and psychological problems
Zirwes 2015^# Germany[56]	–		IV	–	2 mL	Benzene	Died	–	Suicidal	Chronic pain syndrome. Drug intoxication & strangulation
Hasan 2016^ Bangladesh[57]	–		IV	–		Kerosene	–	–	Suicidal	–
Bidaki 2016 Iran[58]	17	M	SC	L forearm	5 mL	Gasoline	Necrotising fasciitis and abscess	Lung involvement was not observed	Suicidal	Cluster B personality air injection into vein, touching electrical wires, cutting body 3 times, pill overdose
Pouwels 2018 Netherlands[59]	52	M	IT	L hemithorax	NS	Benzene & paracetamol 25g	Discharged from psych care after 23 days	A respiratory insufficiency hydropneumothorax CT empyema in the L lung and pectoral abscess	Suicidal	Substance use disorder, alcohol & tobacco, multiple TIA.
Murphy 2018 Ireland[60]	26	M	SC & IM	L forearm	15 mL	Petroleum	Compartment syndrome.	Lung involvement was not observed	Suicidal	Depression, weakness of flexors
Alquraish 2018 Saudi Arabia[61]	30	M	SC	L cubital fossa	6 mL	Kerosene	Cellulitis	Lung involvement was not observed	Suicidal	Prisoner
Asiimwe 2021 Uganda[62]	23	M	IM	L wrist & cubital fossa.	5 mL	Kerosene & rodenticide	Seizure & compartment syndrome L wrist contractures, discharged on day 42	Lung involvement was not observed	DSH	Relationship conflict, depression,
An 2021 China[63]	14	M	IV	Forearm?	5 mL	Insecticide (kerosene & propyl butane)	Ventilated with complete recovery	Pneumonitis	Suicidal	Depression, suicide attempts
Arafat 2024 Bangladesh[64]	30	F	IV	Forearm?	10 mL	Kerosene midazolam amitriptyline	VT, electrolyte abnormalities, DVT, & multi–organ failure. Died	Probably died before pneumonitis	Suicidal	Major depressive disorder.
Bubalo 2024^ Croatia[65]	62	M	IV	Through HD catheter	–	Medical Kerosene	Respiratory, metabolic, lactic acidosis, hypotension, & tachyarrhythmia. Died	Died before pneumonitis	Accidental injection by HCW	CKD on hemodialysis

[a] AKI acute kidney injury, ARDS – acute respiratory distress syndrome, BL bilateral, CT – computed tomography, CTPA pulmonary angiogram, CVVH – continuous venovenous hemofiltration, DIC disseminated intravascular coagulation, DSH deliberate self-harm, DVT – deep vein thrombosis, F female, FOB – fiber optic bronchoscopy, GTC generalized tonic-clonic, HIV human immunodeficiency virus, IM intramuscular, IT intrathoracic, I&D – incision and drainage, IV intravenous, L – left, M male, MI – myocardial infarction, NS not stated, OP – organophosphorus pesticides, R right, SC – subcutaneous, TIA transient ischemic attack, VT ventricular tachycardia, HD – hemodialysis, HCW – health care worker, CKD – chronic kidney disease, # – non-English, * – same patient, ^ – only abstract, original case report cannot be found, in some cases data obtained from secondary sources

ment.[21] Lungs selectively absorb hydrocarbons and excrete them through the alveoli, which is why the kerosene odor is present in the patient's breath. Increasing the minimum surface tension in the alveoli is the central mechanism of pneumonitis caused by injected and aspirated kerosene. This may be due to a direct physical action that interferes with the action of the surfactant or damage to the type 2 pneumocytes, which produce the surfactant. Type 2 pneumocytes are inactivated by kerosene oil, leading to surfactant deficiency, intra-alveolar hemorrhage, inflammation, necrosis and alveolar collapse.[2,3]

Deep vein thrombosis may develop after large vein thrombophlebitis due to the presence of hydrocarbons.[22] In addition, damage to the endothelium caused by intravenous kerosene may have facilitated the formation of thrombuses, a finding that requires further research to validate. Hydrocarbons destroy membrane lipids and disrupt their integrity. It acts as a defatting agent, causing liquefaction necrosis, and can spread along tissue planes.[18] The formation of an abscess at the injection site indicated the consequence of kerosene, leading to inflammatory reactions. Tissue damage and activation of innate immunity will aid in the formation of an abscess. The combination of local tissue damage and impaired immune response creates an ideal environment for abscess formation, requiring surgical intervention for drainage and wound debridement. The abscess was considered sterile based on negative results from aerobic culture.

As kerosene poisoning by injection can lead to multiple complications, vigilance for the development of such issues and prompt intervention may help to minimize morbidity and mortality. Given the widespread availability of kerosene in developing regions, greater awareness, accurate reporting, and more research are needed to understand better the complications, risk factors, and preventive strategies associated with such intentional or accidental injections.

Several limitations should be acknowledged in this case. First, the diagnosis of pneumonitis was solely on clinical presentation and serial chest radiographs, as advanced imaging (CT/HRCT) and lavage-free bronchoscopy were not performed. The patient's rapid clinical improvement justified their omission. Second, although the abscess was presumed to be sterile based on negative aerobic cultures, our setting did not have a comprehensive microbiological evaluation, including anaerobic cultures and PCR for atypical pathogens. These diagnostic limitations underscore the challenges of managing complex toxicological presentations in resource-limited environments, where clinical judgment must often supplement incomplete laboratory data.

Conclusion

Kerosene is a common household chemical that can be used for self-harm, particularly in the developing world. Injection is one of those methods, which can lead to significant morbidity and mortality due to local and systemic complications. Chemical pneumonitis is a common consequence when kerosene enters the circulation, contributing to morbidity. A multidisciplinary approach is crucial for reducing associated complications and improving outcomes. Preventive measures are equally important and require an evaluation of the underlying psychological triggers to identify the root causes. This case highlights the complications of intravenous kerosene injection, including chemical pneumonitis, thrombosis, and abscess formation.

Declarations

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Author contributions

Conceptualization, A.W., P.W. and S.S.; Methodology, S.S.; Software, H.S. and S.S.; Validation, A.W. and T.R.; Formal Analysis, S.S. and T.R.; Investigation, A.W. and P.W.; Resources, A.W., H.S. and P.W.; Data Curation, T.R. and S.S.; Writing – Original Draft Preparation, A.W. and S.S.; Writing – Review & Editing, A.W., P.W., T.R., H.S., and S.S.; Visualization, A.W. and T.R.; Supervision, S.S.; Project Administration, S.S.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

The data that support the findings of this study are available from the senior authors upon request.

Ethics approval

Informed written consent has been obtained from the patient's mother, and ethics approval is not applicable for this manuscript.

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LETTER TO THE EDITOR

Rheumatic mitral stenosis in the perioperative setting – an old acquaintance with new implications

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Dear Editor,
Rheumatic heart disease remains a cause of valvular pathology with significant geographic variation. It is still endemic in Southeast Asia, sub-Saharan Africa, and Oceania, while accounting for approximately 2.5% of valve disease in western countries.[1] Although rare, in developed settings, rheumatic mitral valve disease should still be considered in the perioperative evaluation of elderly patients with a history of valvuloplasty.

We present a clinically relevant case that illustrates the impact of intraoperative transesophageal echocardiography (TEE) in reshaping anesthetic and surgical strategies in a high-risk patient undergoing cardiac surgery.

A 75-year-old woman with hypertension, chronic atrial fibrillation on warfarin (discontinued three days preoperatively), and a history of mitral valvuloplasty (1998) was scheduled for elective mitral valve replacement. Preoperative transthoracic echocardiography (TTE) suggested moderate mixed mitral valve disease and mild tricuspid regurgitation. Intraoperative TEE revealed a markedly distorted anatomy with severe dilated right and left atria (Fig. 1). Detailed evaluation of the mitral valve showed severe rheumatic mitral stenosis with thickened and immobile leaflets, as well as moderate regurgitation, was detected – findings more severe than previously assessed (Fig. 2A). The left atrium was markedly dilated (7×6.3 cm) and filled with dense spontaneous echo contrast, although there was no thrombus in the left atrial appendage, indicating blood stasis and a high embolic risk (Fig. 2B). Moderate tricuspid regurgitation and significant annular dilation (3.9 cm) were also identified (Fig. 3), which was not evident in the preoperative TTE. Pulmonary artery catheterization revealed a mean pulmonary artery pressure of 32 mmHg and a wedge pressure of 16 mmHg.

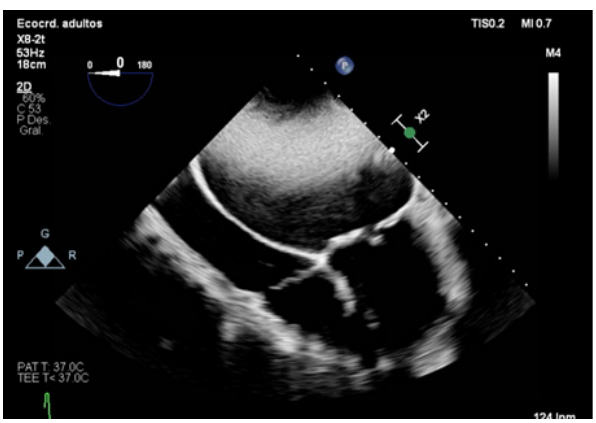

Fig. 1. Transesophageal echocardiogram showing a markedly dilated left atrium as well as an extremely dilated right atria that distorted the normal anatomy with increased difficulty obtaining the planes to evaluate the heart

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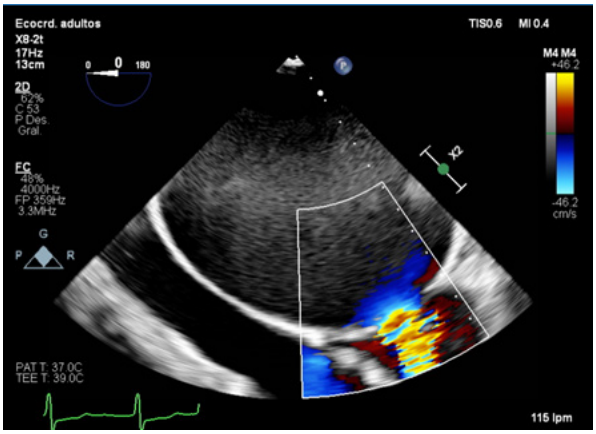

Fig. 2A. Severe mitral stenosis (seen in the figure with the central jet directed to the left ventricle) due to immobile leaflets associated with moderate mitral valve regurgitation differed from the previous assessment of moderate mixed mitral valve disease

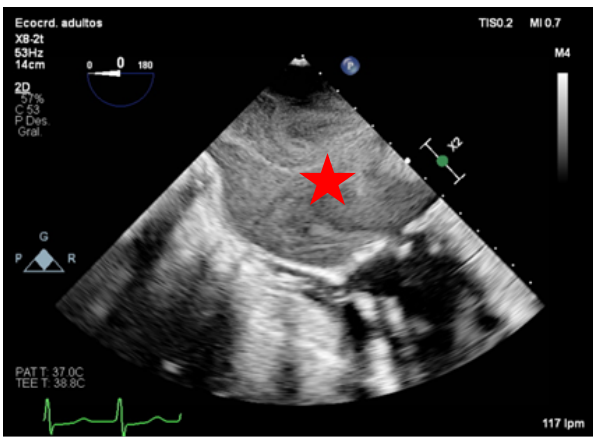

Fig. 2B. Markedly dilated left atrium (7×6.3 cm) filled with dense spontaneous echo contrast, signaled with the red star

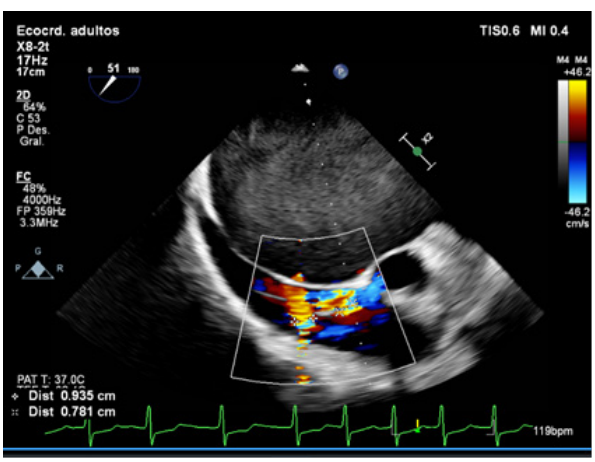

Fig. 3. Central jet of moderate tricuspid regurgitation is seen as a flow directed to the atria, with annular dilation not seen on preoperative transthoracic echocardiography

In light of these findings, cerebral oximetry monitoring was initiated to detect potential embolic events. The surgical plan was modified to include tricuspid valve annuloplasty in addition to bioprosthetic mitral valve replacement.[2] The patient was removed from cardiopulmonary bypass on the first attempt with low dose dobutamine. No paravalvular leakage was observed and only mild central tricuspid regurgitation remained.

The patient was transferred to the intensive care unit, where her postoperative course was uneventful. She was extubated within five hours and progressively weaned off vasoactive drugs without complications. She was discharged on postoperative day six.

Previous studies have shown how TEE influences cardiac surgical decisions in up to 9% of patients.[3] A study by Skinner et al. showed that unexpected findings were observed in 6.3% of patients, of which 68% had changes in the surgical management of their pathology, and where up to 20% of these unexpected findings were due to disease progression.[4]

This case underscores the evolving nature of valvular pathology and the critical role of intraoperative TEE in surgical planning. It also emphasizes the importance of multidisciplinary communication and real-time adaptation in the operating room to ensure optimal patient outcomes. Despite prior interventions and apparent stability, the progression of rheumatic disease may go unnoticed until reevaluation in the operative setting. The presence of spontaneous echo contrast is a valuable marker of stasis and thromboembolic risk, which requires vigilant hemodynamic and anesthetic management.

Rheumatic mitral stenosis may be a long-standing condition, but it remains a condition with significant implications for anesthetic and surgical decision-making, holding new implications for perioperative care.

Declarations

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Author contributions

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Conflicts of interest

The authors declare no competing interests.

Data availability

No datasets are included in this letter.

Ethics approval

This manuscript is a clinical letter based on a single case report in which no identifiable data are disclosed and all data anonymized; therefore, ethics committee approval was not required.

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Reporting guidelines

The guidelines listed below should be followed where appropriate. Please use these guidelines to structure your article. Completed applicable checklists, structured abstracts and flow diagrams should be uploaded with your submission; these will be published alongside the final version of your paper.

Please refer to existing guidelines for reporting methodology; e.g.:

— AGREE guidelines for clinical practice guidelines
— ARRIVE guidelines for *in vivo* animal studies
— CARE guidelines for clinical case reports
— CONSORT guidelines for clinical trials
— PRISMA guidelines for systematic reviews and meta-analyses
— SPIRIT for clinical trials
— STARD guidelines for studies of diagnostic accuracy
— STROBE guidelines for observational studies

Manuscript preparation

Your paper should consist of the following parts. Title page should be supplied as a **separate** file.

Research manuscripts should comprise:

— Title page: Title, Author list, Affiliations, Abstract, Keywords.
— Research manuscript sections: Introduction, Aim, Materials and Methods, Results, Discussion, Conclusions.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions,

Conflicts of Interest, Data Availability, Ethics Approval, References.

Research manuscript sections:

— *Introduction*

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

— *Material and methods*

Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

— *Results*

Results should be clear and concise. The section may be divided into subsections, each with a concise subheading. Tables and figures central to the study should be included in the main paper. Do not use the term "significant" unless p-values are provided. Show p-values to 2 or 3 decimal places. The Results section should be written in past tense.

— *Discussion*

This should explore the significance of the results of the work, not repeat them. Avoid extensive citations and discussion of published literature.

— *Conclusions*

Summarize the work's findings, state their importance, and possibly recommend further research.

Review manuscripts should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords, Literature review sections.
— Back matter: Supplementary Materials, Acknowledgments, Funding Statement, Author Contributions, Conflicts of Interest, Data Availability, References.

Structured reviews and meta-analyses should use the same structure as research articles and ensure they conform to the PRISMA guidelines.

Case reports should comprise:

— Title page: Title, Author list, Affiliations.
— Abstract, Keywords. Case reports should include a succinct introduction about the general medical condition or relevant symptoms that will be discussed in the case report; the case presentation including all of the relevant de-identified demographic and descriptive information about the patient(s), and a description of the symptoms, diagnosis, treatment, and outcome; a discussion providing context and any necessary explanation of specific treatment decisions; a conclusion briefly outlining the take-home message and the lessons learned.
— 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.