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ORIGINAL PAPER

Effect of photodynamic therapy with hypericin on the secretion of selected cytokines of colorectal cancer cells tested *in vitro*

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ABSTRACT

Introduction and aim. Photodynamic therapy is a complex process involving the introduction of photosensitizers into the patient's body and irradiation of them in order to destroy the lesion, and activate the immune system. An important role in photodynamic therapy is played by photobiochemical and physical mechanisms that affect the tumor vessels and lead to the death of the damaged cell. The aim of the study is to determine the effect of photodynamic therapy with the use of Hypericin (Hyp) on the secretion of selected cytokines by colorectal cancer cells.

Material and methods. Two colorectal cancer cell lines SW480 and SW620 were used in the study. Cells treated Hypericin were exposed to visible light. Then cell viability was determined by the MTT assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. Assays were performed for control samples without hypericin and light exposure, with Hyp without light exposure, without Hyp and irradiated with light, and test samples with Hyp and light exposure.

Results. In the experiment we reveal, that Hyp- photodynamic activity does not influence the secretion of cytokines.

Conclusion. The obtaining results confirming the destructive effect of Hyp- PDT on the colon cancer cells, show a possibility of extending the indication for photodynamic therapy using Hyp, qualification of precancerous changes.

Keywords. colorectal cancer, hypericin, photodynamic therapy

Introduction

The photosensitizer (PS) is one of the most important factors responsible for the successful performance of photodynamic therapy (PDT). An ideal PS should have characteristics such as: be a pure chemical substance, commercially available, low toxicity in the dark, but strong photocytotoxicity, have good selectivity for cancer cells, react with the wavelength range – 600–800 nm, which allows for deeper light penetration, should be cleared quickly from the body and be administered in a number of ways: directly to the skin, orally, intravenously or inhaled. *Hypericum perforatum* is a herbaceous perennial, commonly known as St. John's wort. The main photoactive compounds found in *H. perforatum* are hypericin and its analog pseudohypericin (hypericin).[1] Hypericin with the general formula C30H16O8 and molecular weight 504.44 are polycyclic aromatic phenan-throperylenediones. It selective-

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ly accumulates in tumor tissue by diffusion, pinocytosis or endocytosis.[2] Hypericin also exhibits antiproliferative and cytotoxic properties in many cancer cell lines.[3] Although hypericin is classified as a photoactive compound, its use and clinical application is limited due to a decrease in its photosensitizing activity in the presence of serum.[4-9] However, no evidence of phototoxic potential has been found in humans after oral administration of *Hypericum* extract.[10] Hypericin mainly accumulates in the membranes of the endoplasmic reticulum, lysosomes, Golgi apparatus and mitochondria due to its hydrophobic nature. PDT is becoming the routine treatment for some types of non-melanoma skin cancer. The most important components of the method are: photosensitizer, light and molecular oxygen.[11-15] PDT mechanism of tumor cell death is a two-step process in which interactions with cells take place upon activation of PS with light. The physicochemical properties of the photosensitizer have a great influence on the processes of their destruction. Hydrophilic PS interacts with albumin and globulins, while hydrophobic PS tend to bind to low-density lipoprotein receptors. Anionic PSs tend to accumulate in the organelles of cell lysosomes, while cationic molecules are absorbed by mitochondria. Upon activation of PS with a defined wavelength of light, three main mechanisms of cell death are induced: apoptosis, necrosis and autophagy. Two of them, the molecular mechanisms of apoptosis and necrosis, have been thoroughly investigated. Initially, they were considered to be mutually exclusive cellular states.[16] It is now known that there is a balanced interaction between these two modes of cell death. Initiating and effector factors, signaling pathways and subcellular sites were identified as key mediators in both processes. They work by building common modules or as a switch that allows cells to decide which route to take depending on the situation. Autophagy, which is mainly a cytoprotective process, has been associated with both types of cell death, serving as a guide to cell survival or death. The process of cell death after PDT is influenced by: the type of PS and its location in cells, the concentration of PS uptake and its physicochemical features, the concentration of cellular oxygen, as well as the wavelength and intensity of the light used.[17] It is worth adding that there are cases when neoplastic cells become more sensitive to PDT, and sometimes PDT is able to disrupt cellular defense processes, increasing the effectiveness of the therapy. Photodynamic therapy, especially when used in lower doses, has been shown to be effective energy, stimulates the immune response and – by activating the system immune – recognition of tumor antigens (tumor-associated antigens – TAA), even leading to complete remission of adjacent and distant neoplastic lesions from the site of PDT action. These observations prompted the authors to combine photodynamic therapy with immunotherapy, which was anti-cancer effect, and at the same time prevents the recurrence of the disease.[18,19] Interleukin 2 (IL-2) is the most important cytokine stimulating the growth of T lymphocytes, especially those with cytotoxic properties. It means that IL-2 indirectly stimulates the process of programmed cell death (apoptosis) infected with viruses and cancer cells. Stimulation of T lymphocytes increases the production of apoptosis-stimulating molecules on its surface. Interleukin 2 has been considered in research as an anti-cancer drug. Interleukin 4 (IL-4) is of great importance in the process of developing an allergic reaction. It has a broad effect and stimulates many different cells of the immune system. It is produced by basophils, mast cells and Th2 lymphocytes. Due to the stimulation of monocytes and macrophages and the induction of the secretion of pro-inflammatory cytokines, IL-4 is directly and indirectly involved in the formation of an inflammatory focus. Its presence stimulates the activity of macrophages and monocytes. IL-4 is involved in the formation of an inflammatory focus. Positive effect on the production of cytokines stimulating hemopoiesis. An increase in the concentration of interleukin 4 therefore stimulates hematopoietic processes. Interleukin 6 is a pleiotropic cytokine, affecting the innate immune system and acquired, but above all by activating the inflammatory response. Among many functions, the most important role of IL-6 is participation in immunological processes. It is the body's main pyrogen. Mobilization of the immune system to the anticancer defense effect of IL-6 is manifested in the activation of maturation megakaryocytes, bone marrow progenitor cells and growth inhibition cancer cells by increasing the lytic activity of NK lymphocytes and by an increase in the expression of MHC class II proteins. Interleukin-6 stimulates lymphocyte differentiation B targets immunoglobulin-releasing cells of various classes and stimulates lymphocytes antigen-recognizing T, acting synergistically with TNF to induce antitumor response.[18,19]

Aim

The aim of the study was to determine the effect of HYP-PDT on colorectal cancer cells in terms of the secretion of selected cytokines involved in the processes occurring in the tumor environment.

Material and methods

Cell culture

Human colorectal cancer cells SW480 and SW620 were used in the study. They come from the ATCC bank (American Type Cell Culture-ATCC LGC Limited. Queens Road, Teddington, Middlesex, TW11 0LY, UK). Two cell lines of adenocarcinoma colorectal cancer with varying degrees of malignancy were used in the experiments: • SW 480 (ATCC, cat. no. CCL-228)

from a 50-year-old Caucasian male with primary colorectal adenocarcinoma, expresses carcinoembryonic antigen (CEA), transformation factor TGF-β and oncogenes: *myc*, *myb*, *ras*, *fos*, *sis*, *p53*, *abl*, *ros*, *src*, and • SW620 (ATCC, Cat. No. CCL-227) from a 51-year-old Caucasian male with lymph node metastases from colorectal cancer expressing the carcinoembryonic antigen (CEA) of the following oncogenes: *myc*, *myb*, *ras*, *fos*, *sis*, *p53*, *abl*, *ros*, *src*. In the experiment, cells of the SW480 and SW620 lines were used (American-type culture of the ATCC collection. The growth medium of Leibovitz's L-15 culture fluid with the addition of 10% inactivated fetal bovine serum FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin was used to culture cells of the SW 480 and SW 620 lines. The culture was carried out in plastic bottles with an area of 25 cm^2 and 75 cm^2 in a culture medium and then incubated at 37 degrees C in 100% air humidity without carbon dioxide. The cells adhered in the form of a monolayer, the growth medium was exchanged 2-3 times a week. Cells adhered to the bottom of culture bottles were detached from the substrate with 0.25% trypsin solution with 0.53 mM EDTA and incubated for 5-10 minutes at 37°C. After the trypsinization process, fresh growth medium was added to the cell suspension and centrifuged for 5 to 7 minutes (125xg). The cells prepared in this way were suspended in the culture medium, bringing the suspension to the appropriate density, experimentally determined for each line SW 480 500,000/ml, SW 620 250,000/ml, using a Burker hemocytometer, then cells of a certain density were adhered to 96-well plates and incubated at 37°C, in conditions of 100% humidity for 24 hours. Then, the culture medium was removed, rinsed once with PBS, and transferred to a room with limited access to light. Hypericin was added to the culture medium at concentrations of: 1 μM; 0.5 μM; 0.25 μM, 0.1 μM, photosensitizer was aspirated from the monolayer after one hour of incubation. The monolayer was washed 2 times with PBS.

Incubation of cells with Hyp

After 24-hour incubation of the test cells, the growth medium was removed, the cells were washed with calcium and magnesium free PBS solution. They were then treated with hypericin (Hypericin Calbiochem) at 0.1 μM, 0.25 μM, 0.5 μM and 1 μM for 1 hour. After incubation with Hyp, the medium was removed, the cells were washed with calcium and magnesium free PBS twice, and then complete growth medium was added.

Cellular uptake of hypericin

Cellular uptake of hypericin was assessed using an Olympus IX51 inverted microscope with a reflected fluorescence system (Olympus Corp) and a Color View III digital camera with Cell F imaging software (Soft Imaging System GmbH). In the experiment, the intra-cellular fluorescence intensity of Hyp was determined as a function of time with a flow cytometer (Becton Dickinson, LSR II) using the PerCP channel.

Exposure of cells to light

In the next stage of the experiment, cells were exposed to VIS visible light (400-750 nm) from an incoherent light source PDT TP-1 (Fig. 1) (Cosmedico Medizintechnik GmbH, Chwenningen, Germany) equipped with infrared filters and an orange filter, light emission in the 600-720 nm wavelength range was obtained. After adding the culture medium, exposure to light with an intensity of 5 J/cm^2 was started; 10J/cm^2; 20J/cm^2; 40J/cm^2. The photodynamic therapy lamp PDT TP1 (Cosmedico Medizintechnik GmbH, Schwenningen, Germany) was used for irradiation. To avoid hyperthermia with long exposure times, irradiation was conducted through a 270 mL water filter. The following doses were irradiated: 5J/cm^2, 10J/cm^2, 20J/cm^2, 40J/cm^2. Cultivation was then continued at 37°C, 100% humidity without CO_2 for 24 hours.

Cells viability

• Vital staining with 0.2% trypan blue in 0.85% NaCl solution. Trypan blue diluted 1:1 was added to the cell suspension on the glass slide after 3 minutes for the percentage of viable cells that stained white and dead cells that stained blue. Cells stained with trypan blue were counted in the automated counter BioTek Instruments.

• Mitochondrial dehydrogenase measurement – MTT test. After that, the cell viability was assessed with the MTT and LDH test. Cell viability was assessed by the 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM (MTT) bromide conversion method (Sigma-Aldrich, St. Louis, MO, USA). The principle of the method is to assess the ability of living cells to convert the yellow, water-soluble MTT tetrazolium salt into the colored blue formazan under the influence of the osido-reductases present in the mitochondria (Tetrazole Succinate Reductase) and the cytoplasm. The color intensity of the water-insoluble formazan is proportional to the number of viable cells. After culturing, the supernatant from the superadhered cells was removed and then supplemented with culture medium and MTT was added – the final concentration was 1.2 mM, cultured for 4 hours. The supernatant was removed and the formed formazan was extracted with DMSO. MTT was determined in 96-well polypropylene plates (Corning, NY, USA). The formazan absorbance was measured with a Biotek Eon™ microplate spectrophotometer at a wavelength of 550 nm. Measurements were replicated 6 times in 2 independent experiments for each hypericin concentration and light intensity.

Concentration IL-2, IL-4 and IL-6 assessment

The following concentrations in the tested cell culture were analyzed: interleukin

2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6). The concentration of the tested parameters was determined using two types of kits Bio-Plex Pro™ Assay (according to the above-mentioned list of determined parameters) and apparatus Bio-Plex Suspension Array System (BIO-RAD Laboratories Inc, Hercules, CA, USA). Experiment performed in accordance with the kit manufacturer's procedural recommendations. The Bio-Plex system allows the simultaneous determination of up to 100 cytokines in one well 96-well plate for 3 hours in 50 µl of cell culture supernatant. The method is similar to the ELISA enzyme immunoassay technique. Opposites and directed against a specific cytokine are covalently bound on fluorescent dye-encoded polystyrene beads with a diameter of 5.6 µm. Conjugated specific antibodies the bead reacts when incubated with a sample containing a specific cytokine at an unknown concentration. Then, specific detection antibodies are biotinylated, which during the next one incubation binds to other epitopes of the cytokines assayed than coated antibodies on the balls. After carrying out a series of successive washings, in order to remove unbound known antibodies, a complex of streptavidin with phycoerythrins is added to the reaction system, which binds to biotinylated detection antibodies. After another incubation and series washed, the beads with complexed cytokines are resuspended in the appropriate buffer and then introduced into the Bio-Plex instrument for fluorescence reading corresponding to the concentration of individual cytokines assayed. Concentration of each determined cytokine concentration is automatically calculated by the Bio-Plex Manager software based on the domain of the relevant standard curve.

Statistical methods

Measurement results were presented as mean and standard deviation (SD). Statistical significance was determined using Student's t-test (Microsoft Office, Warsaw, Poland). The obtained results were presented in a descriptive form and as tables and graphs. The significance level was set at < 0.05. Graphs contain a description of the coordinate axes and the study groups, a description of the data, numerical values and a graphical representation of standard deviations and statistical significance for the groups compared. The tables show mean values, standard deviation and level of statistical significance measured by Student's t-test. Distribution of variables was checked using the Shapiro-Wolf test.

Results

The levels of the analyzed progression factors

IL-2 concentration was determined using photodynamic therapy in light doses of 1.5 and 10 J/cm^2 and hypericin in concentrations of 0.25 and 0.5 µM. The use of Hyp-PDT did not affect the concentration of IL-2 secreted by SW480 and SW620 cells (Fig. 1 and 2).

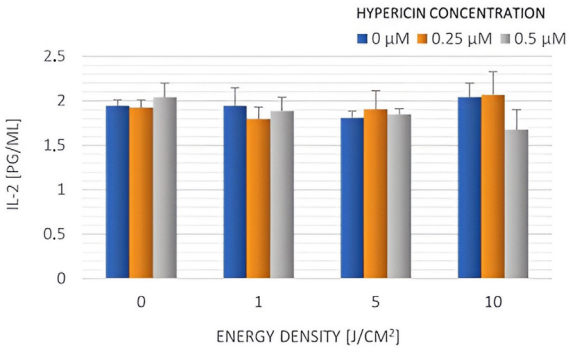

Fig. 1. Diagram presenting the concentration of IL-2 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

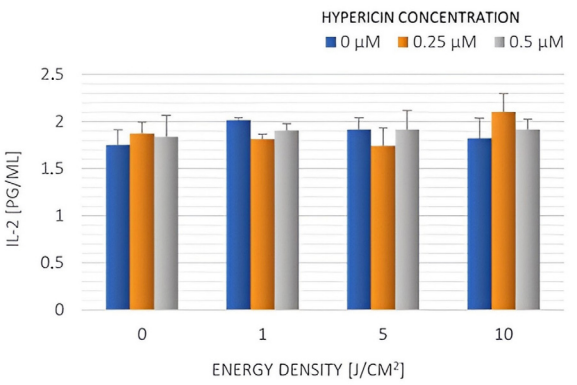

Fig. 2. Diagram presenting the concentration of IL-2 in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

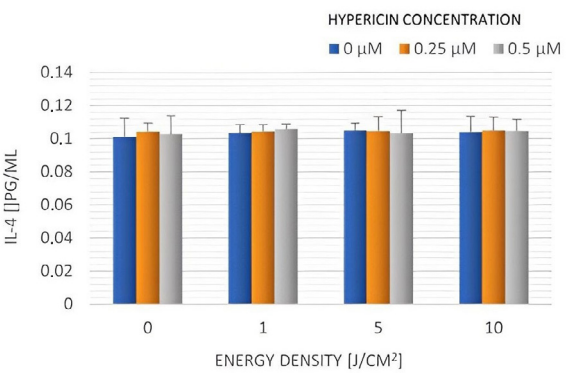

Fig. 3. Diagram presenting the concentration of IL-4 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

IL-4 concentration was determined using photodynamic therapy in light doses of 1.5 and 10 J/cm^2 and hypericin in concentrations of 0.25 and 0.5 µM. The use

of Hyp-PDT did not affect the concentration of IL-4 secreted by SW480 and SW620 cells (Fig. 3 and 4).

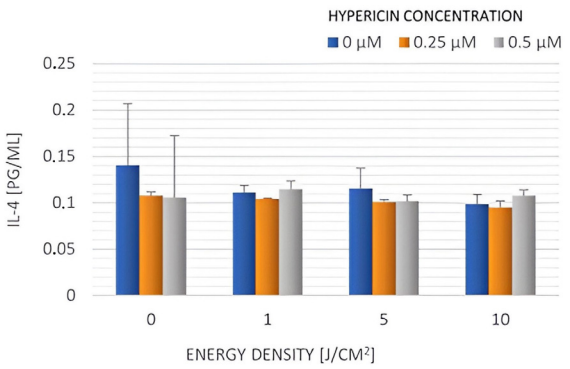

Fig. 4. Diagram presenting the concentration of IL-4 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

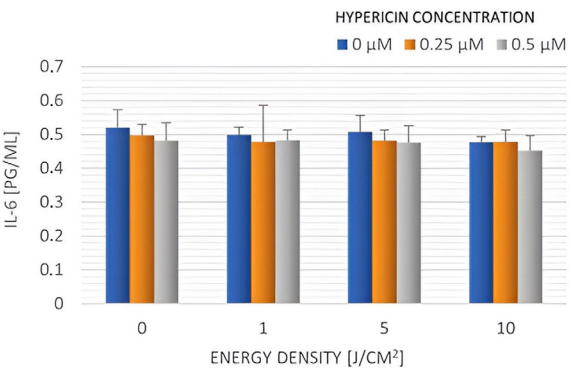

Fig. 5. Diagram presenting the concentration of IL-6 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses.

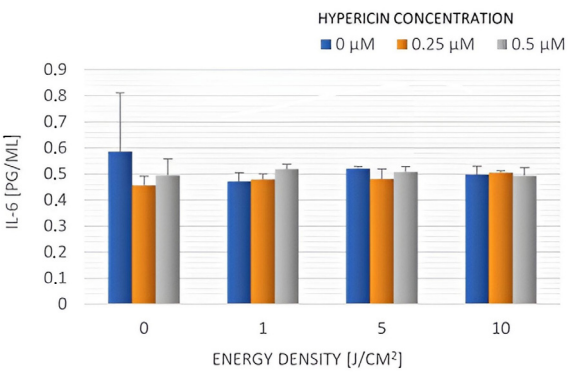

Fig. 6. Diagram presenting the concentration of IL-6 in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

IL-6 concentration was determined using photodynamic therapy in light doses of 1.5 and 10 J/cm^2 and hypericin in concentrations of 0.25 and 0.5 μM. The use of Hyp-PDT did not affect the concentration of IL-6 secreted by SW480 and SW620 cells (Fig. 5 and 6).

Discussion

The action of photodynamic therapy (PDT) is related not only to the direct effect cytotoxic (through necrosis and apoptosis), indirect, using vascular damage, but also immunomodulatory affecting the activity secretion of both immune cells and cancer cells.[17-20] The synergism the cooperation of these mechanisms results in remission in clinical trials not only the primary lesion, but also distant metastases.[21-25] PDT is effective, selective and minimally invasive, that's the above attributes hide possibility its underperformance due to limited penetration light to the tumor tissue. Therefore, the fundamental question remains as to how PDTs may act on the activation of secreting tumor cells that have not undergone cytotoxic action and, thanks to their activity, may cause recurrence of local and distant metastases through auto- and paracrine secretion of active cytokines, growth factors and pro-angiogenic substances.[26-31] The modern cancer treatment strategy is based on knowledge of cell biology cancer, including its secretory activity, especially the factors determining progression diseases. The author determined a necrosis of induced colorectal cancer cells with cytotoxic doses of Hyp PDT and the effect of therapy used in sublethal doses influences the secretion of cells related to the secretion of responsible factors for growth, invasion, angiogenesis, and metastasis. Determination of biomarkers for colon cancer and the influence of photodynamic therapy with Hyp on their secretion may explain the mechanism of tumor progression and determine if Hyp-PDT has a regressive or progressive effect on the remaining colon cancer cells, which have not been destroyed. Interleukins play an important role in cancer progression. Activate them detaching cells from the cancerous tumor, stimulate the production of VEGF by cells cancer, which induces their proliferation and angiogenesis. Research described in the in vitro and in vivo prove that photodynamic therapy affects cell adherence, especially stimulates the process of neutrophils adhesion to cells, also with the participation 2-integrins. Interleukin-6 - through its pleiotropic action - participates in the regulation of many processes, often causing opposite effects. On the one hand, IL-6 takes active participation in the mobilization of the immune system for anti-cancer defense through activation maturation of megakaryocytes, bone marrow progenitor cells, increased activity natural killer cells and by increasing the expression of MHC class II proteins.[31-32]

Taking into account this aspect of IL-6 activity, no differences in IL-6 concentration caused by the Hyp-

PDT may imply unfavorable, in terms of anticancer defense. On the other hand, the participation of IL-6 has been proven in the malignant transformation of tissues characterized by chronic inflammation, and this process very often it is the cause or a phenomenon that accompanies gastrointestinal cancers.[33] The autocrine production of IL-6 by tumor cells reveals its pejorative the nature of the action, manifested in the acceleration of tumor growth, inhibition of the process apoptosis of cancer cells, induction of angiogenesis and participation in the formation metastases. The author showed in the conducted experiment both lines of cancer cells large intestine: SW480 and SW620 produce IL-6. The results of this study confirm earlier reports of IL-6 secretion by colorectal cancer cells and cells many other cancer lines, e.g. breast cancer.[34,35] In the conducted experiment, the author did not show the effect of HYP PDT on the secretion IL-2, IL-4 and IL-6 via both SW480 and SW620 lines. Contrary to our results Jenny Lou et al. found a 46.64-fold and 61.33-fold increase in IL-6 after repeated photodynamic therapy (R-PDT) using porphyrin lipoprotein (PLP) as a photosensitizer.[36] This effect of the R-PDT and combination R-PDT + αPD-1 relative to PBS respectively, suggesting broad innate immune activation. Kawczyk-Krupka et al. demonstrated, apart from the production of IL-6 by both colon cancer lines, cells of the SW620 line react to the use of light energy alone – increased secretion IL-6, while the application of ALA PDT suppresses the release of this cytokine by cells colorectal cancer line SW620, but does not affect its secretion by cells line SW480. ALA PDT-induced IL-6 reduction may work regressively on colorectal cancer cells, which implies additional benefits of using photodynamic therapy in gastrointestinal cancers.[37] Author concluded that the cytotoxic effect of PDT is supported by an additional mechanism that stops proliferation and migration processes and colorectal cancer angiogenesis, which are coordinated by IL-6. In another study Kawczyk-Krupka et al. reveal that PDT performed in hypoxia-like condition in vitro not only effectively destroy malignant tissue, but also used in sublethal dose can develops its anticancer activity through the reduction of IL-6 and IL-10 secretion.[38] In our study we achieved also in certain parameters a cytotoxic effect, however, when using sublethal doses, we did not notice the effect of Hyp-PDT on the secretion of interleukins. Kaleta-Richter et al. described that based on the identification of immunological cancer biomarkers, the therapy of combining various forms of treatment, including immunotherapy and PDT, may be a justified strategy for colorectal cancer treatment that focuses on individualized comprehensive therapy.[39] Researcher pronounced that after Hyp PDT there was a statistically significant amplification of IL-8 secretion during Hyp-PDT in the SW620 cell line and a statistically significant decrease in IL-8 during Hyp-PDT in the SW480 cell line, with no statistically significant differences in IL-10 concentration following Hyp-PDT in the SW480 or in the SW620 cell.[40] Thus, as in our experiments, the effect of Hyp -PDT on cytokine secretion may have different effects, and it may not have any effect on cytokine secretion.

We confirmed that Hyp-PDT can eliminate a primary tumor not only via cytotoxic effects, but due to immunological mechanism. This influence depended not only of the physical condition of photodynamic therapy, but also on photosensitizer type.

Conclusion

Neoplastic diseases are a constant challenge of modern medicine. Due to the constantly increasing incidence and mortality, alternative methods of treatment are sought and improved. PDT as a method without serious side effects is gaining more and more popularity mainly in pediatric studies in children with various types of cancer. PDT with Hyp- successfully inhibits tumor growth via apoptosis and necrosis in various models and clinical trials. What's more it is much cheaper in comparison with the currently used photosensitizers. The photodynamic effect of Hyp is directed at various subcellular organelles, primarily the mitochondria and the endoplasmic reticulum complex. Depending on the conditions of drug administration and light PDT effect leads to cell death which occurs by induction of necrosis, apoptosis, or autophagy-related cell death. Exposure of colorectal cancer cells to Hyp-PDT in sublethal doses did not affect the progression of tumor cells dependent on the release of IL-2,4,6, which is related to the lack of differences in IL concentration for both the SW480 and SW620 cell lines after PDT, compared with controls. In the conducted experiment, the author did not show the effect of Hyp- PDT on the secretion IL-2, IL-4 and IL-6 via both SW480 and SW620 lines, nevertheless, further experiments should be performed, not only in vitro but also in vivo, to fully evaluate the above immunological effect of photodynamic therapy.

Declarations

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Author contributions

Conceptualization, A.M., A.K-K., D.A. and Z.P.C.; Methodology, A.M., A.K-K. and G.C.; Validation, A.M., A.K-K., D.A. and Z.P.C.; Formal Analysis, A.M., A.K-K. and Z.P.C.; Resources, A.M. and D.A.; Writing – Original Draft Preparation, A.M., A.K-K., D.A. and Z.P.C.; Writing – Review & Editing, A.M., A.K-K., D.A., G.C. and Z.P.C.

Conflicts of interest
The authors declare no conflict of interest.

Data availability
Data available on request from the authors.

Ethics approval
Not applicable.

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ORIGINAL PAPER

Effects of hypericin-mediated photodynamic therapy on GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells *in vitro*

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ABSTRACT

Introduction and aim. Photodynamic therapy with hypericin (HYP-PDT) is gaining importance as a potential treatment method for malignant neoplasms. The following study investigated whether HYP-PDT influences the secretion of certain factors of colon cancer cells in vitro.

Material and methods. Two colon cancer cell lines were used in this experiment: SW480 and SW620. The cells were properly prepared and then treated with photodynamic therapy with hypericin at concentrations of 0.25 μM and 0.5 μM and irradiation at the doses of 1 J/cm^2, 5 J/cm^2 and 10 J/cm^2. After using HYP-PDT, changes in the concentrations of four factors: GM-CSF, MIF, VCAM-1 and ICAM-1 were analyzed in the test tubes.

Results. In the case of SW480 cells: a notable decrease in GM-CSF, MIF, VCAM-1 and ICAM-1 secretion was noted after HYP-PDT. In the case of SW620 cells, after HYP-PDT: no effect on GM-CSF secretion was noted; it inhibited the secretion of VCAM-1 and MIF and increased the secretion of ICAM-1.

Conclusion. Photodynamic therapy with hypericin shows immunomodulatory potential in an in vitro cell line experiments. This may indicate its possible ability to modify the course of malignant tumors and therefore requires further research.

Keywords. colorectal cancer, hypericin, photodynamic therapy

Introduction

Malignant tumors are the second leading cause of death, after cardiovascular dis-eases, for people worldwide. In 2020, nearly 2 million cases of colorectal cancer were diagnosed and 935,173 deaths from the disease were registered. Colon cancer ranks as the third most common malignancy in men after lung and prostate cancer and second after breast cancer among women. Thorough understanding of the biology and immunology of tumors and the mechanisms of cancer progression is crucial for the treatment process based on the selection of an appropriate therapy for a given patient, taking into account the total risk, type of molecular changes in tumour cells and effective biotransformation of the applied drug in a given patient. Knowing that the growth of cancerous tumors is controlled by a number of processes occur-

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ring in the tumour niche as well as systemically, it is possible to conduct research using substances capable of influencing immunological processes. Tumour progression factors include many physiologically occurring proteins such as chemokines, cell adhesion factors, vascular growth factors and many other factors involved in promoting tumour growth, cell proliferation and angiogenesis. Furthermore, genetic mutations and disruption of many cell signalling pathways are crucial in tumour genesis and metastasis. These mechanisms lead, among others, to chronic inflammation, which within the tumour mass, together with local immune system activity, are elementary factors in tumour development and disease progression.[1,2]

Photodynamic therapy (PDT), which has already been proven effective in the treat-ment of skin, pancreatic and bladder cancer, as well as non-cancerous diseases such as autoimmune disorders (RA, SLE, psoriasis), acne vulgaris, macular degeneration, and infectious diseases, is one of the promising methods in cancer treatment.[3-13] PDT is a relatively inexpensive and simple procedure, burdened with only few side effects and well tolerated by patients.[14] It is based on an intracellular reaction occurring as a result of an interaction between three factors: a photosensitive substance, light and oxygen. As a result of absorption of light of a specific wavelength by a photosensitive sub-tance, its transition from the basic state to the excited triplet state takes place, which results in the transfer of a hydrogen atom or an electron from the photosensitizer to the substrate with formation of free radicals that react with oxygen. As a result, reactive oxygen species are formed (hydrogen peroxide, superoxide anion radicals, hydroxyl radicals) - type I reaction, or reaction of photosensitizer with triplet oxygen leading to singlet oxygen - type II reaction. PDT has a direct cytotoxic effect leading to cell necrosis or apoptosis, as well as stimulating the immune system and promoting inflammation.[15-20] Effects on the secretory function of tumour cells, thus proliferation, angiogenesis and metastasis formation, is another interesting potential action of PDT.[21-24] Among many photosen-sitizers used in PDT, hypericin (HYP), which is a natural plant pigment present in and extracted from certain plants of the genus Hypericum, most commonly St. John's Wort, is of great interest. Due to its chemical structure, it is classified as an anthraquinone derivative and belongs to naphthodianthrones. The chemical structure of hypericin is shown in Figure 1.

Hypericin is characterized by intensive light absorption at wavelengths above 550 nm, higher affinity for tumour tissues compared to normal tissue, insignificant toxicity in the dark and induces a strongly photocytotoxicity effect in tumour cells, with the strength of this cytotoxicity depending on the concentration of hypericin and the intensity of light and the presence of oxygen. To date, the mechanisms underlying the selective accumulation of hypericin in tumour tissue have not been fully understood, nor have all the mechanisms of its anticancer effects been clearly defined.[25-26] The first reports on the photo-sensitizing properties of hypericin and the possibility of using it in photodynamic therapy and diagnostics date back to the 20th century.[27-28] The first local application of hypericin as a photosensitizer in photodynamic therapy was described in 1996 in a patient with pleural mesothelioma.[29] Promising effects in terms of inhibition of proliferation and destruction of tumour cells following hypericin-mediated photodynamic therapy have been observed among others in glioma, cutaneous melanoma, leukemias, breast, liver, and colorectal cancer.[4,30-37]

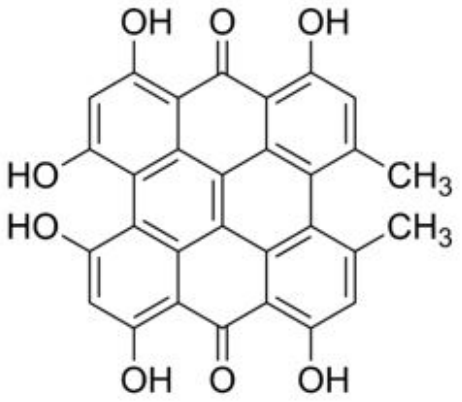

Fig. 1. Hypericin – chemical structure

Aim

Based on literature, author focused on determining the effects of HYP-PDT on colon cancer cells – showing the effectiveness of the therapy after using cytolethal doses and determination of the influence on selected molecular markers responsible for progression (growth, invasion and metastatic tumor) secreted by the cancer cells that have not been destroyed by HYP-PDT.

Material and methods

SW480 and SW620 cell lines

Two colorectal cancer cell lines SW480 and SW620, purchased from American Type Cell Culture (ATCC LGC Limited. Queens Road, Teddington, Middlesex, TW11 0LY, UK) in a frozen state, were used for the experiment. The SW480 cell line was derived from a primary adenocarcinoma in a 50-year-old Caucasian male. The cells were classified as type B in the Dukes classification, and show expression of carcinogen-associated antigen (CEA), transforming factor TGF-P and the oncogenes *myc, myb, ras, fos, sis, p53, abl, ros, Sr*. The cell line SW620 was obtained from the same man, but the cells were collected one year later from a metastatic colorectal cancer lymph node. These cells are characterized by metastatic activity. They were classified as

Dukes' type C, and show expression of carcinogen-associated antigen (CEA) and the oncogenes: *myc, myb, ras, fos, sis, p53, abl, ros, src*.

Cell culture

The SW480 and SW620 cells were cultured in Leibovitz's L-15 medium supplement-ed with 10% inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cultures were maintained in 25 and 75 cm^2 plastic bottles and incubated at 37°C with 100% humidity in a carbon dioxide-free atmosphere. The cells were grown in monolayer as adherent cells. The growth medium was changed 2-3 times per week. The prepared cells were re-suspended in culture medium bringing the suspension to the de-sired density, calculated experimentally beforehand for both cell lines. The determined density was $5x10^5$/ml for SW480 cells and 2.5 x 10^5/mL for SW620 cells. The cell suspension was then loaded into 96-well plates and incubated at 37°C under 100% humidity for 24 hours.

Incubation of cells with hypericin

After 24-hour incubation of the test cells, the growth medium was removed, the cells were washed with calcium and magnesium free PBS solution. They were then treated with hypericin (Hypericin Calbiochem) at 0.1 μM, 0.25 μM, 0.5 μM and 1 μM for 1 hour. After incubation with hypericin, the medium was removed, the cells were washed with calcium and magnesium free PBS twice, and then complete growth medium was added.

Exposure of cells to light

In the next stage of the experiment, cells were exposed to VIS visible light (400-750 nm) from an incoherent light source PDT TP-1 (Cosmedico Medizintechnik GmbH, Schwenningen, Germany) equipped with infrared filters and an orange filter, light emis-sion in the 600-720 nm wavelength range was obtained. The additional filter in the spec-tral range of 600-720 nm wad used for emission detection. Radiation was applied at doses of 1 J/cm^2, 5 J/cm^2 and 10 J/cm^2, and a power density of 1.5 mW/cm^2. The exposures were conducted through a water filter with a 1.5 cm thick water layer to eliminate the risk of developing cell hyperthermia with long exposure times. After exposure, the culture of the test cells was continued at 100% humidity and 37°C, without carbon dioxide for 24 hours.

Assessment of metabolic activity (viability) of the tested cells by MTT assay

The viability of the investigated cells was determined for four different concentrations of hypericin: 0.1 μM, 0.25 μM, 0.5 μM, 1 μM and three doses of surface energy density for exposure: 1 J/cm^2, 5 J/cm^2 and 10 J/cm^2. Cellular viability was verified during the subsequent stages of the experiment by vial staining of the cells with 0.25% trypan blue solution in 0.85% NaCl. Photosensitized and irradiated samples were compared with unexposed controls incubated without photosensitizer, incubated with a photosensitizer and unexposed or incubated without photosensitizer and exposed. Cell viability was determined by the MTT assay (with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which measures mitochondrial dehydrogenase activity. MTT was found to be membrane impermeable. These and other results suggest that MTT is taken up by cells through endocytosis and that reduced MTT formazan accumulates in the endosomal/lysosomal compartment and is then transported to the cell surface through exocytosis. Only living cells have the ability to reduce the yellow 3-[4,5- dimethylthia-zol-2-yl]-2,5-diphenyltetrazolium bromide to water-insoluble formazan. The reaction takes place in the cytoplasm with the participation of $NADH/NAD^+$, $NADPH/NADP^+$. A diagnostic kit from Sigma Chemical Co. (St. Louis, MO, USA) was used to determine mitochondrial dehydrogenase activity. After removal of the supernatant, culture medium with MTT at a concentration of 0.5 μM was added to the adhered cells. After removal of the medium, the resulting water-insoluble formazan was extracted with 100% DMSO. The microplates were shaken for 10 min and centrifuged. Then 150 μL of the supernatant with dissolved formazan was transferred to a 96-well polypropylene flat bottom plate. The absorbance of the extracted formazan was deter-mined at 550 nm vs. DMSO using an ELx 800 microplate reader (BioTek, Winooski, USA). From the results obtained, the percentage of living cells (viability) was calculated according to the formula:

$$\text{Viability\%} = Ab \times 100\ \% / Ak$$

where:
Ab – absorbance of test sample; Ak – absorbance of control sample

Cellular uptake of hypericin

Cellular uptake of hypericin was assessed using an Olympus IX51 inverted microscope with a reflected fluorescence system (Olympus Corp) and a Color View III digital camera with Cell F imaging software (Soft Imaging System GmbH). 100 % DMSO was used in the stock solution of hypericin and hypericin is then dissolved in cell culture media at 0.1%. In the experiment, the intra-cellular fluorescence intensity of Hyp was deter-mined as a function of time with a flow cytometer (Becton Dickinson, LSR II) using the PerCP channel. A 488 nm excitation laser was used to stimulate Hyp fluorescence, and Hyp fluorescence emission was recorded at 600 nm.

The levels of colorectal cancer progression factors

The levels of four different cancer progression factors: granulocyte-macrophage colony stimulating factor (GM-

CSF), macrophage migration inhibitory factor (MIF), vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) were analyzed in the cell culture filtrates. The levels of the tested parameters were determined using the Bio-Plex Pro Assay kit based on xMAP suspension technology (Bio-Rad Laboratories Inc, USA). Assays were performed for control samples without hypericin and light exposure, with 0.25 μM and 0.5 μM hypericin without light exposure, without hypericin and irradiated with light at 1 J/cm^2, 5 J/cm^2 and 10 J/cm^2, and test samples for 0.25 μM and 0.5 μM hypericin and light exposure at 1 J/cm^2, 5 J/cm^2 and 10 J/cm^2. Measurements were performed according to the Bio-Plex kit manufacturer's instructions, starting 24 hours after illumination of the test samples. First, cell culture supernatants were incubated for one hour with reagents containing magnetic beads conjugated with antibodies corresponding to the progression factors studied. After the incubation and washing period, biotinylated detection antibodies were added and samples were further incubated for 30 minutes. Magnetic beads were then washed and streptavidin-phycoerythrin (PE) solution was added to each well for 10 minutes. Then, after washing with buffer to remove un-bound streptavidin-PE, the beads were resuspended in buffer. Beads bound to each cytokine were analyzed in a Bio-plex Array Reader (Bio-Plex 200 System). Fluorescence intensity and cytokine concentrations were assessed using BioPlex Manager software. Standard curves for each cytokine were calculated using a reference cytokine sample provided with the kit. For each type of test sample, tests were performed in triplicate.

Statistical methods

Measurement results were presented as mean and standard deviation (SD). Statistical significance was determined using Student's t-test (Microsoft Office, Warsaw, Poland). The obtained results were presented in a descriptive form and as tables and graphs. The significance level was set at < 0.05. Graphs contain a description of the coordinate axes and the study groups, a description of the data, numerical values and a graphical representation of standard deviations and statistical significance for the groups compared. The tables show mean values, standard deviation and level of statistical significance measured by Student's t-test.

Results

The presence of photosensitizer in the cells

The presence of hypericin in the cells was assessed using an inverted fluorescence microscope. Figures 2 and 3 show photographs taken for both cell lines, showing the fluorescence of the analyzed cells and thus the intracellular accumulation of hypericin. For proper imaging of the cells, a fluorescein isothiocyanate (FITC) filter was used at 200x magnification. Additionally, the extent of cellular hypericin uptake was assessed using a flow cytometer (Becton Dickinson, LSR II) in the PerCP channel by measuring the fluorescence intensity induced by cellular exposure to radiation from a 488 nm laser.

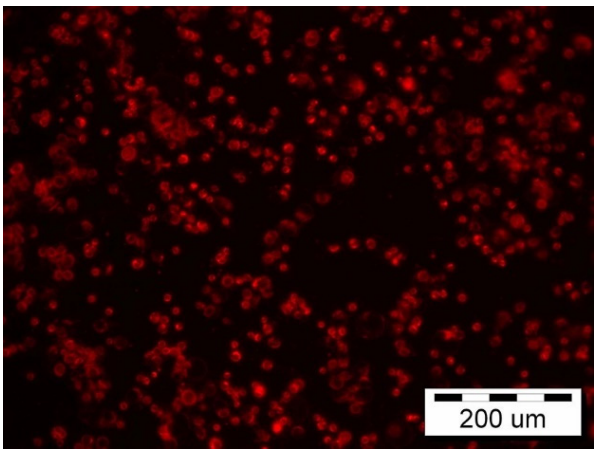

Fig. 2. Fluorescence microscope image of SW480 cell line. Red fluorescence indicates the presence of hypericin

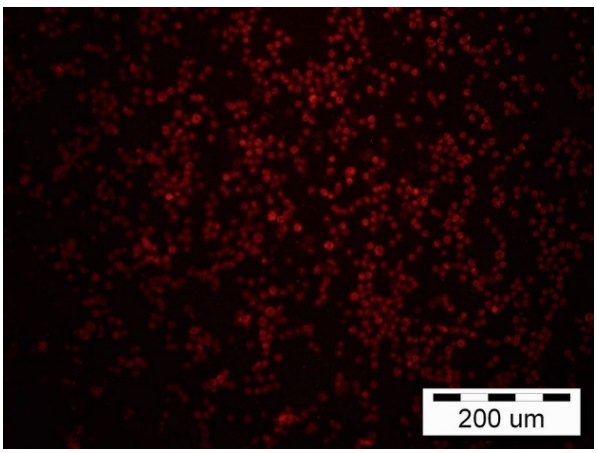

Fig. 3. Fluorescence microscope image of SW620 cell line. Red fluorescence indicates the presence of hypericin

Figure 4 shows graphs representing the escalation of fluorescence of hypericin-treated cells and the correlation between fluorescence intensity and the time and photosensitiser concentration.

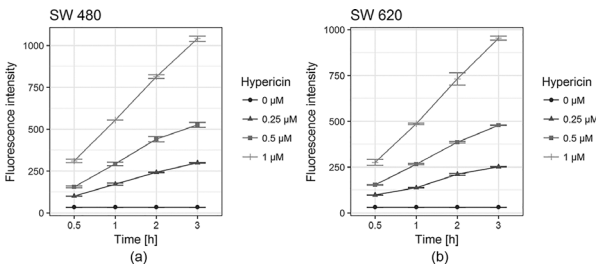

Fig. 4. Diagrams presenting the dependence of the intensity of fluorescence of the tested cells on the time of their incubation with hypericin and on the concentration of photosensitizer: a) for SW480 cells; b) for SW620 cells

Viability of the cells – MTT assay for measuring cell metabolic activity

SW480 cell line

Cell viability in the SW480 cell line after light exposure at 1 J/cm², 5 J/cm² and 10 J/cm² at different doses of hypericin showed no significant differences compared to the control. No significant cytotoxic effect was observed. The results are shown in Figure 5.

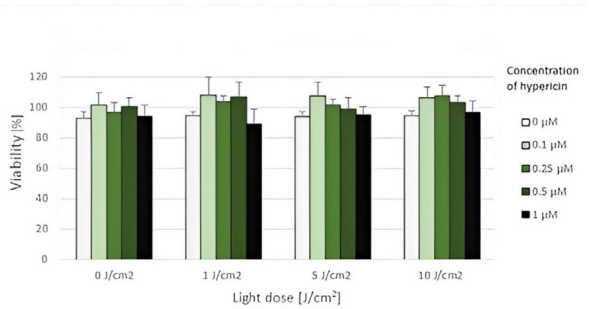

Fig. 5. Diagram presenting the viability of SW480 cell line assesed by MTT test, after HYP-PDT with concentrations of hypericin: 0.1 µM, 0.25 µM, 0.5 µM and 1 µM and light doses: 1 J/cm², 5 J/cm² and 10 J/cm²

SW620

Cell viability of the SW620 cell line after light exposure at doses of 1 J/cm², 5 J/cm² and 10 J/cm² and application of hypericin remained within 100% in most samples. A light dose of 1 J/cm2 and a hypericin concentration of 1 µM led to a reduction in cell viability to 81.88% ($p<0.01$). A 10 J/cm2 dose with 1 µM hypericin resulted in a decrease in viability to 55.57% ($p<0.01$). In view of the significant cytotoxic effect of hypericin at 1 µM, this concentration was not used to assess the secretion of the progression factors tested, as living cells are required for this analysis. The results are shown in Figure 6.

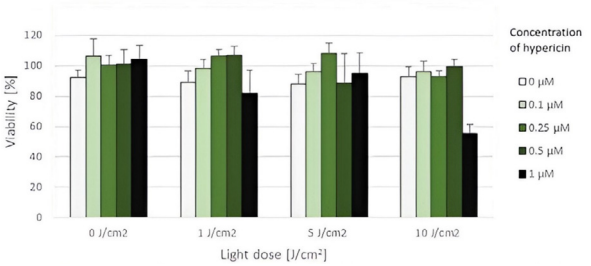

Fig. 6. Diagram presenting the viability of SW620 cell line assesed by MTT test, after HYP-PDT with concentrations of hypericin: 0.1 µM, 0.25 µM, 0.5 µM and 1 µM and light doses: 1 J/cm², 5 J/cm² and 10 J/cm²

The levels of the analyzed progression factors

In this study, the cytokines GM-SCF, MIF, VCAM-1 and ICAM-1 were selected for analysis due to their relatively high concentrations in cancer cell supernatants and their potential role in the process of tumor development. Bio-Plex Pro Assay kit was used to determine the concentration of individual cytokines released from the examined cancer cell lines treated with photodynamic therapy with hypericin. The results of the experiment were presented using descriptive statistics, tables and graphs. In this study it has been observed that light alone and hypericin alone also can affect cytokine release by cells. Statistically significantly lower concentration of cytokines were noted after light doses of 5 and 10 J/cm². A significant effect of hypericin alone was observed in SW480 cells. At the hypericin concentration of 0.25 µM lower concentrations of ICAM-1 were noted, while at the concentration of 0.5 µM lower concentrations of GM-CSF and ICAM-1 were noted. Moreover, the two cell lines were compared by taking control samples (without hypericin and without irradiation) to determine whether cells from primary colorectal cancer (SW480) and metastatic cells (SW620) secrete cytokines to a similar extent (Tab. 1–4).

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm² and hypericin in concentrations of 0.25 and 0.5 µM. The use of Hyp-PDT did not affect the concentration of GM-CSF secreted by SW620 cells. PDT caused a statistically significant decrease in GM-CSF secretion in SW480 cells compared with control ($p<.05$). The results of the tested samples are shown in Figure 7 for the SW480 cell line and in Figure 8 for the SW620 cell line. Moreover, the study of control samples showed that SW480 cell line secrete GM-CSF in higher concentrations ($p<0.02$) than SW620 cell line. GM-CSF results are given in pg/ml. The results are shown in Table I.

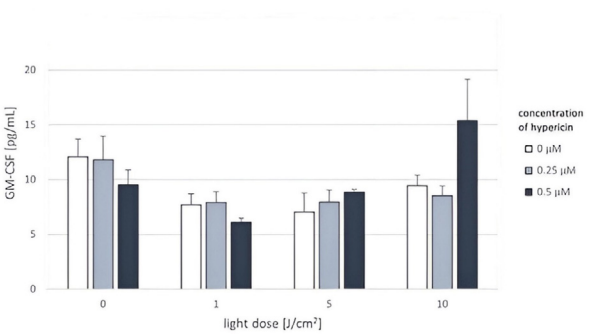

Fig. 7. Diagram presenting the concentration of GM-CSF in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

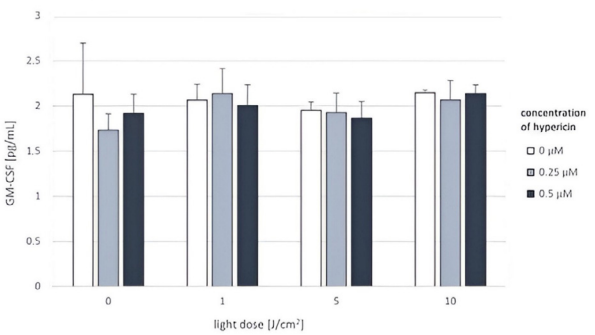

Fig. 8. Diagram presenting the concentration of GM-CSF in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

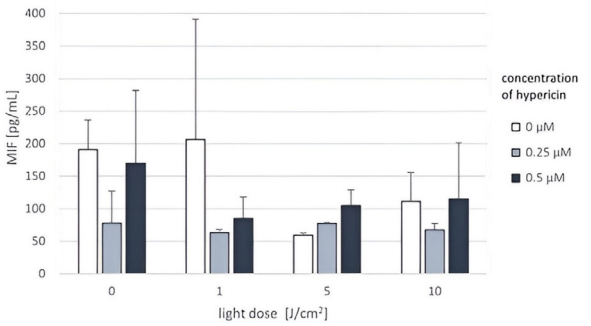

Fig. 9. Diagram presenting the concentration of MIF in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

Table 1. Descriptive statistics showing the difference in the level of GM-CSF secretion between the SW480 and SW620 cell lines in the control samples

GM-CSF				Statistical analysis	
Cell line	Numer of trials	Average concentration (pg/ml)	SD	Compared cell lines	The Mann-Whitney U test
SW480	5	11.37	2.12	**SW480 and SW620**	< 0.02
SW620	5	2.13	0.57		

Macrophage migration inhibitory factor (MIF)

MIF concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm^2 and hypericin in concentrations of 0.25 and 0.5 μM. After Hyp-PDT treatment, lower MIF levels were found in SW480 cell line ($p<0.05$) compared to controls. In SW620 cell line, MIF secretion decreased after Hyp-PDT at hypericin concentrations of 0.25 μM and 0.5 μM with light doses of 5 and 10 J/cm^2 ($p<0.05$). MIF concentration is given in pg/ml. The results are shown in Figure 9 for SW480 cell line and Figure 10 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that there is no statistically significant difference in MIF secretion between SW480 and GM-CSF cell lines ($p>0.05$). The results are shown in Table 2.

Table 2. Descriptive statistics showing the difference in the level of MIF secretion between the SW480 and SW620 cell lines in the control samples

MIF				Statistical analysis	
Cell line	Numer of trials	Average concentration (pg/ml)	SD	Compared cell lines	The Mann-Whitney U test
SW480	5	190.71	45.76	**SW480 and SW620**	> 0.05
SW620	5	148.88	5.20		

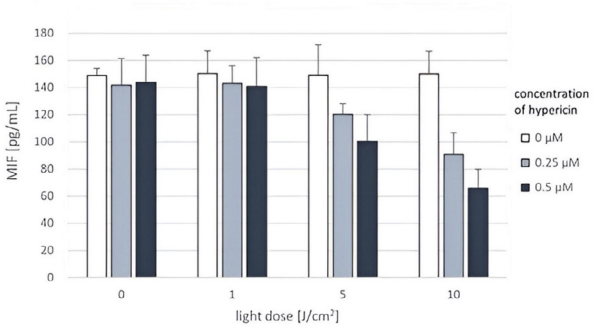

Fig. 10. Diagram presenting the concentration of MIF in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

Vascular cell adhesion molecule-1 (VCAM-1)

VCAM-1 concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm^2 and hypericin in concentrations of 0.25 and 0.5 μM. PDT caused a statistically significant decrease in VCAM-1 secretion in SW480 cells at all light doses and hypericin concentrations ($p<0.05$). For SW620 cells, there were significantly lower VCAM- 1 levels in the samples treated with photodynamic therapy with 0.25 and 0.5 concentration of hypericin and light doses of 5 and 10 J/cm^2 compared to controls. VCAM-1 concentration is given in pg/ml. The results are shown in Figure 11 for SW480 cell line and Figure 12 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that SW480 cell line secrete higher concentrations of VCAM-1 ($p<0.02$) than SW620 cell line. The results are shown in Table 3.

Intercellular adhesion molecule-1 (ICAM-1)

ICAM-1 concentration was determined using photodynamic therapy in light doses of 1, 5 and 10 J/cm^2 and

hypericin in concentrations of 0.25 and 0.5 μM. The use of PDT resulted in a statistically significant decrease in ICAM-1 secretion in SW480 cell line at 10 J/cm² light dose and 0.25 and 0.5 μM hypericin concentrations ($p<0.05$). A significant in-crease in ICAM-1 secretion

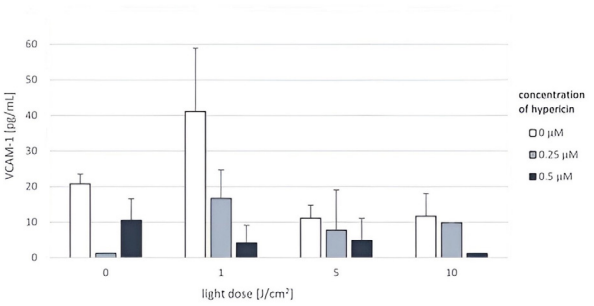

Fig. 11. Diagram presenting the concentration of VCAM-1 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

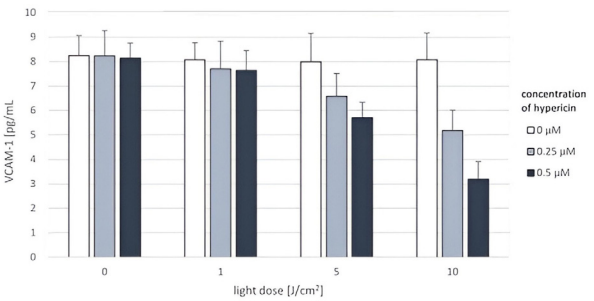

Fig. 12. Diagram presenting the concentration of VCAM-1 in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

Table 3. Descriptive statistics showing the difference in the level of VCAM-1 secretion between the SW480 and SW620 cell lines in the control samples

	VCAM-1			Statistical analysis	
Cell line	Numer of trials	Average concentration (pg/ml)	SD	Compared cell lines	The Mann- Whitney U test
SW480	5	20.72	2.78	**SW480 and SW620**	< 0.02
SW620	5	8.24	0.80		

in SW620 cells ($p<0.05$) was noted after light of 5 J/cm² alone and with Hyp at a concentration of 0.25 μM. ICAM-1 concentration is given in pg/ml. The results are shown in Figure 13 for SW480 cell line and Figure 14 for SW620 cell line. Moreover, the study of control samples (without light and hypericin) showed that SW480 cell line secrete higher concentrations of ICAM-1 ($p<0.02$) than SW620 cell line. The results are shown in Table 4.

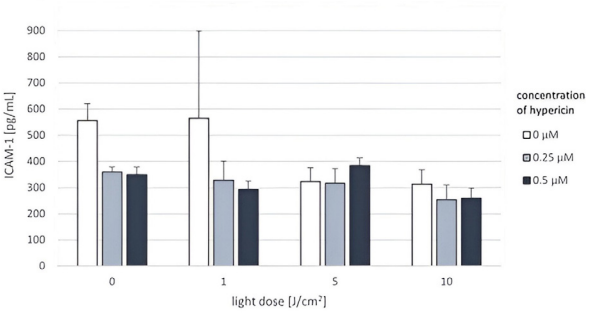

Fig. 13. Diagram presenting the concentration of ICAM-1 in the tested supernatants from SW480 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

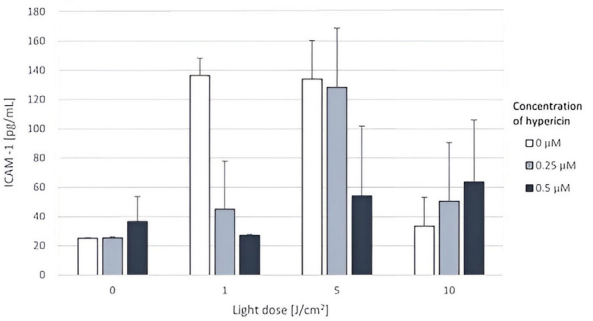

Fig. 14. Diagram presenting the concentration of ICAM-1 in the tested supernatants from SW620 cancer cells under certain conditions: without Hyp and light (control), after treatment with Hyp PDT at various concentrations and after irradiation at various doses

Table 4. Descriptive statistics showing the difference in the level of ICAM-1 secretion between the SW480 and SW620 cell lines in the control samples

	ICAM-1			Statistical analysis	
Cell line	Numer of trials	Average concentration (pg/ml)	SD	Compared cell lines	The Mann-Whitney U test
SW480	5	467.49	132.03	**SW480 and SW620**	< 0.02
SW620	5	35.08	22.33		

Discussion

The use of HYP-PDT in the treatment of colorectal cancer has attracted the interest of researchers for decades. Of particular interest is the immunological effect induced as a consequence of hypericin in photodynamic therapy. In the case of cancer therapy, the ability to influence cellular and immunological processes taking place in the tumour niche is extremely important. In this paper we publish for the first time the results

of a study on the effect of hypericin used in photodynamic therapy on in vitro GM-CSF, MIF, VCAM-1 and ICAM-1 secretion in colorectal cancer cells. GM-CSF is a haemopoietic cytokine whose primary function is to stimulate the growth of granulocytes and macrophages and to influence lymphocytes. In addition, GM-CSF promotes the differentiation of hematopoietic stem cells into dendritic cells and stimulates the growth and proliferation of many other cell types, including eosinophils, monocytes, neutrophils and keratinocytes. GM-CSF is produced and secreted by various groups of cells, including activated B cells, T cells, mast cells, endothelial cells, macrophages and fibroblasts, most commonly in response to inflammatory stimuli. In addition to its function as a hematopoietic growth factor, GM-CSF also has various functions in mature hematopoietic cells, including enhancing phagocytosis and production of pro-inflammatory cytokines, promoting leukocyte adhesion and chemotaxis, and promoting antigen presentation. Antigen-presenting cells play a key role in triggering the anti-tumour immune response. Professional primary antigen presenting cells are dendritic cells. GM-CSF stimulates the growth and activity of dendritic cells by effectively enhancing anti-tumour immunity.[38-39] In addition, GM-CSF modulates collagen metabolism of the extracellular matrix, can promote migration and proliferation of endothelial cells, thus participating in the process of angiogenesis. GM-CSF has also been shown to act as a tumour-derived factor that promotes cancer cell proliferation and migration in various solid tumors and cancer cell lines, thus promoting tumour growth and progression.[40] Constitutive expression and secretion of GM-CSF protein have been observed in some cancer cell research models, and elevated serum GM-CSF levels are considered a potential diagnostic and prognostic marker correlating with poor prognosis in colorectal cancer patients.[41-42] Tumour cells producing GM-CSF promote specific anti-tumour immunity by teaching CD4+ and CD8+ T lymphocytes to recognize circulating tumour-specific antigens, thereby inducing a systemic immune response. In addition to its immune system-stimulating functions, GM-CSF also has direct effects on tumour progression and invasion. In the pathomechanism of breast cancer, it has been shown that GM-CSF participates in the formation of tumour associated macrophages, thus participating in the positive coupling of tumour growth and metastasis formation, while reduced GM-CSF expression inhibits the metastatic process.[43] In our study, we recorded an evident reduction in GM-CSF secretion by colorectal cancer cells of the SW480 cell line, escalating with increasing HYP concentration and light intensity. Furthermore, we showed that SW480 cells secrete more GM-CSF than SW620 cells. A similar result regarding the inhibition of GM-CSF secretion was observed by Du et al., who studied the effect of HYP-PDT on nasopharyngeal carcinoma (NPC).[44] The aim of their study was to analyze the upregulation of matrix metalloproteinase-9 (MMP-9) expression in well-differentiated cells of NPC-derived HK1 cell lines under the influence of HYP-PDT and, among other things, they found that MMP-9 expression was reduced after HYP-PDT by inhibiting GM-CSF production. Colorectal cancer cells can independently synthesize MMP-9, which is involved in the processes of progression and metastasis by, among other things, degrading the extracellular matrix and affecting the formation of organelles associated with cell movement.[45] Moreover, elevated expression of the MMP-9 enzyme in colorectal cancer cells is associated with increased tumour aggressiveness and invasive potential.[46] Thus, HYP-PDT-mediated inhibition of GM-CSF secretion may, by reducing MMP-9 expression, significantly reduce tumour growth processes. Various mechanisms for the effect of GM-CSF on tumour progression have been postulated. Chen et al. identified the function of GM-CSF as a factor that promotes colon cancer progression by inducing epithelial to mesenchymal transition (EMT), which involves the transformation of cells through the loss of cell-to-cell adhesion capacity and through the breakdown of tight and gap junctions, allowing tumour cell expansion.[47]

MIF is a pro-inflammatory cytokine with multidirectional effects. Its main functions are stimulation of interleukin-6, interleukin-1β and TNF-α secretion and stimulation of MHC type II molecules expression on macrophages. Furthermore, MIF promotes cell proliferation, inhibits apoptosis and regulates immune cell processes. Many solid tumors overexpress MIF, which may be involved in regulating the production and expression of various factors, e.g. by increasing VEGF secretion to promote tumorigenesis.[48] The serum level of MIF in colorectal cancer patients and colorectal cancer cells is significantly higher than in healthy individuals and correlates with the presence and extent of metastasis and with an increased risk of metastases, so it may have some potential as a marker for the clinical diagnosis of liver metastases in colorectal cancer.[48,49] Li et al. demonstrated that the proinflammatory cytokine MIF secreted by infiltrating lymphocytes may contribute to the local progression and promotion of metastasis of nasopharyngeal carcinoma through the induction of MMP-9 in the indirect pathway. Furthermore, it may stimulate invasion of nasopharyngeal carcinoma cell lines in vitro, and infiltrating lymphocytes in NPC may be responsible for cancer cell invasion and metastasis.[49] The inhibition of the activity of the p53 protein, which as a transcription product of the TP53 anti-oncogene is involved in many cellular processes aimed at repairing damaged DNA or inducing cell apoptosis in response to DNA damage, is another tumour-promoting mechanism de-

scribed by MIF.[50] The loss of p53 protein function is among the most common turning points in tumorigenesis. Locally high MIF levels cause activation of T cells and enhance the activity of macrophages, which release oxygen radicals and nitric oxide in response to a stimulus. MIF is able to block the p53 response and inhibit nitric oxide-induced macrophage apoptosis.[50] In another study, Wilson et al. argued that MIF promotes intestinal cancer formation, through angiogenesis, and genetic deletion of MIF results in reduced tumour microvessel density.[51] MIF has been associated with numerous cancers, including kidney, prostate, colorectal and glioma, and many reports have documented its key role in activating tumour immuno-suppression.[52-56] In our study, we recorded a significant reduction in MIF secretion by the colorectal cancer cells tested in both SW480 and SW620 cell lines after hypericin-mediated photodynamic therapy. Thus, it can be assumed that therapies inhibiting the MIF function could contribute to an improved efficacy of anticancer treatment.

Adhesion molecules, glycoproteins belonging to the immunoglobulin superfamily, have also been analyzed as factors of progression. Vascular cell adhesion molecule-1 (VCAM-1) is constitutively expressed on tissue macrophages, stromal cells and epithelial cells and on the surface of stimulated endothelial cells. VCAM-1 mediates the adhesion of lymphocytes, monocytes, eosinophils and basophils to vascular endothelium and mediates leukocyte and endothelial cell signal transduction. There is evidence that VCAM-1 is involved in cancer progression and metastasis, by mediating the adhesion of tumour cells to vascular endothelial cells and promoting metastatic processes, including angiogenesis.[57-58] Moreover, it has been identified as one of the most important factors able to promote and sustain the development of tumour vasculature.[59] Generation of new blood vessels plays a key role in tumour progression and is characterized by invasion, migration and proliferation of endothelial cells. The above processes depend strictly on the interplay between cells and components of the extracellular matrix.[57] Several experiments have documented significantly higher levels of VCAM-1 in the serum of cancer patients or in cancer cells in vitro compared with controls.[57,60] Overexpression of VCAM-1 stimulates tumour neovascularization, resulting in tumour growth and metastasis formation. The severity of VCAM-1 expression correlates with disease stage and the presence of metastases and is highly pronounced in endothelial cells, especially in capillaries.[60,61] Maurer et al. demonstrated that colorectal cancer cells overexpress VCAM-1 and ICAM-1.[60] Therefore, it seems logical to undertake studies on ways to inhibit VCAM-1 expression to see if blocking this factor would affect the course of the cancer. In our study, we observed an inhibitory effect of HYP-PDT on VCAM-1 secretion by the colorectal cancer cells in both assessed cell lines.

ICAM-1 is located superficially in the membrane of immune, endothelial and epithelial cells. Physiologically, its expression remains low and is strongly induced by various pro-inflammatory cytokines. ICAM-1 plays an important role in many physiological processes, including immune cell effector functions, T-cell activation, leukocyte movement and elimination of pathogens and dead cells. In addition, ICAM-1 regulates leukocyte movement and adhesion interactions with the vessel wall and directs leukocyte passage through the endothelial layer and mediates intra- and extracellular signals.[62,63] ICAM-1 may similarly act in transformed epithelial and tumour cells, is involved in the activation of pro-inflammatory cascades and mediates multiple signaling pathways such as adhesion, angiogenesis and tumour cell transmigration and immune escape.[2,62] ICAM-1 expression has been found in the vast majority of cells in the tumour microenvironment and has been correlated with aggressive and invasive tumour phenotypes.[62] High expression of ICAM-1 has also been documented in metastases.[64-66] Publications on the effect of photodynamic therapy on the expression and secretion of adhesion molecules VCAM-1 and ICAM-1 are scarce. In an experiment with colon cancer cell lines SW480 and SW620 analyzing the effect of photodynamic therapy with aminolevulinic acid under normoxia and CoCl2-induced hypoxia on the in vitro secretion of adhesion molecules VCAM-1 and ICAM-1 by colon cancer cells, no significant effect of this therapy on these progression factors was observed.[67] On the other hand, a group of Chinese researchers presented a study in which photodynamic therapy increased VCAM-1 expression in glioma cells with a concomitant increase in tumour growth, while PDT combined with a monoclonal antibody directed against VCAM-1 significantly inhibited tumour growth and prolonged survival and significantly reduced VCAM-1 expression.[68] Another report documented a reduction in the number of metastases and a significant decrease in adhesion molecules, including ICAM-1, among colorectal cancer cells tested after PDT with Photofrin and benzoporphyrin derivative mono-acid ring A.[69] In our study, we recorded an inhibitory effect of HYP-PDT on ICAM-1 secretion by SW480 cells, and in the case of SW620 cells, we observed an inducing effect of HYP-PDT on ICAM-1 secretion at a low dose of hypericin and upon irradiation at 5 J/cm^2. Furthermore, we demonstrated that SW480 cells produced ICAM-1 at higher concentrations than SW620 cells.

There are few papers in the literature analyzing the effect of HYP-PDT on the secretion of progression factors. In a study by Majernic et al., the effect of HYP-PDT on the expression of proangiogenic factors was analyzed using different research models.[70] They compared cell culture in a 2D model, 3D model and in experimentally

generated micro-tumors. The cytotoxic effect of HYP-PDT HP was confirmed and the effect was shown to be highly dependent on the research model used. Significant differences in gene expression of the analyzed proangiogenic factors were observed depending on the experimental model and it was found that the level of gene expression did not correlate with the level of protein expression, especially in the 2D model, which was considered to be an effect of damage to organelles responsible for proteosynthesis. It was shown that cells cultured in 2D cell models were significantly more sensitive to treatment than cells in the 3D model. It was concluded that for this type of analysis, the selection of an appropriate experimental model is crucial. Our experiment was performed in a classical 2D culture. There is evidence for limitations of this research model, such as insufficient signaling between cells and between the cell and the extracellular matrix.[71] Important signaling pathways may be disrupted or bypassed under such experimental conditions. The hypoxic state, which is one of the hallmarks of cancerous tumours, can fundamentally affect the effect of a given therapy. Cell culture conditions, depending on in vitro and in vivo experimental models, can significantly affect growth factor gene expression.[72]

Analyzing the available literature on the use of hypericin in photodynamic therapy for colorectal cancer in in vitro and in vivo animal studies, publications proving the effectiveness of this method and showing its multidirectional effects can be found. Blank et al. documented in their in vivo and in vitro experiment on C26 colon cancer cells that photodynamic therapy with hypericin reduced cell viability in a dose-dependent and light-dependent manner and observed extensive vascular damage and tumour necrosis as a result of HYP-PDT.[73] After PDT combining the effects of hypericin with hyperforin on HT-29 colon cancer cell model, increased production of reactive oxygen species (ROS) enhancing anti-tumour activity, induction of apoptosis, inhibition of cell cycle and blockade of expression of matrix metalloproteinases 2 and 9 were reported.[36] Under the influence of photodynamic therapy with hypericin, decreased gene expression of dysadherin, an anti-adhesion molecule known for its pro-cancerogenic and metastasis-promoting function, and changes in the organization of F-actin belonging to cytoskeleton proteins were observed.[35] Austrian researchers performed an in vivo experiment on mice that were injected intravenously with low-dose hypericin and irradiated with red light. After 60 days, the effects of HYP- PDT were evaluated and it was noted that PDT at a standard dose and with a 4-hour interval between drug administration and irradiation resulted in a fourfold delay in tumour growth compared with control groups, while PDT with low doses and a 0.5-hour interval between drug administration and irradiation led to complete tumor eradication in the entire study group.[74]

Due to the high potential of hypericin and its complex mechanisms of action in photodynamic therapy at the molecular level, attempts have been made to combine it with other drugs and various inhibitors to maximize its effects. A group of Slovak re-searchers presented the results of a study on HT-29 colon cancer cells proving that com-bination treatment including HYP-PDT and MK-886, an inhibitor of lipoxygenase, an enzyme involved in multiple immune mechanisms, can improve the therapeutic efficacy of PDT; moreover, it induces changes in the cell cycle and significantly enhances apoptosis under the influence of hypericin photodynamic therapy.[75] Other researchers from the same Slovak Centre showed that pretreatment with proadifen (a non-selective cytochrome P450 enzyme inhibitor with proven apoptotic effects in HT-29 colon adenocarcinoma) affected the function of ABC membrane transporters MRP1 (multidrug resistance protein) and BCRP (breast cancer resistance protein) leading to increased accumulation of hypericin in HT-29 colon adenocarcinoma cells and enhanced HYP-PDT effect.[76]

Although photodynamic therapy is considered an effective method for treating cancer, cancer cells may use certain resistance mechanisms, including up-regulation of apoptosis inhibitors, to overcome the cytotoxic effects of PDT. One such inhibitor of apoptosis is surviving, whose expression is blocked by the small-molecule inhibitor YM155. Application of HYP-PDT together with YM155 sensitizes HT-29 colon cancer cells to HYP-PDT and induces apoptosis.[77] In another report, after application of HYP-PDT with yeast-derived manumycin A, increased sensitivity of HT-29 cells to oxaliplatin and decreased HT-29 cell viability, suppression of cell proliferation, significantly lower levels of apoptosis inhibitors (cIAP1, cIAP2, XIAP, surviving) were observed. This combination resulted in induction of apoptosis and enhanced phagocytosis of cancer cells.[78] These results were confirmed by Chinese researchers, according to whom autophagy and reactive oxygen species are mainly responsible for the increased chemosensitivity to oxaliplatin of cancer cells during HYP-PDT. HYP-PDT at high doses induced autophagic cell death and induced high concentrations of reactive oxygen species and increased cytoplasmic reticulum stress.[79,80] The ABCG2 protein, which belongs to the ABC mem-brane transporters, is another important factor responsible for multidrug resistance in tumors and lower PDT efficacy. The use of a compound called Ko143, which is an ABCG2 inhibitor, increases the efficacy of HYP-PDT as observed in in vitro studies using 2D and 3D models of HT29 and HT116 colon cancer cells.[81] In another study, HYP-PDT induction of MRP-1 protein expression in HT-29 colon cancer cells was observed, suggesting that hypericin may

affect the efficacy of anticancer drugs through interaction with ABC membrane transporters.[82]

The use of hypericin in photodynamic therapy is limited by its hydrophobic properties. In order to improve water solubility and enhance the photodynamic effect, hypericin can be bound to a carrier that will facilitate transport into and within the cell and prevent the formation of aggregates inside the cell. A symmetric triblock copolymer containing poly(ethylene oxide) and poly(propylene oxide), Pluronic P123 (P123), proved to be an interesting nanocarrier for hypericin. The effect of Hyp/P123 was analyzed for colorectal cancer cells Caco-2 and HT-29. A beneficial effect and good efficacy of HYP-PDT was observed after the application of the nanocarrier, which played a significant role in the penetration of Hyp across the cell membrane.[83] In another case, to pre-vent the accumulation of hypericin in a non-target site and to reduce its side effects, Hyp was bound to superparamagnetic iron oxide nanoparticles (SPION) and guided to the target by an external magnetic field. It was evaluated that this strategy could improve the efficacy and specificity of the treatment and could reduce toxic side effects.[84] It has been demonstrated in animal studies that the use of magnetic carriers accumulates the drug more effectively in tumour cells than traditional routes of administration.[85] In another in vitro study, Nano formulation of transferrin with hypericin (HTfNP) was successfully used, preventing premature release of hypericin and improving its availability by better targeting to the tumour site.[86] Similarly, significantly improved performance of HYP-PDT used in a study with HT-29 colon adenocarcinoma cells was achieved by using non-polymeric nanogels formed by a low molecular weight gelator as a hypericin nanocarrier. The nanogels provided free, efficient intracellular transport of hydrophobic hypericin.[87] Chinese researchers have developed a biodegradable nanoparticle, Hyp-NP, to deliver hypericin to the tumour site. It was documented that Hyp-NP showed efficient cellular accumulation with localization in the cytoplasm and retained affinity for fluorescence[88], it may prove useful to enhance the efficacy of Hyp-PDT.

Conclusion

On the basis of the experiments author proved that determination of the growth, invasion and metastasis of the colon cancer cells factors might be useful in the disease progress prognosis, choosing optimal treatment and verifying its effectiveness, as well as in monitoring the patients after the therapy. At the same time, the results confirming the destructive effect of HYP-PDT on the colon cancer cells, show a possibility of extending the indication for photodynamic therapy using HYP, qualification of precancerous changes, including adenoma and early stages of the colon cancer. HYP- PDT influences the secretion of growth, migration, angiogenesis and metastasis factors by the colon cancer cells, mainly by their suppression. In our study, we recorded an evident reduction in GM-CSF secretion by colorectal cancer cells of the SW480 cell line, escalating with increasing HYP concentration and light intensity. Furthermore, we showed that SW480 cells secrete more GM-CSF than SW620 cells. In our study, we recorded a significant reduction in MIF secretion by the colorectal cancer cells tested in both SW480 and SW620 cell lines after hypericin-mediated photodynamic therapy. Thus, it can be assumed that therapies inhibiting the MIF function could contribute to an improved efficacy of anticancer treatment. We noticed an inhibitory effect of HYP-PDT on VCAM-1 secretion by the colorectal cancer cells in both assessed cell lines and an inhibitory effect of HYP-PDT on ICAM-1 secretion by SW480 cells, and in the case of SW620 cells, we observed an inducing effect of HYP-PDT on ICAM- 1 secretion at a low dose of hypericin and upon irradiation at 5 J/cm^2. Furthermore, we demonstrated that SW480 cells produced ICAM-1 at higher concentrations than SW620 cells. These effects of photodynamic therapy using HYP determines the possibility of application of adjuvant therapy, including cell based immunotherapy with respect to the factors responsible for the invasiveness of the colon cancer.

Declarations

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Author contributions

Conceptualization, A.M., A.K-K., D.A. and Z.P.C.; Methodology, A.M., A.K-K. and G.C.; Validation, A.M., A.K-K., D.A. and Z.P.C.; Formal Analysis, A.M., A.K-K. and Z.P.C.; Resources, A.M. and D.A.; Writing – Original Draft Preparation, A.M., A.K-K., D.A. and Z.P.C.; Writing – Review & Editing, A.M., A.K-K., D.A., G.C. and Z.P.C.

Conflicts of interest

The authors declare no conflict of interest.

Data availability

Data available on request from the authors.

Ethics approval

Not applicable.

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ORIGINAL PAPER

Effects of core-stabilization and trunk balance exercises on clinical parameters in patients with non-specific chronic low back pain – a randomized pilot study

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ABSTRACT

Introduction and aim. This study compared the efficacy of core stabilization (CSE) and trunk balance exercises (TBE) with flexibility training on pain-related disability (PRD), psychological status (PS) and fear avoidance belief (FAB) in patients with non-specific chronic low back pain (NSCLBP).

Material and methods. Twenty-eight (28) participants diagnosed of NSCLBP were randomly assigned into CSE, TBE, and control groups (CG). Participants in CSE (n=10); TBE (n=8) and CG groups (n=10) received core stabilization exercise, trunk balance exercise and back care advice respectively. All participants received flexibility training in addition to treatment in their respective groups. Assessment of outcomes were done at baseline, end of 4th and 8th week.

Results. There was significant improvement in all outcomes in the CSE, TBE and CG at 8 weeks; PRD (p=0.005, p=0.008, p=0.005), PS: depression (p=0.005, p=0.008, p=0.007); anxiety (p=0.005, p=0.007) and FAB about work (p=0.005, p=0.007, p=0.005); about physical activity (p=0.005, p=0.018, p=0.006). Comparison of outcomes between CSE and TBE groups showed no significant difference (p>0.05)

Conclusion. Both CSE and TBE with flexibility training are effective in improving PRD, PS and FAB of patients with NSCLBP.

Keywords. exercise therapy, fear, low back pain

Introduction

Low back pain (LBP) is a frequent cause of disability in the community and the leading cause of disability worldwide with a lifetime prevalence of 84% in industrialized countries.[1-3] Non-specific chronic low back pain (NSCLBP) is the most common type of back pain that exists and account for 85% of all cases of back pain.[4-5] The patient with low back pain not only experience pain, but also suffers from impairment which obstructs their day to day activities such as inability to ambulate and dress up.[6]

Core stabilization exercises have been reported as an effective treatment program in reducing physical and psychological symptoms in patients with non-specific chronic low back pain.[7] Balance exercises are designed to improve balance or postural stability. Balance is a dynamic process by which the body's position is in equilibrium, static or dynamic. It is greatest when body's center of mass or center of gravity is maintained within the base of support.[8-9] Trunk balance deficits and muscle impairments could also originate from poor position sense, which has been reported to be present in individuals with chronic low back pain.[9] Poor balance is also a frequent concern reported by patients with chronic low back pain and has been demonstrated through increased

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displacement of the center of pressure while standing upright.[9]

Flexibility is the ability to move a single joint or a series of joints smoothly and easily through an unrestricted pain free range of motion. Flexibility is the extensibility of musculotendinous units that cross a joint, based on their ability to relax or deform and yield to a stretch force.[8-9]

Exercises have been shown to relieve symptoms in patients with NSCLBP.[10] However, it appears there is dearth of empirical data establishing which is more effective between core stabilization exercise (CSE) and trunk balance exercise (TBE) interventions on individuals with NSCLBP. Moreover, there is limited evidence on the impact of the trunk balance exercise on depression, anxiety, and fear avoidance belief in patients with NSCLBP.

Aim

This study therefore compared the therapeutic efficacy of core stabilization and trunk balance exercises with flexibility training on pain-related disability, psychological status (anxiety and depression) and fear avoidance belief in patients with NSCLBP. This study was set to proffer answer to the following question: Would Core Stabilization and trunk balance exercises with flexibility training improve pain related disability, psychological status, (anxiety and depression) and fear avoidance belief in patients with NSCLBP.

Material and methods

Participants

A single blinded randomized controlled pilot study registered with the Pan-African clinical trial registry (PACTR202110750995790) was employed for this study. Approval to conduct the study (CMUL/HREC/02/21/812) was obtained from the health research and ethics committee of the College of Medicine University of Lagos. Informed written consent was obtained from the participant prior to enrolling them in the study. Thirty-three participants were involved in this study; they were patients with NSCLBP seeking treatment from a physiotherapy clinic of a tertiary health institution in Ogun state, Nigeria. Sample size calculation was based on minimum effect size of 0.25 and power of 80% using the G. power software calculator.[11,12] This research was conducted between April 2021 and July 2021. The participants involved in the study were patients diagnosed with recurrent history of non-specific chronic low back pain greater than 3 months with or without pain radiating to one or both lower limbs and patients that scored more than 5 on visual analogue scale. Participants were excluded if they had spinal surgery, history of trauma to the back or specific low back pain. Information on the physical characteristics (age, sex, height, weight, body mass index) were obtained from the participants, while the height and weight were measured following the protocol of the International Society for the Advancement of Kinanthropometry.[13]

Assessment of height and weight of the participants

The participants were instructed to stand erect on the stadiometer with their eyes looking straight forward ahead and their hands held by the side. The height and weight were read and recorded to the nearest 0.1meters and 0.1 kilograms respectively.[13]

Assessment of outcome measures

The assessment of pain related disability, depression, anxiety, fear avoidance belief, were achieved with the pain disability index, hospital anxiety depression scale, and fear avoidance belief questionnaire respectively.

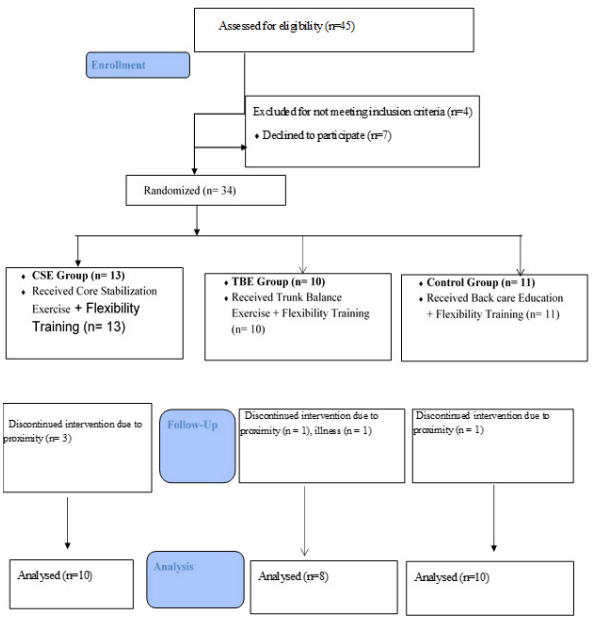

Fig. 1. Flow chart of the study

Randomization

Forty-five (45) patients with complaint of non-specific chronic low back pain were recruited for this study, eleven (11) were not eligible considering the inclusion criteria. Thirty-four (34) participants were allocated into 3 different groups (CSE+flexibility, TBE+flexibility and control) through a random generated number sequence, produced before the recruitment of the participants by the research assistant. Thirteen (13) participants were allotted into CSE+flexibility group, Ten (10) participants into TBE+flexibility group while eleven (11) participants were allotted into the control group that received flexibility and back care advice (Fig. 1). To ensure adequate blinding, allocation of study participants was done by a research assistant who was not involved in the clinical assessment and treatment of patients. Participants and the statistician were blinded to interventions to reduce bias. However, six (6) participants were not

able to complete the study due to proximity and illness (Fig. 1). All the groups received 30 minutes duration of the interventions twice weekly for a period of 8 weeks.

Evaluation methods

The CSE, TBE, back care and flexibility regimens were performed two times a week for 8 weeks. The assessment of pain related disability, psychological status (depression and anxiety) and fear avoidance belief were taken at baseline, and at the end of the 4th and 8th week. The research assistant who was the assessor did not administer any intervention on the participants. The investigators who are physiotherapists (FO and AA) supervised the intervention protocols. The participants and data analyst were also blinded to intervention to eliminate bias.

Outcome measures

Pain disability index (PDI)

This is a 7-item questionnaire used for investigating the magnitude of self-reported pain-related disability, independent from region of pain or pain related diagnosis. The items of the questionnaire are assessed on a 0-10 numeric rating scale in which 0 means no disability and 10 is maximum disability. The sum of the seven items equals the total score of the PDI, which ranges from 0-70, with higher scores reflecting higher interference of pain with daily activities. The PDI measures family/home responsibilities, recreation, social activity, occupation, sexual behaviour, self-care, and life support activity.[14] The PDI has test-retest reliability value of 0.78.[15]

Hospital anxiety and depression scale (HADS)

The HADS is a fourteen-item scale with seven of the items assessing anxiety and seven assessing depression. Each item on the questionnaire is scored from 0-3 that a person can score between 0 and 21 for either anxiety or depression. A score of 0-7 is normal, 8-10 is borderline abnormal and 11-21 is abnormal.[16] It has a high sensitivity value and internal consistency of 0.86.[16]

Fear avoidance belief questionnaire (FABQ)

It has been proven to be a useful clinical tool that demonstrates specific fear avoidance beliefs which are strongly related to work loss due to low back pain.[17] It consists of 2 sub scales, which is reflected in the division of the outcome form into two separate sections. The first subscale (item 1-5) is the physical activity subscale, and the second subscale item (6-16) is the work subscale. Each subscale is graded separately by summing the response respective scale items (0-6) for each item, for scoring purposes, only 4 of the physical activity scale items are scored (24 possible points). The items 2, 3, 4, and 5 are summed for the score of the physical Activity Subscale, while the items 6, 7, 9, 10, 11, 12 and 15 are summed for the work subscale. The FABQ has been demonstrated to be valid and reliable in a chronic low back pain population.[17]

Post intervention assessment was done at the end of 4th and 8th week. All participants were told to abstain from any other treatment intervention for their back pain throughout the duration of the study and to inform the researcher of any complaints they have at any stage throughout the duration of the research.

Protocol for core stabilization exercises

This comprises of abdominal bracing (8 seconds, 30 repetitions), heel slides while bracing the abdomen (4 seconds, 20 repetitions), bridging with abdominal bracing (8 seconds, 30 repetitions), leg lift with abdominal bracing (4 seconds, 20 repetitions), bridging and leg lift with abdominal bracing (8 seconds, 30 repetitions), abdominal bracing in standing position (8 seconds, 30 repetitions), arm lift with bracing in quadruped position (8 seconds, 30 repetitions), leg lift with bracing in quadruped position (8 seconds, 30 repetitions), alternate arm and leg lift with bracing in quadruped position (8 seconds, 30 repetitions).[18]

Protocol for trunk balance exercise

This comprises of kneeling on a pillow and arms abducted to 90°, the trunk was rotated, head and upper limbs to one direction (2 times per direction, maintaining each position for 30 seconds), kneeling on a pillow, the upper limbs were moved in flexion and extension, with a simultaneous movement of the head (3 minutes, performing 6 repetitions of upper limbs movement). Supine with feet resting on the table, the pelvis was lifted up, after reaching maximum hip extension, one lower limb was raised from the table and the knee extended (twice for 30 seconds for each lower extremity), quadruped position, opposite upper and lower limbs were extended, Sitting on the side of the table with unilateral support (1 minute each side), Single-limb kneeling on the edge of the table with a pillow under the knee (30 seconds two repetitions for each limb).[8] The exercise was made more challenging by adding eye closure.

Protocol for flexibility

The participants performed flexibility exercises to the lower extremities such as quadriceps stretching, sitting hamstring stretching, calf muscles stretching, hip adductors, hip abductors, hip flexors/extensors stretch, gluteal muscle stretching. All stretches were held for 15-20 seconds to achieve the maximum benefit This was repeated with both legs 2-3 times.[10]

Protocol for back care education

It was an educational package comprising of instructions and drawings showing how to perform correct lifting and carrying techniques, how to maintain prop-

er posture while in upright position, avoiding prolonged sitting, bending, stooping and squatting and how to perform correct sweeping technique.[19]

Statistical analysis

Statistical Package for Social Sciences (SPSS Inc., Armonk, New York, USA) 25.0 version for Windows package program was used to perform data analysis. Demographic and quantitative data were expressed as mean and standard deviation (SD). Normality test was done with Shapiro Wilk test. One-way ANOVA and descriptive statistics were used to analyse demographic variables. Wilcoxon signed rank test was used to detect any statistically significant differences in the changes within each group pre and post treatment intervention. Kruskal Wallis was used to detect any significant difference across the three groups and Post Hoc analysis was used to detect where the significance lies in the three groups. Mann-Whitney U test was used to compare outcomes across the weeks between groups 1 and 2. All statistical test were performed at 0.05 level of significance (i.e., $p<0.05$).

Results

Forty-five participants with non-specific chronic low back pain were recruited for this study. However, 28 participants completed the study: with 10 (35.7%) of the participants in CSE+flexibility group, 8 (28.6%) participants in TBE+flexibility group and 10 (35.7%) participants in the control group (Figure 1). For the sex distribution, 15 (53.6%) of the participants were females and 13 (46.4%) were males. The mean age of the participants in all the groups was 48.62±1.88 years. The mean weight, height and body mass index (BMI) of the participants in all the groups were 67.26±1.28 kg, 1.62±0.01 m, and 25.49±0.37 kg/m^2 respectively. The groups did not differ significantly in age and height (Table 1).

Table 1. Demographic characteristics of the participants (n=28)*

Variables	All Groups Mean±SD n=28	CSE Mean±SD n= 10	TBE Mean±SD n= 8	Control Mean±SD n=10	F-value	p
Age (years)	48.62±1.88	50.31±3.191	50.40±1.74	45.00±4.16	0.877	0.426
Height (m)	1.62±0.01	1.63±0.02	1.60±0.02	1.62±0.01	0.747	0.482
Weight (kg)	67.26±1.28	71.62±1.32	65.00±2.02	64.18±2.72	4.3	0.022
BMI (kg/m^2)	25.49±0.37	26.87±0.28	25.27±0.67	24.06±0.71	6.899	0.003

* significance level p<0.05; Mean±SD – mean±standard deviation; BMI – body mass index; CSE – core stabilization exercise+flexibility group; TBE – Trunk balance exercise+flexibility group; F-value – One-way ANOVA

Table 2 shows the Wilcoxon Signed Ranks Test which revealed a significant improvement in the outcome parameters in all the 3 groups except for anxiety in the control group (p=0.075). Table 3 shows that there was a significant difference in Fear Avoidance Belief about physical activity score among the 3 treatment groups (p=0.041). Least significant difference Post hoc analysis showed that there was significant difference between the CSE and TBE groups (p=0.03), and the TBE and Control groups (p=0.01), for fear avoidance belief about physical activity.

Table 2. Outcome measure parameters at pre-treatment (baseline) and post-treatment (end of the 8th week) within each group

	Outcome measure	Baseline Mean±SD	End of 8th week Mean±SD	z-value	p
CSE	PDI	42.92±3.91	10.40±1.88	-2.803	0.005*
	Depression	13.85±1.15	2.20±0.93	-2.81	0.005*
	Anxiety	12.23±1.31	1.30±0.5	-2.81	0.005*
	FAB (work)	29.23±1.57	14.6±1.75	-2.81	0.005*
	FAB (physical activity)	22.62±0.61	10.6±1.56	-2.81	0.005*
TBE	PDI	46.4±5.47	17.50±3.96	-2.666	0.008*
	Depression	12.8±1.71	3.25±1.05	-2.673	0.008*
	Anxiety	10.8±1.83	2±1.24	-2.677	0.007*
	FAB (work)	28.4±2.43	13.63±1.73	-2.677	0.007*
	FAB (physical activity)	19.4±1.71	9.25±1.6	-3.371	0.018*
Control	PDI	36.73±4.58	10.00±1.61	-2.805	0.005*
	Depression	7.27±1.12	1.60±0.82	-2.692	0.007*
	Anxiety	6.27±1.44	2.80±1.75	-1.787	0.074
	FAB (work)	27.91±1.97	14.00±1.29	-2.805	0.005*
	FAB (physical activity)	21.45±1.17	12.20±0.81	-2.726	0.006*

* significance level p<0.05; CSE – core stabilization exercise+flexibility group; TBE – trunk balance exercise+flexibility group; PDI – pain disability index; FAB – fear avoidance belief; Z-value – Wilcoxon sign rank test

Table 3. Outcome measure parameters at baseline, end of 4th and 8th week between the 3 groups*

	Outcome measure	CSE Mean±SEM n=10	TBE Mean±SEM n=8	Control Mean±SEM n=10	H-value	p
Baseline	PDI	42.92±3.91	46.40±5.472	36.73±4.581	2.914	0.233
	Depression	13.85±1.154	12.80±1.705	7.27±1.121	11.368	0.003
	Anxiety	12.23±1.307	10.80±1.825	6.27±1.440	8.298	0.016
	FAB (work)	29.23±1.565	28.40±2.432	27.91±1.965	0.139	0.933
	FAB (physical activity)	22.62±0.605	19.40±1.714	21.45±1.171	3.311	0.191
End of 4th week	PDI	25.45±3.730	28.75±5.583	23.73±3.873	0.360	0.835
	Depression	6.55±1.275	6.75±1.800	3.55±1.178	3.538	0.171
	Anxiety	4.45±0.824	4.63±1.487	2.82±0.942	2.258	0.323
	FAB (work)	20.00±1.668	19.63±2.725	20.64±1.636	0.098	0.952
	FAB (physical activity)	17.73±1.356	12.88±1.865	18.73±1.214	6.369	0.041*
End of 8th week	PDI	10.40±1.881	17.50±3.960	10.00±1.606	3.007	0.222
	Depression	2.20±0.929	3.25±1.048	1.60±0.819	1.725	0.422
	Anxiety	1.30±0.496	2.00±1.239	2.80±1.75	0.171	0.918
	FAB (work)	14.60±1.746	13.63±1.731	14.00±1.291	0.372	0.83
	FAB (physical activity)	10.60±1.558	9.25±1.601	12.20±0.814	2.425	0.298

* significance level p<0.05; SEM – standard error of mean; CSE – core stabilization exercise+flexibility group; TBE – trunk balance exercise+flexibility group; PDI – pain disability index; FAB – fear avoidance belief; H-value – Kruskal Wallis Test

Table 4 shows the comparison between the mean score on pain disability index, psychological status (depression, anxiety), fear avoidance belief about work

and physical activity at baseline, mid-treatment, and post-treatment between the CSE and TBE groups. Mann-Whitney U test showed that there was no significant difference (p>0.05) between the outcome parameters of both intervention groups.

Table 4. Comparison between outcome measure parameters at baseline, mid-treatment, and post-treatment between CSE and TBE groups

	Outcome Measures	CSE Mean ±SEM N=10	TBE Mean± SEM N=8	u-test	p
Baseline	PDI	42.92±3.91	46.40±5.472	50.5	0.376
	Depression	13.85±1.15	12.80±1.705	59.5	0.738
	Anxiety	12.23±1.31	10.80±1.825	52.5	0.446
	FAB (work)	29.23±1.57	28.40±2.432	62	0.879
	FAB (physical activity)	22.62±0.61	19.40±1.714	38	0.101
End of 4th week	PDI	25.45±3.73	28.75±5.58	40.5	0.778
	Depression	6.55±1.28	6.75±1.80	43	0.968
	Anxiety	4.45±0.82	4.63±1.49	42.5	0.904
	FAB (work)	20.00±1.67	19.63±2.73	40	0.778
	FAB (physical activity)	17.73±1.36	12.88±1.87	20	0.051
End of 8th week	PDI	10.40±1.88	17.50±3.96	23	0.146
	Depression	2.20±0.93	3.25±1.048	32	0.515
	Anxiety	1.30±0.5	2.00±1.239	39.5	0.965
	FAB (work)	14.60±1.75	13.63±1.731	35	0.696
	FAB (physical activity)	10.60±1.56	9.25±1.601	32.5	0.514

* significance level p<0.05; CSE – core stabilization exercise+flexibility group; TBE – trunk balance exercise+flexibility group; PDI – pain disability index; FAB – fear avoidance belief; U-test – Mann-Whitney U test

Discussion

This study determined the therapeutic efficacy of core stabilization and trunk balance exercises with flexibility training on pain-related disability, psychological status (anxiety and depression) and fear avoidance belief in patients with NSCLBP.

In this study, core stabilization exercise with flexibility training was found to be effective in decreasing pain related disability of patients with non-specific chronic low back pain. This is consistent with a study by Kumar et al[20] which concluded that core muscle strengthening exercise along with lumbar flexibility is an effective rehabilitation technique for all chronic low back pain patients. A previous study has shown that stabilization exercises are more beneficial than conventional treatments to reduce pain and disability in chronic LBP patients.[21] This could be associated with the reestablishment of the normal control of the local muscles of the trunk which, when recruited, stabilizes the spine, and increases activity in the lumbar muscles, and reduce the activity of more superficial muscles such as rectus abdominis, external oblique, and internal oblique. The reduction in pain could be attributed to muscular contraction during spinal stabilization exercises which provides sensory input to trigger different pain inhibitory mechanisms in the central nervous system.[22] These led to a rise in the plasma serotonin level, as a likely means of the spinal stabilization exercises-induced analgesia.[22] This would subsequently cause a reduction in pain-related disability.[7]

In this study, core stabilization exercise with flexibility training was found to be effective in improving depression, anxiety, and fear avoidance belief in patients with NSCLBP. This is in line with findings of Akodu and Akindutire, which reported that core stabilization exercises are very useful in the management of depression and anxiety in NSCLBP patients.[7] This result is also supported by a study done by Akodu et al. which concluded that stabilization exercise is effective in the management of pain-related disability, depression, and anxiety in NSCLBP patients.[10] This could be due to the decline in the pain sensation of the participants' post-treatment. This is also in line with the claim of Balasubramaniam et al[23], who reported that when there is a reduction in the level of perception of pain and disability, the level of depression reduces, as a result, leads to reduction of patients' fear of pain and improvement in avoidance of physical activity. A study by Akodu et al., reported that stabilization exercise is effective in managing fear avoidance belief of patients with non-specific chronic low back pain.[21]

In this study, trunk balance exercise with flexibility training was found to be effective in reducing pain-related disability, improve psychological status (anxiety and depression) and fear avoidance belief in patients with NSCLBP. This is supported by a study done by Gatti et al. which concluded that trunk balance exercises appeared to be effective in reducing disability due to chronic LBP.[8] Trunk balance exercise has a big effect on chronic low back pain patients as it strengthens deep abdominal muscles and improves flexibility and balance.[24] This could be because balance exercises promote recruitment of the trunk musculature. Proper recruitment of these muscles may be lost in patients with CLBP, which may explain the pain, poor postural control and the muscle activation delays and subsequent disabilities. Trunk balance exercises also improves activation of the trunk muscles during both unpredictable and predictable trunk perturbations by providing spinal stability which act through feed-forward and feedback control mechanisms that modulate the stiffness of the spinal muscles to control internal and external forces generated during body movements.[24]

This study also showed that back care plus flexibility exercises was effective in the management of pain disability, psychological status (depression and anxiety) and fear avoidance belief of patients with NSCLBP. This improvement could be due to the reduction in pain and disability level of the participants.[23] This is in line with studies done by Paolucci et al., and Akodu et al., which concluded that back care and stretches has pos-

itive effects on the psychological status of patients with NSCLBP.[10,25]

However, the result of the comparison of both core stabilization exercise with flexibility training and Trunk balance exercises with flexibility training showed that both interventions are both effective in improving pain-related disability, psychological status (depression and anxiety) and fear avoidance belief of patients with NSCLBP as there was no difference in the clinical outcome variables in the two intervention groups after 8 weeks post treatment. This study was limited due to small sample size, lack of gender division, drop out from the study, and short study duration (8 weeks). Caution should also be taken when interpreting the result of this study due to the small sample size, because the result cannot be generalized.

Practical and scientific implication

Core stabilization exercises and trunk balance exercises with flexibility training can be used by physiotherapists along with conventional physiotherapy interventions in the management of patients with NSCLBP.

Conclusion

It can be concluded from this study that both core stabilization exercise with flexibility training and trunk balance exercises with flexibility training were effective in improving pain-related disability, psychological status (depression and anxiety) and fear avoidance belief of patients with NSCLBP. However, when the two interventions were compared, no protocol was found to be superior to the other. It was therefore recommended that core stabilization exercises and trunk balance exercises with flexibility training can be used by physiotherapists in the management of patients with NSCLBP.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare no competing interests.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

Ethics approval

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 health research and ethics committee of the College of Medicine, University of Lagos (CMUL/HREC/02/21/812).

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ORIGINAL PAPER

Whole-body vibration on lower limb flexibility and extensibility – a randomized clinical trial

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ABSTRACT

Introduction and aim. The whole-body vibration has become known for optimizing the production of muscle power due to mechanical oscillations that are dependent on vibration frequency. However, the effects of varying the vibration frequency on flexibility have still been little explored. Compare the effects of two frequencies of whole-body vibration on flexibility and extensibility of the lower limbs.

Material and methods. Randomized clinical trial with a sample of 42 young adult volunteers of both sexes, who performed squatting sessions with individualized load on a platform and distributed into three groups of vibration frequency: control group (CG), with the platform off; low frequency group (LF), with a frequency of 30 Hz; high frequency group (HF), with a frequency of 45 Hz. In total, the intervention was carried out in 12 sessions and lasted 6 weeks, with 2 sessions per week. Flexibility, evaluated before and after the intervention by the sit and reach test (Wells bench) and by evaluating the extensibility of the ischiotibials by goniometry.

Results. No statistical differences were observed for any of the outcomes evaluated.

Conclusion. None of the proposed frequencies produced gains in flexibility and extensibility of the lower extremities and there was no superiority of one frequency over another.

Keyword. musculoskeletal abnormalities, physical therapy modalities, range of motion

Introduction

The term fascia refers to flat layers of dense tissue, such as aponeuroses, joint capsules, dura mater, periosteum, neurovascular sheaths, epimysium, perimysium, and endomysium among others.[1] Taking into account the muscle fascia, the epimysium is the external layer that covers the muscle, making it an individual organ. Internally, the division into fascicles is performed by a continuous connective tissue network called the perimysium; and finally the endomysium encompasses the fibers individually, and these structures connect and coordinate muscle elements through cellular signaling that involves connections between the sarcolemma and the fascia through integrins.[2,3]

In view of its thickness and rigidity characteristics, the main restrictor of passive stretching is the perimysium and, to a lesser extent, the endomysium, which are tissues that undergo adaptations to muscular exercise, disuse, and injury, that is, they limit muscle extensibility and, in this way, flexibility. Joint flexibility is characterized by the range of motion available for the joint to move effectively without producing injury; stretching exercises are generally used for this purpose.[2,4] There are several exercises that can influence flexibility, thus gen-

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erating benefits such as reducing the risk of falls, preventing pain and injuries especially active and passive stretching exercises.[5,6]

Physical exercise produces an increase in local blood flow, leading to an increase in temperature, an important factor for the gain of muscle extensibility.[7–10] One of the forms of physical exercise is whole-body vibration (WBV); the modality has shown improvement in physical performance due to improved synchronization of motor units, potentiation of the stretch reflex, increased activity of synergist muscles and inhibition of antagonists.[11–16] The WBV is an electronic device that generates real possibilities of muscle strength gain and even indicated for flexibility gains.[11,12,17,18] And, despite being controversial, some authors point out that WBV promotes flexibility gains.[18–26]

Aim

Since fascia remodeling can occur due to the stress imposed by physical exercise, the objective of this study was to compare the repercussions of two frequencies of WBV on the flexibility and extensibility of the lower extremities, in nonathletic individuals over the long term.

Material and methods

Study design and recruitment

This study was classified as a blind (participant), parallel, randomized, placebo-controlled clinical trial following the recommendations of the Consolidated Standards of Reporting Trials (CONSORT), was published in the Brazilian Registry of Clinical Trials (ReBEC – RBR-4rrn7cf). The study was approved by the Ethics Committee on Research Involving Human Beings of the Universidade Estadual do Oeste do Paraná and registered under protocol number 5.269.114. All volunteers signed an informed concept for their participation in the study.

Volunteers were recruited in a non-probabilistic and consecutive way. Young adults aged between 18 and 30 years, not practicing systematic physical activities, of both genders were included. The dissemination of the study was done by digital means and personal approaches in the surroundings of the University where the research was developed. Volunteers with neurological and cognitive problems, cardiorespiratory diseases, and a history of joint or muscle injuries in the last six months were not included. The exclusion criterion for data analysis was the withdrawal of the volunteer's authorization to participate in the study.

Randomization and blinding

Volunteers were randomly assigned to three intervention conditions according to the frequency imposed by the WBV. The distribution between the intervention frequencies was paired with respect to sex.

In all, 42 volunteers were randomly separated into 3 interventions, according to the frequency applied on the vibration platform: 30 Hz group (LF), 45 Hz group (HF), and control group (CG).

The randomization process was performed by a researcher not involved in any other stage of the study and assigned to this process only. The randomization list was generated electronically using GraphPad QuickCalcs software.

Outcomes

The endpoints of the study were posterior chain flexibility and hamstring extensibility.

To evaluate the flexibility of the posterior chain, the Sit and Reach test (Wells bench) was applied, and to evaluate the extensibility of the hamstrings, goniometry was performed.

In the evaluation with the Wells bench, the volunteer sat on an EVA tatami with knees extended and bare feet, leaning his entire plantar surface on the Wells bench. After the command, with arms extended, the left one above the right, and the chin close to the chest, the trunk flexion was performed in an attempt to reach the greatest possible distance indicated on the ruler of the Wells bench. The individual performed three trunk flexions without destabilizing or compensating for knee flexion. At the moment of maximum reach, the volunteer held the position for two seconds and returned to the initial posture, repeating the act two more times with a 10 s interval between them. The best reach result among the three was considered.

A universal double arm goniometer was used to analyze the muscle extensibility of the tibial ischii. The goniometer is a valid and highly reliable tool in measuring the range of motion (ROM) of the knee joint. The inter-tester reliability of the goniometer is 0.977–0.982 and the intra-tester reliability is 0.972–0.985.[27] The extensibility of the ischiotibial muscles was measured by the degree of limitation in the ROM of knee extension. Volunteers were placed in the dorsal decubitus that supported the hip joint in 90° flexion. After that, they were asked to extend the knee actively until their maximum fitness, keeping the hip joint fixed at 90°. The goniometer axis was positioned at the lateral epicondyle of the femur, the fixed arm toward the greater trochanter of the femur, and the mobile arm parallel to the fibula toward the lateral malleolus.

Methodological procedures

The evaluations took place on three occasions over time: (EVA1) familiarization, (EVA2) pre-intervention; (EVA3) post-intervention.

Familiarization (EVA1): On the first visit, an interview was conducted to record the physical and functional history of the volunteers, as well as their anthropometric measurements. The following measure-

ments and information were recorded: age (years), body mass (kg), height (m), lower limb length (m), sex, body mass index (BMI), and self-reported level of physical activity. Volunteers were familiarized with the evaluation procedures to minimize the learning effect. The familiarization data was not computed for the statistical analyses. The first visit preceded the second visit by a minimum of 72 h and a maximum of 120 h.

Pre-intervention (EVA2): the volunteers were re-evaluated, in relation to the measures corresponding to the study outcomes, in the same way as during familiarization, between 72 h and 120 h of the latter and before the beginning of the intervention.

Post-intervention (EVA3): the volunteers were evaluated in an identical manner as practiced at familiarization, between 24 h and 48 h after the end of the intervention.

Intervention

In total, the intervention was carried out in 12 sessions and lasted 6 weeks, with 2 sessions per week. Each session lasted an average of 20 minutes, at the same hour of the day.

Squat

The intervention common to all groups was the squat. In the familiarization session the volunteer was asked to perform as many squats as possible, up to the point of fatigue (the goal was for individual protocol planning of training exercises), within a hip range that varied from 180° in the standing position to 70° in the squatting position, delimited by a band attached to two cones.

The volume of squats during the interventions was based on the percentage of the maximum number of squats obtained, individually, in the familiarization assessment. The percentage was increased in the program throughout the 12 sessions. With changes in the percentage of maximum squats from 50% to 80% between sessions one through eight and 80 to 90% for the last four sessions. The rhythm of the movement was controlled by a metronome. In each session a series of squats was performed.

In cases in which the volunteers did not reach the range that delimited the amplitude stipulated for the squat or did it out of the tempo determined by a metronome three consecutive times, it was determined that the volume of squats stipulated for the following week would remain the same during the current week, in order to control the external load.

Vibratory platform

The vibrating platform, the Power Plate® model, was used so that the vibration ran through the entire body of the volunteers during squatting movements, which were performed on the platform.

The volunteers performed a series of squats at an angle of 70º, identical to the baseline evaluation. To adapt to the desired angle, two cones were placed next to the vibration platform, with a strip connected to the top of each, indicating that squatting could not exceed this limit, which was adjusted for each individual, with respect to their body differences. The parameters set for the whole body vibration were an amplitude of 2 mm, at a frequency of 30 Hz for the LF and 45 Hz for the HF. The CG performed the same squatting protocol on the platform, but with it turned off.

Statistical analysis

The SPSS 20.0 software (IBM, Chicago, USA) was used for statistical analysis, with data presented as mean and standard error, and inferential analysis using a mixed univariate ANOVA and ANOVA model, the level of significance adopted was 5% (α=0.05). Cohen's effect size d was also analyzed, based on EV1 and the last assessment (EV3).[28]

Results

Volunteer recruitment took place from March to May 2022, and during those 8 weeks 42 positive responses were obtained. Seven of these volunteers were excluded, 2 due to external musculoskeletal injuries, 1 due to SARS-COV-2 contamination, and 1 due to dropout. The remaining 38 volunteers were distributed in HFD group (n=14; 8 women); in LF (n=13; 8 women) and CG (n=11; 7 women). No significant differences were found in age or BMI. The sample characteristics are presented in Table 1.

Table 1. Sample characterization data (mean and standard deviation values)

	High-Frequency	Low-frequency	Control	p
Age (years)	20.8 ± 0.4	21.2 ± 0.4	21.5 ± 0.4	0.518
BMC (kg/m²)	26.5 ± 1.4	25.1 ± 1.4	24.9 ± 1.5	0.687

No statistical differences were observed for any of the evaluated outcomes (Wells bench - $F(1,453; 50.8)=1.358$, $p=0.189$ (Fig 1A); goniometry - $F(2; 70)=1.652$, $p=0.6$ (Fig 1B)) (Table 1). The effect sizes observed for the Wells bench were: null for HF (ES=0.07) and CG (ES=0.08), very small for LF (ES=0.14). For goniometry the values were: very small for HF (ES=0.17), null for LF (ES=-0.06), and small for CG (ES=0.39). The mean values can be seen in Figure 1.

Discussion

The present study examined the effects of the vibrating platform on the flexibility of the posterior chain and the extensibility of the ischiotibial muscles in healthy young people. The hypothesis of the study was that the use of

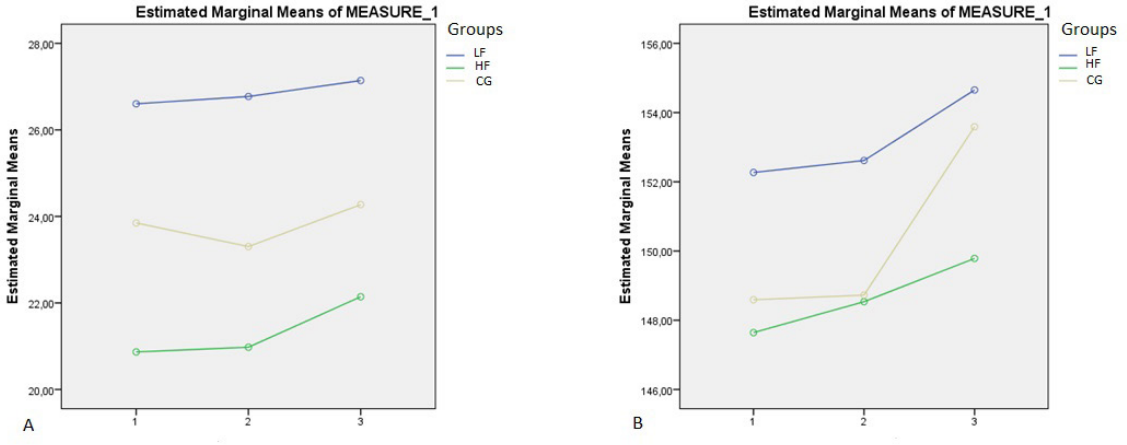

Fig. 1. Graphic representation of the values observed for the different groups (LF, HF, and CG) at the different evaluation times (1, 2, and 3), for the Wells bench (A) and goniometry (B)

WBV, using two frequencies of intervention (30 Hz and 45 Hz), combined with squatting exercise, would contribute to the improvement of the outcomes investigated; however, this hypothesis was not supported because no differences were observed in any of the groups.

The study by Cochrane et al. was positive toward the acute effect of the vibrating platform on flexibility, improving in 8.2% the sit and reach test.[19] The authors justified that the vibration effect promotes the stimulation of the primary endings of the muscle spindles, which in turn activate the stretch reflexes of the motor neurons of the agonist muscle and thus inhibit the antagonist muscle (hamstrings). The authors believe that activation of inhibitory interneurons of the hamstrings decreased the braking force of the hip and lumbar joints, thus facilitating greater reach. Another possible explanation is that vibration can cause muscle heating, which generates an increase in muscle extensibility, so that thixotropism and vasodilation together decrease blood viscosity, and thus increase its displacement. Finally, they mention that vibratory waves reduce the muscle and tendon pain threshold, generating increased tolerance to muscle stretch, allowing greater amplitude. It should be taken into account that the frequency used was 26 Hz and the amplitude was 6 mm, yet the exercises were in different ways, including actively stretching the ischiotibial muscles, i.e., different parameters from those presented in this study.

Dallas et al. analyzed the short-term acute effect of WBV in young gymnasts and observed gains in the sit and reach test (similar to the Wells bench).[29] In another study, Dallas and Kirialanis, again verified gains in flexibility when associating and even without associating WBV with muscle stretching.[23] Dallas et al. verified the effect in divers and again verified flexibility gains at 30 and 50 Hz.[24] Despina et al. used WBV exercises or only resisted exercises in rhythmic gymnasts (30 Hz, 2mm of amplitude) and also reported gains in flexibility.[25] Similarly, Fagnani et al., in a protocol of 8 weeks (three times a week) of WBV (35 Hz and 4 mm of displacement) with 26 competitive athletes of different sports, observed improvements in strength, jumping, and flexibility, evaluated by the sit-and-reach test.[20]

Of the studies cited above, it should be taken into consideration, that the volunteers were athletes, unlike the present study in which the volunteers were not athletes, which may be a factor that led to different results. In healthy, but elderly individuals, Tseng et al. report gains in flexibility after three months of treatment and even in the segment in six months, but it is worth mentioning that it is a population with characteristics of loss of amplitude (elderly), that is, they have greater possibilities of gaining still within the flexibility considered normal.[22] Still, analyzing nonathletes without underlying diseases, Gerodimos et al. analyzed the acute aspect of WBV (15, 20 and 30 Hz, 6 mm amplitude) on flexibility, indicating immediate advantages that lasted up to 15 minutes, but the gap of the long-term effects remained.[26] In the study by Marmitt et al., with three volunteers using WBV (30 Hz) aiming to increase muscle mass and flexibility, during 60 days, twice a week, performing: squat, back squat, sumo squat, bridge over the shoulders, and toe lifting.[21] They did not observe differences between the initial and final time, similar to what was found in this study.

Although some studies show that resistance exercise combined with the vibration platform can help increase flexibility, the present study did not show improvement. Therefore, we speculate that the WBV parameters, such as frequency, amplitude, exercise protocol, and even previous conditioning of volunteers, can interfere with the gain of muscle extensibility that would reflect in joint flexibility, noting that in this study there was no stretching before or after the application of the vibration re-

source. We suggest that future studies not only evaluate the association with stretching, but also analyze whether different frequency ranges can interfere with disease processes such as osteoarthritis.

Conclusion

None of the proposed frequencies produced gains in lower limb flexibility and extensibility, and there was no superiority of one frequency over another. However, one must take into consideration limitations such as the study population, the performance of only subacute analyses, without long-term follow-up.

Declarations

Funding

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Author contributions

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

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

The data remains in the possession of the authors and can be presented if requested.

Ethics approval

The study was approved by the Ethics Committee on Research Involving Human Beings of the Universidade Estadual do Oeste do Paraná and registered under protocol number 5.269.114.

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ORIGINAL PAPER

The dynamics of hypertension and renal function in CKD and non-CKD patients affected with COVID-19 – final results of BIRCOV trial

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ABSTRACT

Introduction and aim. There is evidence in the literature about a change in the effectiveness of inhibitors of the renin-angiotensin system (iRAS) in people with COVID-19. Considering different mechanisms of pressure reduction by different iRAS groups, one can expect differences in people with COVID-19 receiving these drugs. The aim of angiotensin-converting enzyme inhibitors (ACEi), angiotensin II receptor blockers (ARB) and direct renin inhibitors (DRi) usage in COVID-19 (BIRCOV study) was to pinpoint clinical and laboratory differences in people with hypertension who received iRAS and suffered coronavirus infection.

Material and methods. An open prospective trial of 108 patients was performed in subjects suffering from COVID-19 who have been receiving iRAS: ACEi, ARB or DRi as basic antihypertensive therapy. The disease follow-up was 12 and 24 weeks. A blood pressure (BP) measurement was performed the week before COVID-19 and up to 24 weeks from the disease onset. Subanalysis in patients with chronic kidney disease (CKD) was performed.

Results. In patients with COVID-19, a change in the effectiveness of antihypertensive therapy depending on the type of drug in the iRAS group has been documented in the first 4 weeks from the onset of the disease. The use of ACEi had significantly increased the risk of severe hypotension, unlike ARBs that do not cause hypotension. The synchronous decline of estimated glomerular filtration rate (eGFR) and systolic BP was more pronounced in CKD patients followed by albuminuria incidence. The greatest decrease in eGFR was in people taking ACEi.

Conclusion. People with grade 1-2 hypertension who are constantly receiving RAS inhibitors suffering from COVID-19 may develop hypotension with ACEi. COVID-19 leads to transient albuminuria and decreased glomerular filtration rate, which is especially dangerous for people with CKD 4-5.

Keywords. ACEi, ARB, BIRCOV trial, COVID-19, DRi, iRAS

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Introduction

The global COVID-19 pandemic evoked certain changes in approach to chronic kidney disease (CKD) patients with hypertension. Since the beginning of the COVID-19 pandemic, there have been reports of increased mortality among patients, receiving treatment for hypertension and the role of the renin-angiotensin system inhibitors (iRAS) has been discussed. It is well-known that SARS-CoV-2 uses an angiotensin-converting enzyme 2 (ACE2) receptor and furin site of S glycoprotein facilitating virus entry into host cells.[1-4] Given that ACE2 levels may vary in hypertensive subjects, the severity of COVID-19 disease and blood pressure levels might be different and it's natural to assume that SARS-CoV-2 affects the state of the renin-angiotensin system.[5] Available data remain controversial indicating positive, neutral, or negative effects of iRAS in COVID-19 infected patients in the clinical setting.[2,3,6,7] Current international guidelines suggest continuing the usage of antihypertensive drugs, in particular iRAS, in people with hypertension who become ill with COVID-19 with no reported differences between different classes of antihypertensive agents.[8,9] Although most studies do not point out a negative effect of the virus on blood pressure levels, there is information about the different iRAS classes' effects. Mandeep R et al (2020) found some differences between the effects of angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) and a possible difference in direct renin inhibitors administration (DRi) is presumed.[5,10] Considering the different mechanisms of pressure reduction by iRAS, one can expect differences in people with COVID-19 receiving these drugs.

Aim

In this regard, in March 2020, we initiated a study which was aimed to pinpoint possible clinical and laboratory differences in people with hypertension who received iRAS and suffered coronavirus infection.

Material and methods

Ethics approval

Local ethic commission (21.02.2020 №1) and Shupyk National Healthcare University of Ukraine, Kyiv, Ukraine (24.04.2020 №16) approved the study.

Study design

BIRCOV trial (AR**B**, ACE**I**, D**R**i in **CO**VID-19) registered in ClinicalTrials.gov (NCT04364984) was accepted and completed. The study began on April 1, 2020, primary completion was achieved on July 24, 2021, and final results were available on August 1, 2021 (https://clinicaltrials.gov/ct2/show/results/NCT04364984?term=NCT04364984&cntry=UA&draw=1&rank=1). The study protocol originates from POEM (Patient-Oriented Evidence that Matters) (https://wilkes.libguides.com/c.php?g=191942&p=1266516) intervention that was performed as an open prospective randomized two medical center trial in subjects suffering from COVID-19 who have been receiving iRAS: either ACEi, ARB or DRi as basic antihypertensive therapy.

120 people with hypertension and confirmed COVID-19 infection have been screened and 112 Caucasian patients with confirmed COVID-19 and stage 1-2 hypertension receiving iRAS at the onset of COVID-19 had been included and inspected for 24 weeks. The stage of hypertension has been assessed according to the 2018 ESC/ESH Guidelines.[11] The sampling method was of a non-probability type, including male and female patients with an age range of 18-90 years old. The inclusion criteria were hypertension, stage 1-2 and confirmed COVID-19 infection. The exclusion criteria were hypertension stage 3, HF (NYHA) 3-4.[12] The cohort has been subdivided into three groups based on the iRAS type prescribed.

COVID-19 infection was confirmed by a PCR test and defined into alpha, beta, gamma, and delta subtypes according to CDC (www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). Detailed features of the detected coronavirus SARS-CoV-2 strains were presented as following variants (strains):

1. del69-70, N501Y, 144del, A570D, D614G, P681H, T716I, S982A, D1118H Alpha B.1.1.7 British Variant 50% increased transmission Potential increased severity based on hospitalizations and case fatality rates No impact on susceptibility to EUA* monoclonal antibody treatments Minimal impact on neutralization by convalescent and post-vaccination sera.
2. E484K, N501Y, del242-244, D80A, D215G, K417N Beta B.1.351 South-African Variant 50% increased transmission Significantly reduced susceptibility to the combination of bamlanivimab and etesevimab monoclonal antibody treatment, but other EUA* monoclonal antibody treatments are available. Reduced neutralization by convalescent and post-vaccination sera
3. E484K, N501Y, L18F, T20N, P26S, D138Y, R190S, K417T, D614G, H655Y, T1027I Gamma P.1, B.1.1.28.1 Japan-Brazilian Variant No impact on transmissibility Significantly reduced susceptibility to the combination of bamlanivimab and etesevimab monoclonal antibody treatment, but other EUA* monoclonal antibody treatments are available Reduced neutralization by convalescent and post-vaccination sera.
4. T19R, T95I, G142D, E156-R158G, L452R, T478K, D614G, P681R, K417N Delta B.1.617.2 /Delta Plus AY.1&2 Indian Variant increased transmissibility Potential reduction in neutralization by some

EUA* monoclonal antibody treatments Reduced neutralization by post-vaccination sera

To date, 45 patients (42%) have received the vaccinations against COVID-19 before the disease's onset.

The BIRCOV trial establishment sought to analyze the clinical data (Clinical arm) of included patients with the follow-up checkpoints of 12 and 24 weeks. Additionally, we highlighted a group of CKD patients from the whole cohort (Kidney arm), where the CKD stage had been assessed by eGFR according to 2012 KDIGO guidelines.[13] All the patients were randomized from the family doctor (clinical arm) and nephrology clinic (kidney arm).

The disease follow-up had two checkpoints: 12- and 24-weeks. The primary outcome measure was blood pressure (BP) levels in mm Hg, measured using ambulatory BP monitoring (ABPM), or home BP monitoring (HBPM) one week before COVID-19 infection and tested during the disease onset on weeks 2, 4, 12, 24 using in-clinic monitoring in case of hospitalization. Low blood pressure was defined below 90/60mmHg.The secondary outcome measures were: the number of patients with fever (above 37.2°C) up to 3 weeks after COVID-19 onset, the number of patients with cough (12 weeks after onset), the number of patients with throat pain (2 weeks after onset), the number of patients with diarrhea (2 weeks after onset) and the number of patients who needed hospital admission and intensive care unit (24 weeks after onset). The additional outcome measures for the Kidney arm were the estimated glomerular filtration rate (eGFR) measures as primary and the albuminuria levels as secondary. Figure 1 represents the summary of the study design.

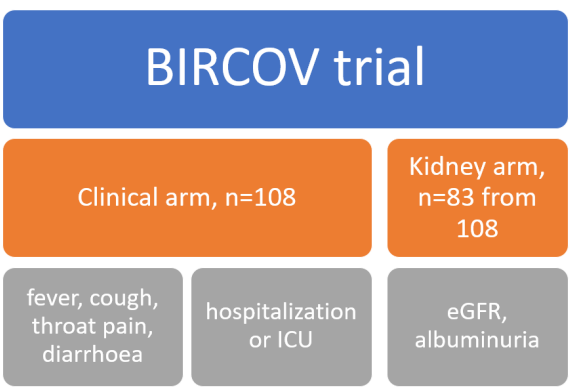

Fig. 1. The summary of the Study design. Total number of patients admitted (108) were included in the Clinical arm of the design, where 83 patients were eligible for the Kidney arm. The endpoints included fever, cough, throat pain, diarrhea, hospitalization or intensive care unit (ICU) admission, estimated glomerular filtration rate (eGFR) and albuminuria levels

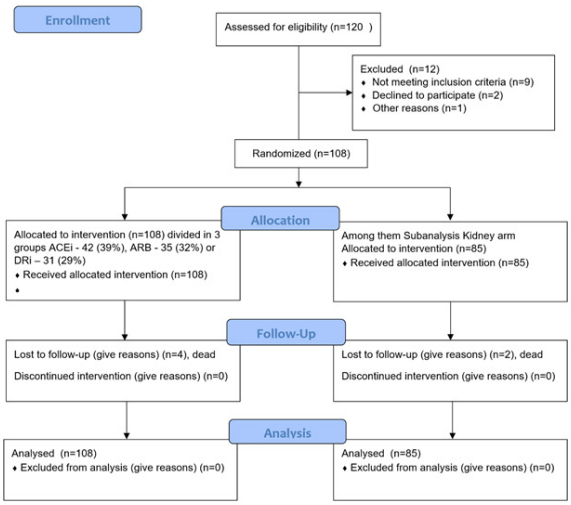

Fig. 2. The Diagram of Statistical Analysis Plan (CONSORT – transparent reporting of trials)

Additionally, the Hydration status of patients according to Ivanova et al. was assessed.[14]

Statistical evaluation of the research results was carried out in the package of medical statistics (https://www.gigacalculator.com/calculators/). The type of statistical test was superiority. The tool used for statistical analysis of outcome measure data and the calculated p-value was ANOVA. The estimation parameter used was Cox Proportional Hazard, parameter dispersion type was standard deviation.

The risk of progression to kidney failure requiring dialysis or transplantation (using the Kidney Failure Risk Equation) (https://qxmd.com/calculate/calculator_308/kidney-failure-risk-equation-4-variable) had been calculated for all patients of the Kidney arm on 2, 4, 12 and 24 weeks from COVID-19 onset.

All patients gave their consent to submit their personal data.

The complete diagram of the statistical analysis plan is presented in Figure 2.

Results

We have conducted a screening of 120 outpatient subjects with COVID-19 and hypertension; 112 were enrolled; 108 (96%) completed the study; 60 (56%) males and 48 (44%) females, mean age of 55±1,12 (18-87; coefficient of variation 0.210514, coefficient of asymmetry -0.261873) years old. Four (3,7%) patients (2 males, 2 females) had died during the first 2 months of COVID-19 onset.

Coronavirus SARS-CoV-2 strains of enrolled patients are represented in Table 1.

Among 108 hypertensive patients enrolled in our trial, 35 (32%) had stage 1 hypertension, and 73 (68%) had stage 2.

Eighty-three (77%) subjects had CKD, ranging from 1 to 4 stages: CKD 1 – 23 (27%), CKD 2 – 46 (56%), CKD 3 – 10 (12%), CKD 4 – 4 (5%).

All patients were randomized into 3 groups who received iRAS: ACEi - 42 (39%), ARB - 35 (32%), or DRi – 31 (29%). Among the participants of the study, 11 (10%) people were over 65 years of age, among them, from each group, 6 people (14.29%) received ACEi, 4 (11.4%) ARB, and one (3.23%) DRi. Eighty-four subjects (78%) received the combined therapy of iRAS with calcium channel blockers (CCB) and diuretics, 17 (16%), combined iRAS with B-blockers, 7 (6%) received iRAS monotherapy.

Table 1. Characteristics of the type of coronavirus strains revealed in enrolled patients

Coronavirus SARS-CoV-2 strains	Number of people, n=108
Alpha, abs, %	16 (15%)
Beta, abs, %	23 (21%)
Gamma, abs, %	22 (20%)
Delta, abs,%	47 (44%)

Clinical arm

The reason for the prescription of iRAS and its combination with other antihypertensive agents was the presence of hypertension itself. Among 108 (96%) hypertensive persons who finished the trial 35 (32%) previously had stage 1 hypertension, and 73 (68%) had stage 2. Thus, a week before the development of COVID-19, the mean blood pressure was 137±0.9/83±0.6 mm Hg (coefficient of variation 0.067728, coefficient of asymmetry 1.029771). The dynamics of changes in blood pressure by control points are shown in Table 2 and Figure 3.

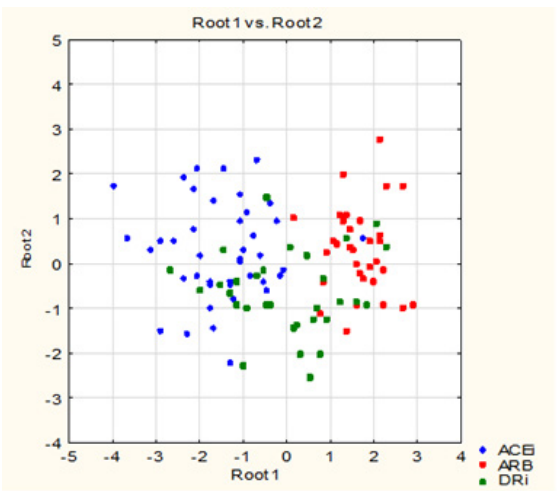

Fig. 3. Systolic and diastolic blood pressure values 2 weeks after the onset of COVID-19. Root 1, diastolic BP; Root 2, systolic BP, 0- 120/80 mm Hg, step - 10 mm Hg, ACEi – angiotensin-converting enzyme inhibitors; ARB – angiotensin receptor blockers; DRi – direct renin inhibitors

Table 2 which presents the baseline BP numbers in dynamics according to weeks and groups of antihypertensive treatment has shown that the BP changes did not have significant statistical differences between the chosen medicine one week before enrolment.

Table 2. The baseline BP mm Hg values before the COVID-19 onset and with a follow-up of 2, 4, 12 and 24 weeks in ACEi, ARB and DRi groups*

Drug group/ Week	-1	0	2	4	12	24	p (1-0)	p (0-2)
ACEi. n=42	138±1.1/ 83±1.2	126±1.2/ 77±0.7	104±0.9/ 68±0.6	114±1.1/ 72±0.7	128±1.2/ 77±1.0	137±1.2/ 81±1.2	<0.01	<0.01
ARB.n=35	136±1.1/ 82±1.2	132±1.0/ 78±0.7	131±1.0/ 77±0.6	133±1.0/ 78±0.6	135±1.1/ 79±0.9	137±1.2/ 82±1.2	0.02	<0.01
DRi. n=31	134±1.4/ 82±1.2	127±1.2/ 79±0.6	115±0.9/ 70±0.6	121±0.9/ 74±0.6	125±1.0/ 79±0.8	129±1.2/ 80±1.2	<0.01	<0.01

* ACEi – angiotensin-converting enzyme inhibitors; ARB – angiotensin receptor blockers; DRi – direct renin inhibitors

However, we had a documented trend of BP lowering in the first two weeks of the COVID-19 disease (Figure 3) with its gradual return to baseline values up to the 12th week. Twenty-three (21%) patients had withdrawn medicine for up to 2 weeks due to severe hypotension. The BP values after COVID-19 in most subjects however remained lower than the baseline for 4 weeks of follow-up.

The analysis of individual data demonstrated that 16 (38%) patients with hypertension taking ACEi had to cancel the medicine or lower the dosage in the first 10-14 days of the COVID-19 disease due to pronounced hypotension development. From the group of patients taking DRi, 7 (23%) had a mildly softer decline in BP. Patients in the ARB group had little to no decline in BP. This decline had no relation to dehydration or fever.

The data obtained indicate that the use of ACE inhibitors significantly increases the risk of withdrawal compared to DRi (RR 1.648 95% CI 0.772–3.519, NNT 7.0) and ARB (RR 13.023 95% CI 1.815–93.426, NNT 2.9) due to COVID-19.

No less interesting was the restoration of normotensives after the onset of coronavirus infection. It turned out that in the group of those taking DRi, after 4 weeks there were practically no significant differences from the starting BP, and after 12 weeks the consequences of hypotension were completely eliminated. On the contrary, in people who took ACEi, lower blood pressure values were still maintained in the post-COVID period.

The secondary outcomes measures: number of patients with fever (above 37.2C) (follow-up: 12 weeks), number of patients with cough (up to 12 weeks), number of patients with throat pain (up to 2 weeks), number of patients with diarrhea (up to 2 weeks) and number of patients who needed hospitalization and intensive care unit (up to 24 weeks), representing the course of COVID-19 in the BIRCOV study, are shown in Table 3.

Analysis of clinical symptoms did not reveal any dependence on the type of antihypertensive therapy with an iRAS. The mortality rate was 3.7% (4 patients). Two

of the patients received an ACEi and two received an ARB. The absolute risk for ARB compared to DRi was 0.057, for ACEi – 0.048 versus DRi. Thus, the absolute risk of death in people with COVID-19 receiving ARB was higher than in people taking ACEi, despite the presence of more severe hypotension in the first 4 weeks from COVID-19 onset.

Table 3. The secondary outcomes characteristics with a follow-up of 2, 4, 12 and 24 weeks in ACEi, ARB and DRi groups

Number of participants with clinical presentation, % / time frame in weeks	Onset	2 weeks	3 weeks	4 weeks	12 weeks	Relative risk
Fever (above 37.2°C)	101 (ACEi – 39, ARB – 32, DRi – 30) (90%)	12 (11%)	0	2 (2%)	0	Onset – 3 weeks: RR 8.417 95% CI 4.926–14.382, NNT 1.213
Cough	87 (ACEi –30, ARB– 29, DRi– 28) (78%)	78 (70%)	0	0	3 (3%)	
Throat pain	56 (ACEi – 21, ARB 19, DRi –16) (50%)	1 (1%)	0	0	0	
Diarrhea	8 (ACEi– 4, ARB –3, DRi –1) (7%)	0	0	0	0	
Hospital and intensive care unit	4 (ACEi – 2, ARB – 2, DRi – 0) (3.5%)	18 (16%)	4 (4%)	1 (1%)	0	Onset – 2 weeks: RR 0.222 95% CI 0.078–0.635, NNT 7.714
Died			3 (2.6%)	1 (1%)	0	3 to 4 weeks: RR 3.00 95% CI 0.317–28.390, NNT 54.00

ACEi – angiotensin-converting enzyme inhibitors; ARB – angiotensin receptor blockers; DRi – direct renin inhibitors

Other complaints in people with COVID-19 included fatigue (58%), headache (44%), attention deficit disorder (27%) and shortness of breath (24%) in the first 2 weeks after the disease onset. Significant differences were found only on the clinical complaint of fatigue between ACEi and ARB groups (Fisher's criterion (bilateral) 0.00135, p<0.01; RR 2.197 95% CI 1.294–3.731, NNT 2.658).

In addition, a common manifestation of COVID-19 in people taking iRAS were severe symptoms of poor odor recognition (77%), partial loss of taste (18%), nasal vasculitis (6%) and persistent angioedema (3%).

Kidney arm

Characteristics of the causes of CKD were determined according to KDIGO, 2012 criteria.[13] They were presented by diabetes mellitus types 1 and 2 – 30 (36%), arterial hypertension – 15 (18%), kidney disease of unknown reason – 10 (12%), polycystic kidney disease 5 (6%) as ADPKD 3 and ARPKD 2 persons and other causes – 23 (28%), presented by urology diseases – 17 (CACUT syndrome – 6, chronic pyelonephritis – 11), glomerulonephritis – 5 (the kidney biopsy has been performed to 4 from 5 patients), and 1 with lupus nephritis class IV.

Mean arterial pressure did not differ statistically in the group of patients with CKD compared with the entire sample of people included in the study.

Twenty-three (21%) patients required dose reduction or discontinuation of antihypertensive drugs for up to 2 weeks due to severe hypotension. Among them, 16 (70%) people were taking ACEi and 7 (30%) – DRi. Post-COVID-19 BP values remained below baseline for the majority of subjects for the next 4 weeks. A more significant decrease in BP was observed in patients with grade 1 hypertension: 20 (57%) versus 29 (39%) with grade 2 hypertension (RR 1.438 95% CI 0.962–2.152, NNT 5.742) and in people with CKD: 62 (75 %) vs. 9 (36%) without CKD (RR 2.075 95% CI 1.212–3.552, NNT 2.584). This decrease was not associated with dehydration due to hyperthermia. Patients in the ARB group did not experience a significant decrease in blood pressure.

Findings show that the use of ACEi significantly increases the risk of discontinuation compared with DRi (RR 1.648 95% CI 0.772–3.519, NNT 7.0) and ARB (RR 13.023 95% CI 1.815–93.426, NNT 2.9) in patients affected with COVID-19.

To date, in this group of patients, normotension was restored after the onset of coronavirus infection. In the group taking DRi after 4 weeks there were practically no significant differences from the initial pressure, and after 12 weeks the effects of hypotension were completely eliminated. In contrast, people who took ACEi still had lower blood pressure values in the post-COVID-19 period.

Of 4 people who died during the study, two of the patients received an ACEi, and two received an ARB, and each group featured one CKD patient respectively. The risk of death was lowest for those receiving DRi, the absolute risk for ARB versus DRis was 0.057 (number of patients to be treated (NNT) 17,500), for ACEi versus DRi 0.048, and the number of patients to be treated – 21.000; the absolute risk for anti-ACEi ARBs was 0.057 (RR 1.200 95% CI 0.178–8.087, NNT 105.0).

Table 4 represents the baseline eGFR values with a follow-up every 2, 4, 12 and 24 weeks in ACEi, ARB and DRi groups.

The synchronous decline of eGFR and systolic BP was more pronounced in CKD patients. The greatest decrease in eGFR was noted in people who had been taking ACEi, weeks 0–24: the correlation coefficient (r) is 0.815, the relationship between the studied features was direct, the strength of the relationship according to the Chaddock scale was high, the number of degrees of freedom (f) is 3, the Student's t-test was 2.432, although the dependence of the features was statistically insignificant (p=0.1356).

The individual analysis demonstrated that eGFR decline correlated directly with the advancement of CKD. The drop in eGFR ranged from 23% in CKD 1 to 45% in CKD stage 4. Two people required short-term dialysis.

The analysis of secondary outcome points demonstrated that 23% of people without 3-12 months available preceding albuminuria had developed the A2 range albuminuria according to KDIGO, 2012 (13). During 12 weeks of observation, 81% of patients had spontaneous albuminuria withdrawal. Post COVID-19 (above 12 weeks) albuminuria remained in 19% of patients, and 90% of them have had CKD.

Table 4. The changes in eGFR (ml/min/1.73 m^2) and ACR (mg/mmol) are presented in dynamics according to weeks and groups of antihypertensive treatment*

Drug/week	0	2	4	12	24	p (0-2)	p (0-4)
ACEi, n=42	69±1.7	52±1.1	51±0.9	58±2.0	68±1.9	<0.01	<0.01
ARB, n=35	72±1.7	70±1.8	73±1.5	70±1.6	71±1.8	not reliable	
DRi, n=31	71±1.8	70±1.6	69±1.5	72±1.7	70±1.7		
Urine/week, interquartile range	0	2	4	12	24		
ACR, mg/mmol	226.5	473.5		550.5	372.0	p (0-2, 0-12) <0.01	

* ACEi – angiotensin-converting enzyme inhibitors; ARB – angiotensin receptor blockers; DRi – direct renin inhibitors

Patients with preceding CKD had an increase in albuminuria in 78% of cases and its return to the past results were observed only in 24% of patients by the 12th week and 49% in 24 weeks respectively. The interquartile range of albumin/creatinine ratio (ACR) was estimated in 24 patients with CKD. The ACR ratio in patients treated with ACEi, ARB, and DRi was 530, 161.5, and 9, respectively, but the mean values were not statistically distinguishable due to the large scatter of values due to varying degrees of severity of the primary renal process. The risk of a three-fold increase of ACR in the first 2 weeks from the onset of COVID-19 was 2.068 (95% CI 0.816-5.241, NNT 3.043) in the ACEi group, 0.75 95% CI 0.270-2.080, NNT 8.000) in the ARB group and 0.422 (95% CI 0.069–2.596, NNT 3.654) in the DRi group.

The post-COVID-19 syndrome was presented by the development of albuminuria in patients that were previously clear of it, and worsening albuminuria in patients that had it previously.

In people with COVID-19, by the second week from the onset of the disease, there had been a decrease in eGFR and, probably a reciprocal, increase in the level of uric acid in the blood, significantly higher from the baseline. Comparison of the two indicators in dynamics revealed a correlation coefficient of -0.871, the relationship between the studied features was inverse, and the strength of the connection according to the Chaddock scale was high, but the dependence of the features is not statistically significant (p=0.0914).

Discussion

The SARS-CoV-2 virus is ingested into the body through ACE2 receptors in the nose, penetrating the cells in other areas afterwards, de-allocating in other ACE2 receptors sites: the intestines, blood vessels and heart. These, possibly, explain the symptoms of heart disease, acute kidney injury and intestinal symptoms.[15,16] In children, who are known to have a lower expression of ACE2, the illness usually does not manifest with significant symptoms.[17]

If the SARS-CoV-2 virus inhibits the activation of ACE2 receptors in the muscles, they are expressed at low to medium levels, including ligands, explaining that in children, mature people without hypertension, and especially older people without hypertension, the symptoms of the disease can show up under the hour of COVID-19.[18]

ACEis perform their effect through the ACE1 receptor, while the SARS-CoV-2 uses the ACE2 receptor. Sequential metabolism of angiotensin 1–9, then angiotensin 1–7 goes two ways: 1) acts as an agonist through the Mas-1 receptors leading to vasodilation; 2) acts as an antagonist of angiotensin AT 1 receptor, enhancing vasodilation.[19]

Thus, the SARS-CoV-2 may be similar in ARB action, which explains hypotension in the acute period of coronavirus infection documented in BIRCOV trial. If the patients had taken ACEi and caught SARS-CoV-2 we documented the largest blood pressure decrease, and those who had taken ARB had practically no effect on blood pressure. This was some trend in increasing the risk of death in people with COVID-19 who are taking ARBs as an antihypertensive agent.[5]

Cohen et al. presented three possible mechanisms of the effect of RAS inhibitors, two of which, representing the detrimental role of ACEi and the neutral or beneficial role of ARB were replicated in the data of the BIRCOV study.[20] At the same time, a small triple-blind study has shown no reduction in blood pressure when using ACEi/ARB medications in hypertensive COVID-19 patients. Perhaps, observations in intensive care units and hospitals do not allow us to clearly depict the features that were established by us on an outpatient basis.[21]

There is a well-grounded idea that if the virus implements RAS changes, it should be done before the body's mechanisms for regulating bradykinin go out of whack. Receptors for bradykinin are restored, and the body also ceases to efficiently break down bradykinin (ACE ruins bradykinin, but if the virus suppresses it, you can't work it so effectively.) As bradykinin storm develops, we observe moderate arrhythmias and a decrease in BP.[22] Possibly, a stronger bradykinin storm led to an increase in the side effects of COVID-19 in apparently subacute angioedema in three patients in the BIRCOV study.

According to the data of the Italian registry, prospectively investigating 566 COVID-19 patients taking ACEi or ARB, those in ARB group have reduced the death rate of hospitalized patients, but this was not the case for the ACEi administration. Mortality due to both causes and hours of hospitalization was the primary outcome.[23]

However, these data are contradictory. The results of ACEI-COVID trial have shown that the discontinuation of RAS blockers in COVID-19 may lead to faster and better recovery.[24] Jia et al. (2021) concluded that patients with COVID-19 and hypertension may benefit from using ACEIs/ARBs.[25]

Most publications indicate the absence of negative effects of iRAS in people with COVID-19.[24,26] However, according to Reyes et al., COVID-19 patients with hypertension were more likely to suffer severe outcomes, hospitalizations and deaths compared with those without hypertension.[27] Wherein, the use of either beta-blockers, calcium-channel blockers, or diuretics was associated with a higher risk of COVID-19 hospitalization and mortality compared to ACEi use (adjusted OR (95%CI): 1.66 [1.43–1.93]) and ARB use (1.53 [1.30–1.81]).[28]

Adverse effects during the development of COVID-19 develop mostly in people with comorbid conditions which was confirmed in the present study.[29] The second most important result of the BIRCOV study was a transient decrease in renal function by eGFR for healthy people and quite pronounced for people with CKD, accompanied by an increase in albuminuria. These data are in good agreement with the known ones, claiming higher morbidity and mortality in patients with CKD.[8,30]

One of the most common COVID-19 side effects is the development of acute kidney injury (AKI). Simple risk scores using age, sex, a complete blood cell count, C-reactive protein and D-dimer are highly predictive of AKI and death and can help simplify and better inform clinical decision-making.[15] In the present study, 2 patients were identified and had a sharp deterioration in kidney function. At the same time, ACEi usage might help individualize pharmacological treatment and improve clinical outcomes,[31] which does not contradict our data. Polymorphism of ACE genes may also be of some importance: additional meta-analyses uncovered that both ACE1 rs4646994 DD-genotype and ACE2 rs2285666 GG-genotype carriers had a significantly increased risk of developing severe COVID-19 (OR=2.06, 95% CI: 1.45, 2.93; OR=2.14, 95% CI: 1.26, 3.66; respectively). Genetic polymorphisms of ACE1 rs4646994 DD-genotype, ACE2 rs2285666 GG-genotype, and TMPRSS2 rs12329760 CC-genotype and C-allele may serve as predictive models of COVID-19 severity.[32]

The BIRCOV study supports the available data on the absence of a negative effect of iRAS inhibitors on COVID-19[20]. Further studies are required the profoundly discover the characteristics of the course of COVID-19 infection in people with concomitant diseases, including hypertension and CKD in the ongoing clinical trials and meta-analyses of randomized trials to elucidate the optimal use of iRAS in patients with COVID-19.

Study limitations were determined by unavailable tools of outpatient ACR and uric acid measurements for some subjects.

- ***What is already known about this subject:***

1. It is known that SARS-CoV-2 uses an ACE2 receptor facilitating virus entry into host cells.
2. There are three known possible effects of ACEi and ARB in COVID-19 in clinical practice: condition worsening, neutral or helpful.
3. Considering the different mechanisms of iRAS pressure reduction, one can expect differences in people with COVID-19.

- ***What this study adds:***

1. People with mild hypertension, infected with COVID-19, develop hypotension while constantly receiving iRAS.
2. People with CKD stage 4-5 are at the highest risk of kidney function loss while infected with COVID-19.
3. Of the iRAS group, ACEi have demonstrated the highest risk of severe hypotension, eGFR decline and onset of albuminuria.

- ***What impact this may have on practice or policy:***

1. Patients with hypertension and/or CKD should be carefully monitored when receiving iRAS administration during COVID-19 infection.
2. We should carefully follow the BP, eGFR and albuminuria levels in CKD patients, receiving iRAS administration during COVID-19 infection.

Conclusion

COVID-19 has been shown to induce reversible hypotension in outpatients if they receive an ACEi for hypertension. The working hypothesis indicates DRi as the safest antihypertensive treatment drug in 24 weeks' follow-up observation with the least volatility of BP and mortality. The nature of BP reduction in people with hypertension of grades 1-2, taking iRAS, allows comparing the effect of SARS-CoV-2 with the action similar to ARB, i.e. in people taking ACEi, the effect of BP reduction was the most sufficient. Hypertensive patients, affected by COVID-19, may experience a transitory renal function

deterioration with an incidence of albuminuria but this statement needs more research in parallel groups. In patients with hypertension and CKD, these effects were more pronounced and correlated with eGFR levels. Not all patients with CKD had a return to baseline albuminuria and renal function after COVID-19.

Declarations

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Author contributions
Conceptualization, D.I. and A.G.; Methodology, D.I.; Software, T.C.; Validation, T.C.; Formal Analysis, T.C.; Investigation, Z.I. and D.I.; Resources, Z.I., M.I. and D.I.; Data Curation, Z.I.; Writing – Original Draft Preparation, D.I.; Writing – Review & Editing, D.I and M.I.; Visualization M.I.; Supervision, D.I. and A.G.; Project Administration, M.I.; Funding Acquisition, Z.I.

Conflicts of interest
The authors declare no conflict of interest.

Data availability
Data available on request from the authors.

Ethics approval
Local ethic commission (21.02.2020 №1) and Shupyk National Healthcare University of Ukraine, Kyiv, Ukraine (24.04.2020 №16) approved the study.

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ORIGINAL PAPER

Satisfaction with perinatal care in women giving birth during the COVID-19 pandemic

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ABSTRACT

Introduction and aim. The role of a medical team during the perinatal period is significant, since it not only focuses on the patients' physical health, but also impacts mental wellbeing. The aim of this study is to compare the level of satisfaction with the quality of care provided by healthcare professionals during pregnancy and perinatal period before and during the COVID-19 pandemic.

Material and method. The study was conducted among 102 women who had at least two births, one in the pre-pandemic period and the other during the pandemic. An original questionnaire (53 items) was used to assess the quality of medical care.

Results. The assessment of the quality of medical care, and the emotional and informational support received from medical personnel during pregnancy and perinatal care was significantly higher before the COVID-19 pandemic ($p<0.001$). During the pandemic, the respondents experienced significantly more anxiety about their health ($p=0.027$) and their baby's health ($p=0.028$) as well as anxiety caused by the lack of a partner during labor ($p<0.001$).

Conclusion. It is necessary to further evaluate the quality of medical care for pregnant and perinatal women in order to determine the best possible procedures for the functioning of health care in the time of a pandemic.

Keywords. COVID-19 pandemic, pregnancy, quality of perinatal care

Introduction

The perinatal period is a novel situation for the patient accompanied by wide range of emotions including fear and anxiety for the health of her and the baby. It is also associated with fear of the pain and suffering that awaits them during labor. Therefore, appropriate medical care by healthcare staff is of great importance.[1] The World Health Organization reported that about 830 women die every day from preventable pregnancy and delivery-related causes, with 99% of all maternal deaths occurring in developing countries.[2]

Perinatal care is a multidisciplinary activity aimed at providing medical care together with health promotion and treatment in the preconception period, during pregnancy, childbirth and the postpartum period.[3] The role of the medical team, which includes an obstetrician-gynecologist attending pregnancy, a midwife, a nurse and a physiotherapist, is not limited only to the physical sphere of a woman during pregnancy and puerperium. Its significant influence on the mental sphere has also been demonstrated in the literature. Patients' satisfaction with medical care is perceived as the degree of compliance between their expectations regarding the quality of services and the actual reception of medical care. It depends i.e. on the degree of respect for the patient's rights, providing emotional support, showing in-

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terest and care, or responding to the patient's requests. The sense of satisfaction also depends on the provision of appropriate medical and information support regarding the course of labor, the procedures and measures used, as well as information about the baby's health condition and the correct application of professional skills by medical personnel.[1,4]

The success of the appropriate perinatal preparation for the birth of a new human being depends on the comprehensive involvement of the medical team and the family.[5] Thanks to the activities in the perinatal care undertaken by i.e. midwives or physiotherapists in childbirth classes, women gain valuable knowledge about the physiological changes during pregnancy, the course of childbirth and the postpartum period, as well as skills in the care of a new-born baby. In addition, they learn the principles of pregnancy hygiene and proper diet, acquire information related to teratogenic factors, and learn how to fight ailments that appear during and immediately after pregnancy.[5,6] Women who obtained comprehensive information on the course of labor from medical personnel had a greater sense of security and showed less stress before childbirth.[1] On the other hand, the prevention of postpartum mood disorders, known as "baby blues", includes counteracting all negative factors by implementing protective factors in the form of good interpersonal relationships with family and medical staff, providing the patient with a sense of security and a support network, reducing social isolation.[1,7]

Viral pandemics threaten the general population, including pregnant women. COVID-19 is caused by the SARS-CoV-2 coronaviruses, which are positive sensitivity single-stranded RNA viruses. Generalizing pregnancy as a state of immunosuppression or an increased risk of infection is a misleading concept. Pregnancy is a unique immune state that is modulated but not suppressed.[8] Research by Chivers et al. found that some women postponed or skipped antenatal visits because of fear of contracting COVID-19, and considered giving birth at home. Women experienced a higher level of isolation by preventing their partner from being present during routine examinations and the delivery.[9] Currently, most maternity units and scientific bodies express the opinions that a supporting person should be allowed to be present during labor, provided that they have no signs of COVID-19 disease, wear a surgical mask, and follow hand-washing and disinfection procedures.[10,11]

The study by Hui et al. demonstrated that measures to reduce the spread of COVID-19 have significantly increased the number of patients with postpartum depression, which was related to the inability to have hospital visits, preventing undertaking childcare activities, or the inability to organize celebrations related to the birth of a baby. Moreover, a low level of satisfaction with medical care was observed due to the lack of access to non-pharmacological pain relief measures, such as childbirth massage.[12] In women suspected or confirmed to have COVID-19 disease, appropriate caution should be exercised in childbirth. Labor management should be carried out in a safe manner, with minimal staffing requirements to reduce exposure, but with the ability to provide urgent obstetric, anesthetic and neonatal care when necessary.[13]

Aim

The aim of this study was to compare the level of satisfaction with the quality of care provided by healthcare professionals during pregnancy, labor and the postpartum period before and during the COVID-19 pandemic.

Material and methods

Ethical approval

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszow No. 2022/004.

Material

The study was conducted from December 2020 to October 2021 and enrolled women who had at least two births. The on-line questionnaire was posted in groups for pregnant women on the social networking site.

The eligibility criteria for the study were: age from 20 to 45 years, minimum two births, including one in the pre-pandemic period and the other in the COVID-19 pandemic period. Exclusion criteria from research and analyses were: COVID-19 occurrence during perinatal hospitalization, leaving the questionnaire items unanswered.

Research tool

The study used the proprietary questionnaire, which includes items regarding the assessment of the quality of perinatal care before and during the COVID-19 pandemic. The questionnaire consisted of 54 items divided into five domains. The first part contained 4 items on socio-demographic data. The second part (13 items) focused on questions about the course of the pregnancy. The next part included 26 items related to hospitalization and the course of labor, the fourth part contained 4 items about the postpartum period. The subjects provided responses to the questions about the quality of medical care in a 5-point scale, ranging from: 1 – definitely positive, 2 – rather positive, 3 – no opinion, 4 – rather negative to 5 – definitely negative. The last part of the questionnaire focuses on issues related to the physiotherapy of patients during pregnancy and in the postpartum period (6 items).

Statistical analysis

The statistical analysis of the collected data was performed with the Statistica 13.3 statistical program (StatSoft Inc. Tulsa, OK, USA).

The basic statistical description includes mean values ($\bar{x}$) with standard deviation (SD), and median values (Me). The analysis was performed using the non-parametric Wilcoxon pairwise test, which assessed the significance of changes in the answers given by the respondents to the same item in relation to two different periods – the time before the pandemic and during the COVID-19 pandemic. Qualitative data were presented in the form of frequencies numerical (N) and percentage (%). The level of statistical significance was adopted at $p<0.05$.

Results

One hundred and two women with the mean age of 30 years (in the range from 20 to 45 years, Me=29) were qualified for the analysis. Among the respondents, the largest percentage (82.35%) were married women, while 13.73% of women declared themselves unmarried, and 3.92% as divorced. The largest percentage (31.37%) of women lived in rural areas, followed by the group (29.41%) of urban residents of cities up to 50,000 inhabitants, while the smallest percentage (6.86%) lived in large cities with more than 250,000 inhabitants. Among the respondents, the greatest number had secondary education (44.4%), followed by higher education (27.38%), with basic vocational education (26.05%), and the smallest number with lower secondary education (2.17%).

Table 1. Assessment of availability of the attending physician and other specialists / tests during pregnancy*

Availability of physicians	Specialist physicians/ tests during pregnancy				Physician attending the pregnancy			
	Before COVID-19		During COVID-19		Before COVID-19		During COVID-19	
	n	%	n	%	n	%	n	%
Definitely positive	15	14.7	3	2.9	19	18.6	17	16.7
Rather positive	55	53.9	13	12.7	52	51.0	48	47.1
No opinion	22	21.6	29	28.4	21	20.6	22	21.6
Rather negative	10	9.8	45	44.1	9	8.8	11	10.8
Definitely negative	0	0	12	11.8	1	1	4	3.9
Total	102	100	102	100	102	100	102	100
p	Z=7.37; p<0.001				Z=1.46; p=0.142			

* n – number; % – percent; Z – value of the Wilcoxon Matched-Pairs test; p – value of the probability of the test

Before the pandemic, 16.7% of the respondents had a problem with finding a suitable doctor in charge of pregnancy, compared to on average twice as many women 35.3% who encountered that issue during the pandemic. This difference was statistically significant (p=0.001). Availability of the doctor in charge of pregnancy did not differ significantly in the period before and during the COVID-19 pandemic (p=0.142), while the availability of other specialist doctors / tests during pregnancy was significantly better assessed before the pandemic than during the COVID-19 pandemic (p<0.001) (Table 1).

Both the quality of prenatal care by the attending physician and the quality of hospital care during labour by medical staff were assessed at a significantly higher level in the pre-pandemic period compared to care during the COVID-19 pandemic (p<0.001) (Table 2).

Table 2. Assessment of the quality of antenatal care by the attending physician and hospital care during childbirth by the medical staff*

Assessment of the quality	Antenatal care by the attending physician				Hospital care during childbirth by the medical staff			
	Before COVID-19		During COVID-19		Before COVID-19		During COVID-19	
	n	%	n	%	n	%	n	%
Definitely positive	33	32.4	16	15.7	25	24.5	12	11.8
Rather positive	50	49	36	35.3	50	49	37	36.3
No opinion	9	8.8	26	25.5	18	17.6	28	27.5
Rather negative	9	8.8	20	19.6	8	7.8	19	18.6
Definitely negative	1	1	4	3.9	1	1.0	6	5.9
Total	102	100	102	100	102	100	102	100
p	Z=4.70; p<0.001				Z=4.27; p<0.001			

* n – number; % – percent; Z – value of the Wilcoxon Matched-Pairs test; p – value of the probability of the test

The possibility of a partner being present during labor was much more frequent in the period before the pandemic (66.7%) than during the COVID-19 pandemic (2.9%) (p<0.001). The impact of not having a partner during labor on the well-being of women was comparable in the period before and during the COVID-19 pandemic. For 80% of women giving birth before the pandemic and 74.2% of women during the pandemic, the lack of a partner deteriorated their sense of wellbeing during childbirth.

Table 3. Assessment of emotional and informational support just before, during and after childbirth from medical staff*

Assessment of support	Emotional				Informational			
	Before COVID-19		During COVID-19		Before COVID-19		During COVID-19	
	n	%	n	%	n	%	n	%
Definitely positive	27	26.5	22	21.6	29	28.4	11	10.8
Rather positive	54	52.9	44	43.1	53	52	39	38.2
No opinion	18	17.6	23	22.5	19	18.6	24	23.5
Rather negative	1	1	12	11.8	1	1	24	23.5
Definitely negative	2	2	1	1	0	0	4	3.9
Total	102	100	102	100	102	100.0	102	100
p	Z=3.03; p=0.002				Z=6.04; p<0.001			

* n – number; % – percent; Z – value of the Wilcoxon Matched-Pairs test; p – value of the probability of the test

The respondents assessed both the emotional and informational support received immediately before, during and after childbirth from medical staff significantly better before the pandemic than during the COVID-19 pandemic. The difference in emotional support was statistically significant at p=0.002, and the difference in information support was statistically significant at p<0.001 (Table 3).

The level of stress related to childbirth differed statistically significantly among women in the period before and during the COVID-19 pandemic (p<0.001). Pre-pandemic stress levels amounted to approx. 5.01 (SD=2.28; Me=5) points/10 points, while during the COVID-19 pandemic it was on average 7.18 (SD=2.22; Me=7) points/10 points. The causes of stress in the perinatal period were statistically significantly different in the period before and during the COVID-19 pandemic. During the pandemic, significantly more often than before, the respondents felt anxiety about their own health (p=0.027), anxiety about the child's health (p=0.028) and anxiety caused by the lack of a partner during labor (p<0.001). In addition, they felt the fear of contracting COVID-19 and the fear of the lack of a doctor, e.g. due to being in quarantine (Table 4).

Table 4. Causes of stress in the perinatal period*

Causes of stress in the perinatal period	Before COVID - 19		During COVID - 19			
	n	%	n	%	Z	P
Fear of childbirth / pain	65	63.7	54	52.9	1.72	0.085
Concern for one's health	34	33.3	49	48.0	2.21	0.027
Concern for the baby's health	91	89.2	100	98.0	2.2	0.028
Anxiety caused by having to stay in the hospital for several days	26	25.5	19	18.6	1.03	0.302
The anxiety of not having a partner in childbirth	8	7.8	42	41.2	4.94	<0.001
Fear of contracting COVID-19	0	0	53	52.0	-	-
Fear of doctor's absence due to, for example, being in quarantine	0	0	26	25.5	-	-
Other	1	1	1	1	0	1

* n – number; % – percent; Z – value of the Wilcoxon Matched-Pairs test; p – value of the probability of the test

In the postpartum period, visits by the midwife in the patients' home after childbirth took place during the COVID-19 pandemic on average twice less frequently than before the pandemic. This difference was statistically significant (p<0.001). A similar number of respondents during the pre-pandemic and the COVID-19 pandemic indicated the need for a physiotherapist's assistance during or after pregnancy. Both before and during the pandemic, the same number of women had the opportunity and benefited from the physiotherapist's assistance, and the intended effects of physiotherapy were achieved, respectively in 66.7% and 83.3% of the respondents.

Discussion

Research conducted on human coronaviruses, before the outbreak of the COVID-19 pandemic, indicates that a pregnant woman and her fetus can be considered a high-risk group due to a number of physiological changes. This is due to the burdens generated by the developing pregnancy on the female body, from the circulatory and respiratory system to the immune system.[13,14]

The COVID-19 pandemic has led to a significant reduction in the number of antenatal and postnatal care services. A study by Kotlari et al. in the USA showed that one third of pregnant women had an increased level of stress due to changes in prenatal appointments. The number of antenatal visits has dropped to an average of six. Women also resigned from visits due to lack of transport, family pressure to remain in isolation and personal fear of the virus.[1,15] In the Netherlands, the initial visit to the doctor was made at 10-12 weeks of pregnancy for blood tests and ultrasound. Subsequent visits were made via phone or online. A similar situation occurred in France and Great Britain, where consultations were held by telephone.[2,16] In Poland, women indicated limited access to health services, such as the possibility of undergoing recommended tests or seeing a doctor.[11] This is also confirmed by our study, the results of which indicate that the availability of specialist doctors and tests performed for the needs of patients was significantly better assessed before the pandemic than during the COVID-19 pandemic (p<0.001).

Maternity facilities in European Union countries often severely limited the number of hospital visits. Some hospitals in Great Britain stopped allowing the presence of partners during childbirth, which influenced the emotional changes of pregnant women. In the Netherlands, on the other hand, one person could be present during childbirth, even if she had symptoms of COVID-19, however, they had to carry personal protective equipment and keep a distance. In France, a partner could be present during childbirth, as long as he was wearing a protective mask.[2] In Poland, however, the presence of an accompanying person was initially impossible, and later they had to perform a PCR test for COVID-19 up to 72 hours before delivery.[11] Our study showed that more than 40% of women experienced anxiety caused by the lack of a partner during childbirth.

Pregnant women undergo many physical and mental changes during pregnancy, and their susceptibility to various life stresses increases during this period. Pregnancy is considered to be one of the most stressful periods in a woman's life. During a pandemic, pregnant women experience increased levels of stress. It consists of many factors, from concerns about health and well-being to the lack of presence and support from loved ones. A woman who has had history of postpartum and perinatal complications, or has high risk pregnancy, is at an even higher level of stress. In such a case, a pregnant woman requires greater attention of the staff, often consultations with specialists in various fields, and therapeutic assistance.[17] Studies by Zhao et al. and Grigoriadis et al. have shown that the intensity of stress during Covid-19 in the last trimester is greater than in the first 6 months of pregnancy. Therefore, in this period the chances of developing anxiety and depressive disorders increase.[9,10,18,19] In our study, the level of stress related to childbirth differed statistically significantly in the period before the pandemic ($\bar{x}$ =5.01 points/10 points)

and during the COVID-19 pandemic ($\bar{x}$ =7.18 points/10 points), (p<0.001). The respondents declared that during the pandemic they felt fear for their health (48%), their baby (98%), the fear of not having a partner during childbirth (41.4%) or even of the lack of a doctor in charge of the pregnancy who, for health reasons caused by the coronavirus, could be on leave or in quarantine (25.5%). The above observations are also in line with the studies by Mariño-Narvaez et al. and Lebel et al., which showed that during a pandemic, a high level of anxiety and depression symptoms occurs in the majority of pregnant women. They experienced great anxiety for their own and their child's lives. These concerns were intensified by insufficient medical care, relationship tension, as well as social isolation caused by the COVID-19 pandemic.[20,21]

One of the important factors indicated in studies influencing the satisfaction of women with childbirth is the support provided during its duration.[22] In our study, the respondents assessed the emotional and informational support as well as the overall quality of care received from medical personnel just before, during and after childbirth significantly better (p=0.002, p<0.001 and p<0.001, respectively) before the pandemic than during the COVID-19 pandemic. Positive assessment of emotional and informational support was given by 14.7% and 31.4% less women, respectively during the pandemic than before the pandemic, and the overall hospital care was perceived positively by 25.3% less respondents. This may indicate the limitation of contacts between the staff and the patient due to the minimization of COVID-19 infections in health care facilities, which, however, has negative consequences in terms of the sense of quality of medical care. In the study by Kraśnianin et al., pre-pandemic nursing care was assessed positively by 91% of Polish patients. The respondents proposed the following forms of improving perinatal care: more accurate information about the child's condition, faster response to requests and comments, avoiding routine at work, better information between the therapeutic team, more medical staff, and better equipment in the delivery room.[23] Iwanowicz-Palus et al., analyzing the factors determining satisfaction of women after labor with the perinatal care provided, showed that those with higher education, having one child, giving birth naturally - positively assessing the course of pregnancy, childbirth, and experiences related to the delivery itself, assessed the provided perinatal care higher and better.[4]

The COVID-19 pandemic is a difficult moment in the perinatal period. There are many negative aspects with numerous consequences. Some of the participants managed to find some positives. Many partners due to social distancing requirements were transferred to work remotely at home. This provided support and companionship during this difficult period. In a study by Kolker et al., one of the respondents explained: "His presence provided companionship and support ... it was almost like a blessing in misfortune".[24]

Conclusion

The subjective assessment of the quality of medical care received during pregnancy and childbirth and the emotional and informational support provided by medical personnel was significantly higher before the COVID-19 pandemic than during it.

The level of stress related to childbirth significantly increased in women during the COVID-19 pandemic, and was mainly related to anxiety about the child's health and their own health, as well as the possibility of obtaining support from a partner during childbirth.

It is necessary to further evaluate the quality of medical care for pregnant and perinatal women and to verify the factors influencing it in order to determine the best possible procedures for the functioning of health care in the time of the COVID-19 pandemic in order to meet the expectations of women and improve the medical care provided.

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Declarations

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Author contributions

Conceptualization, A.B. and N.W.; Methodology, A.B., W.B., M.M., M.N., K.P., M.R., A.S. and N.W.; Software, W.B., M.M., M.N., K.P., M.R. and A.S.; Formal Analysis, A.B.; Investigation, A.B., W.B., M.M., M.N., K.P., M.R., A.S. and N.W.; Resources, W.B. and M.M.; Data Curation, M.N. and K.P.; Writing – Original Draft Preparation, W.B., M.M., M.N., K.P., M.R. and A.S, Writing – Review & Editing, A.B. and N.W.; Supervision, A.B. and N.W.; Project Administration, M.R. and A.S.

Conflicts of interest

The authors have no conflict of interest. Additionally, there is no relationship of interest with any company in the study we are responsible for. No support was received from any project or company for the research.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Bioethics Committee of the College of Medical Sciences of the University of Rzeszow No. 2022/004.

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ORIGINAL PAPER

Pandemic awareness and caring behaviors of nurses working in intensive care unit – a multicenter study

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ABSTRACT

Introduction and aim. In the COVID-19 pandemic, which is a global threat, the awareness levels of intensive care nurses who meet all the care needs of patients can affect the quality of care. In this study, pandemic awareness and care behaviors of nurses who undertook the patient's care needs in intensive care units were examined.

Material and methods. The research was carried out with 317 nurses working critical care units of 12 hospitals in different provinces. "Nurse Characteristics Form", "Pandemic Awareness Scale" and "Caring Behaviors Inventory-24" were used as data collection tools. Data were collected between March and August 2022 in the middle of the COVID-19 pandemic process using an online survey.

Results. It was determined that 75.4% of the participants were female and the mean age was 25.56±4.49. Pandemic awareness scale score was 3.04±0.62 (0.11-3.67) and caring behaviors inventory score was 5.48±0.84 (1.00-6.00). A significant, positive, weak relationship was found between nurses' pandemic awareness and all sub-dimensions of caring behaviors ($p \leq 0.05$).

Conclusion. It was seen that the caring behaviors of nurses with high pandemic awareness were also positively affected. Although it was the first time they had experienced the pandemic, nurses were found to have good caring behaviors.

Keywords. pandemic awareness, caring behavior, intensive care, nurse

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, an unprecedented global epidemic, has spread to all continents worldwide, affecting various aspects of economic and social life, and has claimed the lives of millions of people.[1-3] However, the coronavirus pandemic has affected the whole world and has created such a great social impact, suggesting the need for attention and preparation against new infectious diseases that may arise at any time.[4]

The current pandemic moment, which imposes on humanity to raise awareness of behavioral changes related to social isolation/removal as well as preventive measures such as hand washing, personal and environmental hygiene, adequate nutrition for patients and hospital has revealed the importance of nurses' more qualified, ethical, technical and scientific roles every day. It is known that intensive care nurses are an indispensable element of the health army that fights on the front lines in times of crisis and epidemic diseases in environmental and humanitarian disasters, especially in the current pandemic.[5] Disease conditions and life risks arising from COVID-19, as well as the working conditions to which health workers are exposed, the sickness of professionals and the inadequate functioning of care services are directly reflected in the quality of health care services pro-

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vided. Pandemics, a global health problem, are a threat to nurses and all other health workers.[6] Intensive care nurses spend more time with patients and are directly responsible for patient care.[7] Global health institutions are aware of the necessity of nursing care in their efforts to prevent and respond to epidemics, and underline that its importance is increasing day by day.[8-9] Caring behaviors can be directly affected by the health care provider, organizational factors, nursing care delivery model and cultural differences based on common values in the society.[10] For this reason, it is necessary to carry out studies to determine the factors affecting caring behaviors and care by taking into account the current conditions in each country.[11] Pandemics are a new experience involving the care of highly contagious patients, and little is known about it.[5]

Aim

In this study, pandemic awareness and care behaviors of nurses who undertook the patient's care needs in intensive care units were examined.

Research questions:

- What is the level of pandemic awareness of nurses working in intensive care?
- What is the level of care behavior of nurses working in intensive care?
- Is there a relationship between the awareness of the pandemic and care behaviors of nurses working in the intensive care unit?
- What are the factors affecting the awareness of the pandemic and care behaviors of nurses working in intensive care?

Material and methods

Ethical approval

In order to conduct a research, written ethics committee permission was obtained from Istanbul Arel University Ethics Committee Commission (E-69396709-050.01.04-208684 Number and Decision No: 08) and written institutional permission was obtained from the health group of the hospital where the research was conducted. The nurses constituting the sample of the study were informed about the purpose, duration and what was expected from them and approval was obtained for participation in the research in line with the principle of willingness and volunteerism. The data was created through Google drive during the pandemic, the survey invitation was given an online survey link via WhatsApp, making it clear that the participation was voluntary. The questionnaire response time was completed between 8-12 minutes. In this study, all procedures were performed in accordance with the ethical standards, and by the Helsinki Declaration.

Study design and participants

This study used a descriptive cross-sectional research design. The population of the study consisted of 564 nurses working in general intensive care, neonatal intensive care, cardiovascular surgery intensive care and coronary intensive care of 12 hospitals in different provinces within a special health group between 4.03.2022 and 04.08.2022. The sample was planned to include 306 nurses at a 99% confidence interval. 23 nurses did not want to participate in the study and 9 nurses filled the questionnaire incompletely.The research was completed with 317 nurses who volunteered to participate.

Data collection tools

In the study, "Nurse Characteristics Form", "Pandemic Awareness Scale" and "Caring Behaviors Inventory-24" were used as data collection tools.

Nurse characteristics form

In this form prepared by researchers; There are 12 questions to evaluate nurses' age, gender, marital status, education level, year of employment in intensive care, status of having an intensive care certificate, opinions on the reasons that may affect the care provided in intensive care and whether they are considering resigning during the pandemic.

Pandemic awareness scale (PAS)

The scale, which was developed by Arpacı (2022) and studied Turkish validity and reliability, is applied to 14-82 age groups. The score that can be obtained from the five-point likert type scale varies between 9 and 45. There are two inverse items on the scale (item 1 and item 3). The scale consists of a single sub-dimension and nine items. Clause 1 and Clause 3 are inversely coded. The Cronbach α value is 0.89[12]. For this study, it is 0.74.

Caring behaviors inventory-24 (CBI-24)

Wu et al. CBI-24, which was developed by CBI-24, was conducted by Kurşun and Kanan in 2012 for our country.[13] The scale consists of 4 subgroups and 24 items: assurance, knowledge and skills, respectfulness and connectedness. 6-point Likert-type scale (1: never, 2: almost never, 3: sometimes, 4: usually, 5: most often, 6: always) is used for the responses of CBI-24. For the scale and each sub-dimension, a score between 1-6 is obtained by dividing the score obtained by adding the scores of the items to the number of items Cronbach determined the coefficient as 0.97, while in this study it was determined as 0.99.[14]

Statistical analyses

Continuous variables are expressed as means ± SD, and categorical variables are expressed as percentages. Baseline demographic and occupational characteristics of the groups were compared with Chi-Square or

Fisher exact test for categorical data and student's t test and one- way analysis of variance (ANOVA) for continuous variables. Relationship between variables analyzed by using Pearson correlation analyses. For all tests, two-sided P values $p<0.05$ were considered significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 24.0 for Windows (SPSS Inc, Chicago, Illinois, USA).

Results

The mean age of the nurses participating in the study was 25.56±4.49 (min.20-max.54). 75.4% of the participants were women, 73.2% were single and 72.2% were high school graduate nurses. Most of the nurses (69.1%) work in internal intensive care units (coronary, reanimation, general, COVID) and 70.7% had 1-5 years of working experience. 90.5% of the nurses stated that they worked willingly in intensive care units and 77.9% cared for 1-3 patients (Table 1).

Table 1. Nurse's socio-demographic and professional characteristics*

	n (317)	%
Age (years)		
20-24	187	59
25 and over	130	41
Gender		
Female	239	75.4
Male	78	24.6
Marital Status		
Married	85	26.8
Single	232	73.2
Education Status		
High school	229	72.2
License	83	26.2
Postgraduate (master's/doctorate)	5	1.6
Working year in intensive care		
1-5 years	224	70.7
6-10 years	72	22.7
11 years and over	21	6.6
Have an intensive care nurse certificate		
Yes	40	12.6
No	277	87.4
Willingly work in intensive care		
Yes	309	97.5
No	8	2.5
The ICU where he works		
internal medicine (coronary, reanimation, general, COVID)	219	69.1
Surgery (CVD, general surgery)	38	12.0
Neonatal ICU	60	18.9
Number of patients per nurse		
1-3 Patients	247	77.9
4 Patients and above	70	22.1
Thinking that the ICU has a sufficient number of nurses		
Yes	75	23.7
No	242	76.3
Sufficient time for ICU care		
Yes	174	54.9
No	143	45.1
Thinking about resigning		
Yes	103	32.5
No	214	67.5

* ICU – intensive care unit

As stated in Table 2, the average pandemic awareness score of nurses was determined as 3.04±0.62. The mean of the sub-dimension of nurses' nursing behavior assurance was 5.50±0.86; knowledge skill sub-dimension average was 5.58±0.83; the mean sub-dimension of respectfulness was 5.45±0.75; the adherence sub-dimension average was 5.36±0.89 and the total score average of caring behavior were 5.48±0.84.

Table 2. Nurse's pandemic awareness and care behaviors

	Mean ± SD (min.-max.)
PAS	3.04±0.62 (0.11-3.67)
CBI-24	
Assurance	5.50±0.86 (1-6)
Knowledge and skills	5.58±0.83 (1-6)
Respectfulness	5.45±0.75 (1-6)
Connectedness	5.36±0.89 (1-6)
Total	5.48±0.84 (1-6)

* Mean ± SD (min-max); PAS – pandemic awareness scale; CBI-24 – caring behaviors inventory-24

In Table 3, it was found that while the units where nurses worked did not make a difference in their caring behavior, they affected pandemic awareness ($p\leq0.05$). The awareness level of nurses working in internal intensive care units was found to be higher than those working in surgical and neonatal intensive care units. It was determined that the fact that nurses had sufficient time for ICU care affected only the connectedness dimension from the sub-dimensions of the caring behaviors scale ($p\leq0.05$). There was no significant difference between the fact that nurses did not have an intensive care nurse certificate, worked willingly in intensive care, the number of patients per nurse, the lack of sufficient number of nurses in the ICU, sufficient time for care, nurses did not intend to resign during the pandemic process, and the sub-dimensions of the caring behaviors scale and the mean scores of total caring behavior.

A significant, positive, very weak relationship was found between the pandemic awareness score average of nurses and all sub-dimensional scores of caring behaviors (Table 4) ($p\leq0.05$).

Discussion

In this study, the relationship of intensive care nurses who provide care during the pandemic with pandemic awareness and caring behavior and some factors affecting it was examined. The results showed that nurses had high mean scores on the caring behavior and pandemic awareness scales and had good caring behavior. In the literature review, no similar study was found to discuss the results of this study, and it seems that this study is the first to address this issue. The pandemic that entered our lives with COVID-19 has adversely affected all our life behaviors. This research, which examines the awareness levels of intensive care nurses who are health care

Table 3. Factors affecting nurses' caring behaviors inventory and pandemic awareness *

	CBI-24										PAS	
	Assurance		Knowledge and skills		Respectfulness		Connectedness		Total			
	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p
Age (years)												
20-24	5.57±0.82	0.1	5.62±0.81	0.36	5.50±0.73	0.18	5.42±0.86	0.13	5.53±0.81	0.16	3.07±0.6	0.4
25 and over	5.41±0.9		5.53±0.85		5.36±.0.77		5.27±0.94		5.40±0.87		3.01±0.65	
Gender												
Female	5.47±0.87	0.58	5.57±0.85	0.72	5.46±0.74	0.9	5.37±0.89	0.7	5.47±0.85	0.87	3.05±0.63	0.73
Male	5.55±0.81		5.61±0.77		5.45±0.77		5.32±0.91		5.49±0.81		3.02±0.6	
Marital Status												
Married	5.40±0.95	0.2	5.49±0.91	0.24	5.37±0.83	0.24	5.30±0.94	0.45	5.39±0.92	0.28	3.07±0.6	0.62
Single	5.54±.0.82		5.62±0.8		5.48±0.71		5.38±0.88		5.51±0.81		3.03±0.63	
Education Status												
High school	5.54±0.78		5.61±0.76		5.47±0.71		5.40±.0.82		5.51±0.77		3.04±0.59	
License	5.40±1.04	0.39	5.51±1	0.61	5.40±.0.86	0.74	5.26±1.05	0.44	5.40±1.01	0.54	3.03±0.69	0.88
Postgraduate (master's/doctorate)	5.32±0.91		5.44±0.82		5.50±0.56		5.20±1.19		5.35±0.88		3.18±0.97	
Working year in intensive care												
1-5 years	5.54±0.81		5.61±0.79		5.48±0.71		5.40±.0.85		5.52±0.8		3.06±0.6	
6-10 years	5.38±0.98	0.34	5.50±0.96	0.6	5.36±.0.87	0.47	5.24±1.04	0.38	5.36±0.97	0.37	2.99±0.73	0.74
11 years and over	5.46±0.82		5.59±0.78		5.46±0.68		5.30±0.82		5.45±0.79		3.06±0.43	
Have an intensive care nurse certificate												
Yes	5.32±0.93	0.15	5.46±0.88	0.33	5.33±0.81	0.25	5.17±0.93	0.15	5.30±0.9	0.16	3.11±0.73	0.43
No	5.53±0.84		5.60±0.82		5.47±0.74		5.39±0.89		5.50±0.83		3.03±0.6	
Willingly work in intensive care												
Yes	5.50±0.86	0.64	5.58±0.84	0.68	5.45±0.75	0.80	5.35±0.9	0.54	5.47±0.85	0.57	3.04±0.62	0.61
No	5.64±0.38		5.70±0.4		5.52±0.49		5.55±0.41		5.64±0.36		3.15±0.59	
The ICU where he/she works												
Internal medicine (coronary, reanimation, general, COVID)	5.53±0.79		5.63±0.75		5.47±0.71		5.39±0.84		5.51±0.77		3.11±0.55	
Surgery (CVD, general surgery)	5.44±0.93	0.6	5.50±0.98	0.38	5.36±0.80	0.76	5.21±1	0.53	5.39±0.94	0.52	2.88±0.55	0.01
Neonatal ICU	5.42±1.04		5.48±1.00		5.42±0.85		5.34±1.02		5.41±1.00		2.90±0.83	
Number of patients per nurse												
1-3 Patients	5.48±0.91	0.41	5.55±0.89	0.19	5.44±0.79	0.43	5.35±0.93	0.77	5.46±0.89	0.39	3.02±0.65	0.31
4 Patients and above	5.57±0.61		5.70±5.58		5.52±0.6		5.38±0.75		5.55±0.61		3.11±0.51	
Thinking that the ICU has a sufficient number of nurses												
Yes	5.45±1.01	0.57	5.45±1.01	0.12	5.41±0.85	0.52	5.34±1.03	0.86	5.42±1	0.46	2.99±0.69	0.45
No	5.52±0.8		5.62±0.76		5.47±0.71		5.36±0.85		5.50±0.78		3.06±0.6	
Sufficient time for ICU care												
Yes	5.55±0.83		5.58±0.84		5.50±0.71		5.46±0.85		5.53±0.83		3.06±0.62	
No	5.45±0.88	0.3	5.59±0.82	0.89	5.39±0.79	0.19	5.23±0.93	0.02	5.42±0.85	0.25	3.02±0.62	0.6
Thinking about resigning												
Yes	5.41±0.92	0.18	5.54±0.84	0.53	5.41±0.81	0.48	5.28±0.91	0.29	5.41±0.88	0.30	3.08±0.58	0.39
No	5.54±0.82		5.60±0.82		5.47±0.72		5.39±0.88		5.51±0.82		3.02±0.64	

* significant difference at p<0,05; Bonferroni post-hoc analiz

practitioners, has an important advantage because it is the first research conducted in intensive care nurses in our country in terms of determining the level of awareness not only about COVID-19 but also about pandemic epidemics in general.

Table 4. The relationship between nurses' pandemic awareness and caring behaviors inventory

		CBI-24				
		Assurance	Knowledge and skills	Respectfulness	Connectedness	Total
PAS	R	0.162	0.152	0.144	0.17	0.178
	p	<0.001	0.001	0.001	<0.001	<0.001

* R – correlation coefficient; using Pearson's corelation analyses

Due to the limited literature on COVID-19 pandemic awareness, the results were discussed with similar research. Al-Dossary et al. in terms of of the awareness level of nurses about COVID-19, 96.85% had excellent awareness about the virus.[25] In terms of preventive practice (awareness and skills) of nurses in dealing with COVID-19, 83.2% reported the highest prevention, while 38 (7.6%) had low prevention. More than half of respondents (60.4%) had a high positive attitude towards providing care to COVID-19 patients. Meanwhile, in terms of the perception of nurses towards COVID-19, more than half of the nurses (69.2%) had a very high perception. Several findings were highlighted in the study. The mean score of pandemic awareness of intensive care nurses was found to be high. When the results were examined, the awareness level of women was higher than that of men. The level of awareness of those who were married was higher than those who were single. As the level of training increased, the level of awareness of nurses increased. The awareness of nurses with a graduate education level was higher than that of nurses who graduated from undergraduate and high school. The level of

awareness of nurses who were between 1-5 years in terms of working years was higher than those who were 6-10 years, and those over 11 years. The awareness of nurses who had intensive care-specific training certificates was higher than those who did not. As the number of patients cared for by intensive care nurses increased, the level of pandemic awareness increased.

This study, the value obtained from the CBI-24 total score average of the intensive care nurses showed that their perceptions of caring behaviors were high. In a similar study conducted by Kızılırmak and Bulut, the total score of the scale was found to be 5.36±0.5. When the mean scores of nurses' caring behavior were examined in the study, the highest mean score was found in the knowledge-skill sub-dimension (5.58±0.83) and the lowest mean score was in the sub-dimension of connectedness (5.36±0.89).[15] Efil et al. nurses' levels of caring behaviors were high (5.4±0.6).[16] Similarly, in studies conducted with nurses working in different units in the literature, it was seen that nurses got the highest score in the knowledge skill sub-dimension and the lowest score in the connectedness sub-dimension.[11-24] However, in Gülpınar's study, the sub-dimension of being respectfulness and the sub-dimension of assurance in Şanal's study had the lowest average score.[20,22] In the studies, it was thought that the reason for the higher sub-dimension score of knowledge and skills was the widespread specialization in nursing education and the years of experience of the nurses participating in the study were between 1-5 years.

In this study, intensive care nurses who provided care during the pandemic pointed to high knowledge and professional skills among nurses despite the conditions of the country's hospitals. This finding highlights the impact of having knowledge and awareness on caring behaviors. For this reason, it is necessary and recommended to develop nursing knowledge and practices through continuous education in order to empower nurses. In this study, it was expected that the pandemic awareness and caring behaviors of intensive care nurses would be very high. However, the findings showed that their behavior was good when it came to providing care to patients, especially COVID-19.

Study limitations

Although sampling was done with the consent of the staff, working environment conditions such as participants' workload, stress and fatigue can affect the quality of their responses. Future studies are proposed to clarify the relationship between research variables in the wider statistical population.

Conclusion

The findings showed that the pandemic that entered our lives with COVID-19 and the pandemic awareness and caring behaviors of intensive care nurses who cared for very highly contagious patients were good. Despite these results, relevant managers need to pay special attention to awareness training of health professionals in order to improve their caring behaviors.

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Author contributions

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

Conflicts of interest

The authors declare that there is no conflict of interest regarding this article.

Data availability

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

Ethics approval

In order to conduct a research, written ethics committee permission was obtained from Istanbul Arel University Ethics Committee Commission (E-69396709-050.01.04-208684 Number and Decision No: 08) and written institutional permission was obtained from the health group of the hospital where the research was conducted.

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ORIGINAL PAPER

Nurse academicians' experiences in the pandemic and their perspectives on future pandemics – a qualitative study

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ABSTRACT

Introduction and aim. The COVID-19 pandemic, which affects the whole world, has also significantly affected nurses, nursing students and nursing academicians. This study aims to determine the experiences of nurse academicians and their perspectives on possible future pandemics.

Material and methods. The study is a descriptive qualitative type. It was conducted in September-December 2022 in the Department of Nursing, Faculty of Health Sciences, of a university located in the west of Turkey. The sample of the study consisted of 11 nurse academicians. Personal Information Form, Interview Form on COVID-19 Pandemic Experiences and voice recorder were used to collect data. Data analysis was done with content analysis.

Results. As a result of the analysis of the data obtained from the interviews, four main themes emerged: (1) nurse academicians and nursing education in the pandemic, (2) the impact of the pandemic on life, (3) the gains in the pandemic, and (4) suggestions for future pandemics**.**

Conclusion. As a result of this study, it was determined that nurse academicians' families, social, and academic lives, and health were deeply affected during the pandemic, but they also found opportunities during the pandemic. It is thought that guidelines and action plans are needed to ensure the safety of nursing education for future pandemic-like situations and to minimize the problems experienced by academic nurses. For this, it is recommended to reconsider the technical possibilities and methods of education.

Keywords. distance education, nurse academicians, nursing education, pandemic

Introduction

The COVID-19 (Coronavirus 2019) pandemic, which emerged in China in the last months of 2019 and shook the whole world at the beginning of 2020, has affected the world population in many ways, spiritually, physically, socially, economically, and politically.[1–4] As the geographic area where the virus spreads, cases have increased rapidly and there has been a rapid increase in the number of virus-related deaths.[4] In this whole process, the health system and the education system are at the forefront of the systems most affected by the virüs.[2] In addition to nurses who have an important place in the health system, nurse academicians, and student nurses have been affected in many ways.[5] During the pandemic process, many measures have been taken to prevent the spread of the epidemic around the world, such as quarantine, social distance, social isolation, curfew and interruption of education.[6,7] While these measures implemented in Turkey interrupted collective activities, many routine activities in daily life, they also affected education and training activities.[8,9]

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In the COVID-19 pandemic, the distance education process has been started in higher education institutions in Turkey as of March 23, 2020.[10] The transition to the distance education system within the scope of complying with the quarantine and social distance rules has affected the educational activities and working conditions of the academicians working in higher education institutions. In addition, this unexpected situation required academic nurses to adapt quickly.[10,11] Nurse academicians who carried out the face-to-face education system in classroom settings and clinical practices found themselves under new conditions. These rapid changes in education have forced academic nurses to make important changes such as planning theory lessons, making new plans for applied lessons, measurement and evaluation in a very short time.[5,10,12] Along with these rapid changes, it has been observed that the pandemic process has been difficult for academics who have to continue to carry out academic studies and projects.[10,12,13]

In the literature, it is seen that there are studies on how nursing education, nursing students and clinician nurses are affected mostly. Although it is seen that academicians as well as nurses and nursing students were affected significantly during the pandemic process, the inadequacy of studies on this subject draws attention.[12]

Aim

The aim of this study is to learn the experiences of nurse academicians about what they have experienced in their nursing education and life in the COVID-19 pandemic and to determine their perspectives as nurse academicians regarding future pandemics. In the literature, studies that deal with what nurse academicians experience during the pandemic process are quite inadequate. It is thought that the results of this study will guide the plans to be made to realize and reduce the negative effects of the COVID-19 pandemic, whose footprints have not been erased from our lives yet, and pandemic-like situations that may occur in the future, which may cause problems in the lives of nurse academicians and nursing education. In the light of all these findings, it is aimed to create a road map regarding the measures that can be taken against extraordinary situations that may arise in the future and the aspects that need to be developed.

Material and methods

Ethics approval

Ethical approval was obtained from the Human Research Ethics Committee of Zonguldak Bülent Ecevit University (Date 01.07.2022 and number 185771) to conduct the research. Written permission of the institution was obtained on 20.07.2022 from the faculty where the research was conducted. Verbal and written consents were obtained from the academic nurses participating in the study.

Study design

The report of this study was written using the COREQ (Consolidated Criteria for Reporting Qualitative Research Guidelines), a guide to writing qualitative research.[14]

This study was conducted in a descriptive qualitative type. Descriptive qualitative design is frequently used in studies in the field of health. Descriptive qualitative studies are seen as the most appropriate method to describe the different experiences of the participants and the subjective nature of the problem in accordance with the research question.[15]

Setting

The research was conducted between September 2022 and December 2022 in the Department of Nursing, Faculty of Health Sciences, of a university located in the west of Turkey.

Participants

The population of the study consisted of nurse academicians in the Nursing Department of the Faculty of Health Sciences of a university located in the west of Turkey. There are 23 nurse academicians in total in the Nursing Department. Purposive sampling method was used in this study. Since this study was in a qualitative design, the sample size was determined when it reached the saturation point. In qualitative research, a sampling approach is used, which requires continuing to collect data until the stage (saturation point) when the concepts and processes that may be the answer to the research question begin to repeat.[16] When the emerging concepts and processes start to repeat each other, it is decided that a sufficient number of data sources have been reached.[16] 11 nurse academicians formed the sample of this study.

Inclusion criteria for this study; to work as a nurse academician in the faculty where this study was conducted, to be a nurse academician during the pandemic period, and to volunteer to participate in this study. Exclusion criteria from this study: not volunteering to participate in the study, starting to work as an academic nurse after the pandemic.

Data collections

"Personal Information Form" and "Interview Form Regarding COVID-19 Pandemic Experiences" were used to collect the data of the study. A voice recorder was used to record the interview data.

Personal Information Form

It was prepared by the researchers by scanning the literature, and questions of academic nurses' age, gender, marital status, education level, income status, title, family type, presence of chronic disease, academic year of study are included.[12]

Interview Form Regarding COVID-19 Pandemic Experiences

This form was created by researchers to better understand the experiences of academic nurses during the pandemic process by scanning the literature. Semi-structured interview technique was used in the interview. In this technique, an interview form containing the questions planned to be asked beforehand was prepared. On the other hand, depending on the flow of the interview, sub-questions were asked during the interview (Table 1). The questions used in the interview were prepared by the authors according to the aim of the rstudy by scanning the literature. Before starting the study, it was used in the study sample after taking the opinions of two academicians from different faculties regarding the interview questions.

Table 1. Semi-structured Interview Questions

Questions
– Could you tell us a little bit about what it was like to be an academic nurse during the COVID-19 pandemic?
– Do you think your life has been affected by the COVID-19 pandemic? How?
– Has your health been affected by the COVID-19 pandemic? How?
– What kind of problems did you experience in nursing education during the COVID-19 pandemic?
– What are the gains of the COVID-19 pandemic for you?
– As an academic, what are your suggestions for investments to be made in academics in the upcoming pandemics?

An announcement was made to the nurse academicians working in the faculty where the study was conducted, from the partner deprtmant network. After the announcement, an interview appointment was planned with the nurse academicians who volunteered to participate in the study and interviews were started. The interviews were conducted by the first researcher. The data were collected by in-depth individual interview method with nurse academicians who met the criteria for inclusion in the sampling. At the beginning of the interview, the nurses were informed about the purpose of the research and how to do it. After informing about the use of a voice recorder, verbal and written consents were obtained. With the permission of the nurse academicians, the notes of the data were kept.

Data analysis

In the analysis of the data obtained with the interview form, a three-stage method suggested by Creswell was followed.[17] In the first stage, the audio recordings of the interviews were first transcribed, field notes were added, and the transcripts were read several times to gain a deep understanding. In the second stage, the transcripts were divided into meaningful codes according to the purpose of the research in order to describe and interpret the data. The codes obtained in the third stage were evaluated and conceptually similar codes were classified. After the classification, the themes were determined. The analysis was carried out individually and independently by two researchers.[17] After the individual analyzes, the researchers came together and discussed the themes and sub-themes by critically evaluating the data in accordance with the research purpose. They agreed on the themes that best described the findings. During the analysis, the researchers did not allow their own values and beliefs to influence the analysis.

Rigor and reflectivity

The principles of credibility, transferability, consistency and confirmability were used to strengthen Rigour.[18] Long-term interaction is important in ensuring internal validity. Interviews with nurse academicians in the study lasted an average of 31 minutes. The researchers clearly presented the sufficiency of the data they obtained to answer the research question to the reader in the findings section. The interviews were concluded in accordance with the aim of the study and at the saturation point (depth-focused data collection). In order to ensure external validity, the researchers arranged the data according to the emerging themes and transferred them without adding comments (thick description). Nurse academicians included in the study were determined by purposive sampling method in accordance with the aim of the study. In ensuring internal reliability, the researchers were consistent in all interviews by using the same voice recorder and interview form. In order to ensure external reliability, all data collection tools, audio recordings, raw data, codes and themes created during the analysis were examined by the expert and stored for use when necessary. All of the researchers are women and the first researcher (PhD) is an academician in the field of mental health and psychiatric nursing, and the second (PhD) is an academician in the field of obstetrics and gynecology nursing. Researchers are experienced in qualitative method. The nurse academicians participating in the study work in the same faculty as the researchers, and the participants were informed about the aim of the study before starting the study. Also as the researchers themselves were academic nurses, they were therefore interested in the research topic.

Results

The average age of the academician nurses participating in the research is 39.72±7.19 years. All of the academic nurses are women, 6 of them are married, 7 of them are doctoral and 9 of them live in a nuclear family. The average working year of academic nurses varies between 6 months and 25 years (Table 2).

As a result of the analysis of the data obtained from the interviews, four main themes emerged as (1) nurse academicians and nursing education in the pandemic,

Table 2. Sociodemographic and academic characteristics of nurse academicians

Nurse academicians no	Age	Gender	Marital status	Family Type	Educational status	Academic title	Department	Years of working as an academic	Having a chronic illness
N1	44	Female	Married	Nuclear Family	PhD	Associate professor	Pychiatric nursing	18	No
N2	48	Female	Single	Nuclear Family	PhD	Assistant professor	Child Health and Diseases Nursing	22	Diabetus mellitus Hypertension
N3	37	Female	Married	Nuclear Family	PhD	Assistant professor	Nursing Fundamentals	9	Psoriasis
N4	55	Female	Married	Nuclear Family	Master	Lecturer	Public Health Nursing	25	Rhythm disorder
N5	42	Female	Single	Nuclear Family	PhD	Lecturer	Public Health Nursing	18	No
N6	40	Female	Single	Nuclear Family	PhD	Lecturer	Child Health and Diseases Nursing	1	Crohn's disease, Ankylosing spondylitis
N7	30	Female	Married	Nuclear Family	Master	Lecturer	Public Health Nursing	<1	No
N8	38	Female	Single	Alone	Master	Lecturer	Internal Medicine Nursing	7	No
N9	34	Female	Married	Nuclear Family	PhD	Research Assistant	Surgical Diseases Nursing	5	No
N10	34	Female	Married	Nuclear Family	PhD	Research Assistant	Nursing Fundamentals	9	No
N11	35	Female	Single	Alone	Master	Research Assistant	Child Health and Diseases Nursing	1.5	No

(2) the impact of the pandemic on life, (3) the gains in the pandemic, and (4) suggestions for future pandemics.

Theme 1

Nurse academicians and nursing education in the pandemic

Two sub-themes were discussed under this theme. Nurse academicians defined being an academic nurse during the pandemic period and evaluated nursing education in the pandemic process from the perspective of academicians.

Sub-theme 1. Being an academic nurse in the pandemic

Nurse academicians expressed their perceptions about being an academic nurse during the pandemic period. Four of them expressed being an academic nurse during the pandemic as a difficult, challenging and worrying process.

"Being a nurse academician during the pandemic period was a difficult and worrying process that made us very difficult. Because we carry out most of our training in clinical practice, we ate the biggest ax from here….." (N8)

"It was difficult, there were technological difficulties. The student's adaptation was difficult, our adaptation was difficult. There were concerns, of course. Do students attend classes? Can I transfer the knowledge and skill I want to convey to the student?" (N10)

Two of them stated that they were confused when they thought of their colleagues in the field during the pandemic period, and there were questions about how to prepare nursing students for the profession in this situation.

"Being an academic nurse…. So it was like, what are we going to do now? I wonder if we go to the clinic and get support? When I saw how hard they (the nurses in the field) were struggling, that faltering really happened to me…"(N3)

A few of them stated that they perceived being an academician nurse during the pandemic as a dream, as if it was a dream. Another stated that he felt lucky to be at home during this period.

"I felt lucky, it was a chance for me to be a nurse academic. I joined more organizations both academically and was at home. We were away from most risks" (N9)

Nurse academicians expressed various metaphors about being a academic nurse during the pandemic. They explained being a academic nurse in the pandemic with the concepts of empowerment, self-sacrifice, warrior, dedication, chaos, iceberg, luck, and being strong.

"Iceberg, I think it was an iceberg… The iceberg has a background. Maybe we spent 3-4 times the effort we spent in normal education during the pandemic. We had many sleepless nights. From the outside, even though it is said, "oh how comfortable you are, you are at home, you have worked from a desk", there are parts of the iceberg that are not visible." (N8)

"Devotion. Like this; Online education was a process that we were not used to at all. We had to learn it. At the same time, we made an effort to convey everything we know to the student." (N11)

Sub-theme 2. Nursing education in the pandemic

Nurse academicians evaluated nursing education during the pandemic period. All of them mentioned the negative impact of the pandemic on nursing education, nursing educators and nursing students.

All of them expressed the negative aspects of nursing education, especially the practice part of it, during the pandemic process, and the majority of them described the practice part of education as a loss. They stated that they experienced internet connection problems and lack of technological infrastructure while teaching in distance education. Five of them stated that they had difficulty in joining the students to the online courses, three of them stated that they had no face-to-face interaction with the students in the lessons, and they had anxiety

about the effectiveness of the online course. One nurse academician stated that as an academician, he could not get any satisfaction in this process.

"As an academic, I love face-to-face contact, face-to-face communication. But in online classes, it's like you're really lecturing on the wall. No reaction, no interactivity…." (N6)

It was stated that the other problem experienced by nurse academicians in distance education is about the evaluation of students. Two of them stated that online assessment of students threatens the safety of the exam, and three of them stated that the success results cannot be obtained by measuring enough.

"There were connection problems. There were systemic infrastructure problems. There was an atmosphere of chaos. Exams were a little challenging. It was not an environment for measuring knowledge… The number of students is high. Classic or test? It was very difficult to distinguish and evaluate this." (N7)

Nurse academicians expressed the difficulties they experienced in providing distance education at home. One of them stated that there was no suitable environment to teach the lesson at home and that he could not concentrate on conducting the lesson. Nurse academicians also expressed the negative impact of distance education on nursing students. In this direction, one participant stated that nurses with communication weakness were trained during the pandemic process, one participant stated that students could not attend their classes due to housework at home, and two participants stated that students had problems in accessing the internet and connecting to the lesson.

"Nursing is a profession that includes human relations... When this process progresses through the technological infrastructure, our students have deficiencies in this sense." (N7)

Theme 2. Impact of the pandemic on life

Three sub-themes were discussed under this theme. Academic nurses talked about the impact of the pandemic period on family and social life, on their own health and academic life.

Sub-theme 1. The effect of the pandemic on family and social life

Nurse academicians, who had to carry out quarantine processes and education activities from home during the pandemic period, stated that their family and social lives were adversely affected at that time. Three of them stated that they experienced loneliness, longing and loss of motivation because they could not meet with their family members and close friends for a long time due to the prohibition of intercity transportation, while one of them stated that he could not meet with them face to face due to the anxiety of infecting his family.

"Even if you are not COVID, you have to think about the other person. You have to limit your relationships. Emotionally, it really pushes you into the void..." (N6)

"We were providing video interviews with the technological infrastructure, but we found the end of the process very pessimistic both for my family and for me… Since I couldn't see them, I couldn't be motivated.. "(N7)

Some of the nurse academicians stated that they lost their relatives due to the pandemic and could not attend the funerals of their relatives.

"I lost three of my relatives due to COVID. Funeral procedures and burial were very troublesome. You can't go to the hospital with them, as if you are a foreigner or I don't know, it's very serious like that you will never be approached." (N5)

Some of the nurse academicians stated that their workload at home increased due to being at home during the pandemic and that they had difficulties due to the lack of spousal support during this period. They stated that they had difficulties due to the combination of responsibilities such as housework, following the lessons of the children, and conducting their own education and training activities during the pandemic.

"Then you feel very stuck, involuntarily. The burden of both the children and the house. You never go out. Of course, all the food, cleaning, etc., child and patient of the house are on you. It was actually a very tiring period for me." (N9)

Sub-theme 2. Impact of the pandemic on health

Nurse academicians mentioned that their physical and mental health were adversely affected during the pandemic process. They stated that they experienced vision and sleep problems due to long hours in front of the computer, they gained weight, the course of their chronic diseases worsened and they were tired.

"In terms of education, I was at the computer for eight hours from morning to evening. In the meantime, I really felt my eyes and pupils tremble… Being in front of the screen all the time made me tired." (N1)

"I slept late at night, there were times when I had to get up very early in the morning. The thing that impressed me the most was the recurrence of my chronic diseases a little more. Because I have gained weight. This triggered my diabetes a lot. I had to switch to insulin during this process." (N2)

During the pandemic period, the nurse academicians especially stated that their mental health deteriorated. It was determined that they experienced especially anxiety, fear, lack of motivation, loneliness, psychological fatigue, feeling of being stuck and felt depressed during the pandemic. An academician stated that he started using antidepressants due to the mental problems he experienced during this period.

"Psychological fatigue, I didn't want to do anything. We worked 24 hours at home, we never stopped. There are

lessons during the day, after the end of the lessons we have meetings with the school. So it was very tiring." (N3)

Sub-theme 3. The effect of the pandemic on academic life
Nurse academicians talked about the impact of the pandemic on their graduate education, scientific studies, thesis and research processes.

The majority of nurse academicians stated that they could not get permission from the institutions where they would conduct scientific research due to the pandemic, they could not collect data, they had to collect data online, they had to change the methods of ongoing research, the academics whose thesis continued had to change their thesis from the beginning and the research processes were disrupted. Some stated that they had to take a break from the postgraduate education process.

"I was in the data collection process of my doctoral thesis, I was nearing the end, and when there were sudden closures, my academic process in the doctorate was interrupted and prolonged." (N3)

Nurse academicians with children, on the other hand, stated that they could not spare time for academic studies, focus and lack academic motivation due to caring for the child and housework in quarantine at home.

"Some academics may have completed their unfinished work in this pandemic, there was less workload at home, but for me, for all academics with small children, I think it was difficult to work at home."(N9)

Theme 3. Gains in the pandemic
Nurse academicians also talked about the positive impact of the pandemic period on their lives. A few stated that they had opportunities for their academic and personal development while in quarantine, and one considered this period as an opportunity to improve the methods used in nursing education.

"I got life coaching and EFT (Emotional Freedom-Technique) certificate to keep myself busy during the pandemic process, which supported my academic studies"(N2)

"We had an opportunity to update ourselves and keep up with the times in terms of teaching methods."(N1)

Some stated that during the pandemic period, a new field of research related to the pandemic emerged, there was time to produce projects, they increased their international connections through online communication, and they had the opportunity to participate in online international congresses.

"Right now, I can easily get an education online where I can spend thousands of liras and spend my days and stay there. I think this is a very good achievement. Because the time of us academics is very valuable." (N2)

"Thanks to online trainings, webinars, congresses, and online training environments have postponed the difficulties of physical conditions. Especially now, I am looking at the face-to-face congress fees are really high. Access to information has become easier." (N7)

Theme 4. Suggestions for future pandemics
Nurse academicians evaluated our state of readiness for a possible pandemic and similar crisis in the future in terms of nursing education and academics. All of them stated that the difficulties brought by the pandemic were forgotten due to the decrease in the visible effect of the pandemic and that the preparations for possible pandemics were insufficient. In this direction, most of the academic nurses have discussed three situations and made suggestions for future pandemics and similar crisis situations. These three situations: should nursing students be in the clinic during the pandemic, are we ready for a new possible pandemic? What should the new pandemic preparations include as an academic nurse in nursing education?

The majority of nurse academicians stated that they should continue their education in the clinic when they are sure that the student is ready after the necessary precautions are taken for the students in pandemic situations.

"Most of our students who graduated and were appointed during the pandemic period suddenly found themselves caring for a pandemic patient… Actually, stopping our (clinical) education made no sense in this regard." (N9)

All of the academic nurses stated that we are not ready enough for a new pandemic in terms of nursing education and overcoming the difficulties experienced by academic nurses.

"We are not ready for the (new) pandemic, we are not ready technically, in terms of our cooperation in hospitals, the consumables that students will use here, their extra special conditions, the entrance and exit of teachers and our permission processes in clinical practices"(N1)

Nurse academicians have made some suggestions in order not to experience the problems experienced in education again in case of a new pandemic. Some of these suggestions are the creation of clean areas for clinical applications, the preparation of emergency action plans and guides, the preparation of students for clinical applications, the development of telemedicine, telenursing and simulation applications.

"Right now we are pretending that the pandemic is over, everything is over, but the pandemic is not and will not end. It's not something that can be accomplished with individual efforts. Socially, so it's organizational. We can have associations. I think we should sit down and talk about what we are going to do about education in the pandemic by establishing a cooperation, a consensus. Or, for this, emergency action plans and our pandemic guides need to be formed." (N1)

"Even if there is no pandemic, the number of students is high, the number of teachers is low, and the clinical

practice areas are limited. In other words, the number of simulation laboratories should increase, we need to work on more scenarios and adapt the student to the clinic at school first"(N2)

Discussion

In this study, it is aimed to determine the experiences of nurse academicians and their perspectives on possible future pandemics in the COVID-19 pandemic. As a result of the data obtained in the study, four main themes emerged.

The first finding under the first theme obtained from this qualitative study is that being an academic nurse during the pandemic period is a difficult, challenging and worrying process and requires being a warrior and self-sacrifice. In the literature, studies that determine the meaning of being an academic nurse in the pandemic are quite limited. As a result of a similar study conducted with 14 academic nurse educators in the USA, it is seen that the experience of being an academic in the pandemic is defined as "chaotic", "a rollercoaster consisting of tasks and emotions".[12] Conducting studies that determine the experiences of academicians about being an academic nurse during the pandemic can guide the planning to prevent similar difficult experiences in future pandemics. The other finding under the first theme obtained from this qualitative study is the negative effect of the pandemic on nursing education from the perspective of academicians. It has been determined that academic nurses have problems connecting to the internet while conducting distance education courses, they have a lack of technological infrastructure, they have difficulty in adding students to the courses, and they are worried about the teaching of online courses. Another finding is that conducting the applied courses of the students remotely in nursing education is seen as a lost time in learning the profession. It is seen that there are similar problems in the limited study in this dimension in the literature.[19–21] As a result of a similar qualitative study conducted with 12 nurse educators and 7 nursing students in Iran, nurse educators mentioned many difficulties such as infrastructure problems in the virtual education system, time-consuming preparation of the educational content and low teacher-student interaction.[19] In the literature discussing the impact of the pandemic on nursing education, the concerns of nurse academicians about the quality of education regarding accelerated curriculum and reduced clinical practice hours draw attention.[22] As a result of a similar study conducted in the USA, it was determined that academic nurses sought to find out how to teach students online during the pandemic.[12] Clinical practice in nursing education is a learning opportunity for nursing students and is very important in developing their professional identity and focusing on the nursing role.[22] It is stated that the professional development of students has been affected significantly due to the closure of universities in nursing education in the pandemic, the suspension of clinical practices and the replacement of face-to-face courses by distance education.[22] As a result of the similar study conducted by Tolyat et al. with nursing students and academician students during the pandemic, it was determined that nursing students had insufficient self-esteem in gaining professional clinical competence in the pandemic.[19] Although the impact of the COVID-19 pandemic on the clinical competence of students has not been objectively investigated, it can be deduced that students did not acquire the necessary clinical competences during the pandemic period when viewed from the perspective of academic nurses.[19,22] For this reason, studies should be carried out on innovative methods that can efficiently provide student competence, especially for clinical applications, in nursing education for possible pandemics in the future.

In this study, under the second theme, the effects of the pandemic on the family, social and academic life and health of academics were revealed. Due to the quarantine practices taken during the pandemic, it was determined that nurse academicians experienced loneliness, longing and anxiety in their family and social life, lost their family relatives from COVID, and increased workload and responsibilities at home. Studies that determine how the family and social life of academic nurses are affected during the pandemic are quite limited.[10] As a result of a similar study conducted with 102 academic nurses in Turkey, it was determined that the majority of nurse academicians had the opportunity to spend more time with their families during the pandemic period, although they missed spending time with friends and relatives. In the same study, it was determined that academic nurses were worried that something would happen to the people around them, and they spared time for housework rather than academic studies.[10] In a similar study conducted with academics, it was determined that their social lives were negatively affected during the pandemic.[23] As a result, it can be said that due to the pandemic, the workload of academics at home has increased more and this situation affects their academic activities.

In this study, it was determined that academic nurses experienced vision and sleep problems, physical health problems such as weight gain, worsening of the course of chronic diseases, and mental health problems such as anxiety, fear, loneliness, psychological fatigue, and depression. The studies on the effect of the pandemic on the health of academic nurses could not be reached. In the literature, it is seen that there are studies investigating the effect of the pandemic on the health of academicians.[23,24] In a qualitative study examining the effects on mental health, it was determined that ac-

ademicians experienced anxiety, fear, stress, depression towards themselves and their family members, resulting in sleep and weight gain problems, and an increase in existing health problems.[23] As a result, it is seen that the pandemic experience greatly affects the health of academicians. Another result of this study is that nurse academicians have problems in obtaining institutional permission for scientific research, collecting data, and maintaining ongoing research during the pandemic. In addition, it was determined that they could not allocate time for academic studies, focus and lack academic motivation due to the increased burden of children and household chores at home. It is seen that similar results were obtained in a limited study in the literature. As a result of a similar study, it was determined that the majority of academic nurses experienced the anxiety of not being able to continue their projects due to the pandemic and not being able to collect research data, which negatively affected their academic performance.[10] It was determined that the inability to collect data and the inability to continue academic studies due to circumstances negatively affected nurse academics as well as other academics.[25] In a similar study conducted in Turkey with midwifery academicians, it was determined that the majority of them had problems during the research process, especially during the data collection phase, due to the pandemic.[26] As a result, finding solutions to these problems experienced for a possible pandemic is necessary for academicians to continue their research processes.

Under the third theme of this qualitative study, the achievements of nurse academicians during the pandemic period were discussed. Some of the nurse academicians stated that they had opportunities for their academic and personal development in the pandemic despite the negativities and chaos brought by the pandemic. Similar results are found in the limited studies conducted with academic nurses in the literatüre.[10,12] In similar studies, the majority of academic nurses saw the pandemic period as an opportunity to finish the postponed works, to plan new research, to write book chapters and articles. And they stated that they can participate in free education programs.[10,21] It is also clear that the COVID-19 pandemic process has brought many opportunities for universities and scientific communities. It is seen that with online trainings, academician nurses gain experience and prepare to enter the educational age in which new methods are used.[27]

Under the fourth theme obtained from this study, academic nurses made evaluations and made suggestions on nursing education related to possible pandemic-like crisis situations. It has been determined that there are suggestions for nursing students to continue their clinical education after taking the necessary precautions in pandemics, to continue their nursing education efficiently, to prepare for a new pandemic, to develop telemedicine, telenursing and simulation applications in order to overcome the difficulties experienced by academic nurses. Looking at the ICN 2022 theme reveals the importance of investing in nursing for global health.[28] Therefore, the basis of investment in nursing starts with a good nursing education. The COVID-19 pandemic, which has seriously threatened global health for the last three years, has had a significant negative impact on nurses, students, nursing academics, and therefore nursing education, and has shown the importance of investing in nursing.[5] The quality of undergraduate, graduate and postgraduate education programs in nursing is very important for global health. For this reason, it is clear that it is necessary to be prepared for possible pandemic-like crises in the future.

Study limitations

The present study has several limitations. First, due to the nature of qualitative research, the sample size of this research is limited. In addition, all of them are female academic nurses. The experiences of male academic nurses should be investigated.

Conclusion

In this present study, the difficulties experienced by nurse academicians in nursing education, family and social life, academic life, and mental and physical health during the pandemic draw attention. Despite these difficulties, it is seen that nurse academicians are trying to provide nursing education in the most efficient way by using innovative technological education methods during the pandemic period, and they can catch opportunities to improve themselves academically and individually. All of the academic nurses participating in the study stated that due to the decrease in the visible effect of the pandemic, the difficulties brought by the pandemic were forgotten and the preparations for new pandemics were insufficient.

Nursing academics especially drew attention to the necessity of preparing for new possible pandemic-like crises. For this reason, it is seen that there is a need for clear guidelines and action plans that can be used in situations similar to the new pandemic, taking into account the needs of current and future nursing students in order to ensure the safety of academic nurses and nursing education, which will significantly affect global health. It is recommended that nurse academicians consider the technical possibilities and methods necessary for education, which lays the foundation for the feeling of inadequacy in education that arises with many problems experienced in the distance education process, especially in the distance education process, without waiting for a new pandemic-like situation to occur.

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Author contributions

Conceptualization, S.Ç.İ.; Methodology, S.Ç.İ. and Y.D.; Software, S.Ç.İ.; Validation, S.Ç.İ. and Y.D.; Formal Analysis, S.Ç.İ. and Y.D.; Investigation, S.Ç.İ. and Y.D.; Resources, S.Ç.İ. and Y.D.; Writing – Original Draft Preparation, S.Ç.İ.; Visualization, S.Ç.İ. and Y.D.; Supervision, S.Ç.İ. and Y.D.; Project Administration, S.Ç.İ.; Funding Acquisition, S.Ç.İ. and Y.D.

Conflicts of interest

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

Data availability

Data available on request from the authors.

Ethics approval

Ethical approval was obtained from the Human Research Ethics Committee of Zonguldak Bülent Ecevit University (Decision date 01.07.2022 and decision number 185771) to conduct the research. Written permission of the institution was obtained on 20.07.2022 from the faculty where the research was conducted. Verbal and written consents were obtained from the academic nurses participating in the study.

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ORIGINAL PAPER

The effect of weight loss on serum ceruloplasmin levels in obese patients

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ABSTRACT

Introduction and aim. Serum ceruloplasmin level may be a biomarker associated with obesity and cardiovascular risk. We aimed to evaluate the effect of body weight lost by diet and exercise program on metabolic parameters and serum ceruloplasmin levels in obese patients.

Material and methods. A total of 120 obese patients with BMI ≥30 kg/m^2 were enrolled in a 16-week balanced diet program with the goal of losing 10% or more of body weight while maintaining a daily energy deficit of 500-1000 kcal/day.

Results. Mean weights of the patients decreased from 93.2±15.1 kg to 83.2±13.1 kg ($p<0.001$) and mean BMI decreased from 35.8±5.6 kg/m^2 to 31.9±4.9 kg/m^2 ($p<0.001$). Mean ceruloplasmin decreased from 25.2±4.7 mg/dL to 23.6±4.9 mg/dL ($p<0.001$), mean total cholesterol from 191.8±37.1 mg/dL to 153.8±28.7 mg/dL ($p<0.001$), mean LDL from 120.3±31.4 mg/dL to 91.1±27.7 mg/dL ($p<0.001$) and mean fasting blood glucose from 108.2±35 mg/dL to 103.3±81.1 mg/dL ($p<0.001$). There was a statistically significant and weak correlation between the change in ceruloplasmin and the change in BMI ($p=0.016$, $R=0.233$). There was a statistically significant and weak correlation between ceruloplasmin change and weight change ($p=0.010$, $R=0.251$).

Conclusion. Obese patients' serum ceruloplasmin levels were found to decrease with weight loss.

Keywords. ceruloplasmin, obesity, weight loss

Introduction

Obesity is a chronic metabolic disease characterized by an increase in body fat stores caused by an excess of energy intake over energy expenditure. Obesity has been linked to type 2 diabetes, dyslipidemia, metabolic syndrome, atherosclerosis, and cardiovascular disease.[1] Obesity considered to be one of ten most dangerous diseases by the World Health Organization (WHO) has also been found to be intricately linked to cancer in recent studies conducted by the same organization.[2]

In the report prepared by the Commission on Methods and Measures to Combat Obesity, it was announced that 34% of the population of Turkey was overweight in 2021, and the obesity rate was 39.1% in women and 24.5% in men. Because obesity is one of the leading causes of morbidity and mortality worldwide, numerous studies have been conducted to better understand the pathophysiologic mechanism of obesity and related diseases.[3,4] The most prominent of these mechanisms is that obesity-induced inflammation causes metabolic disorders and chronic diseases.[5] A host of metabolic abnormalities, oxidative stress, mitochondrial dysfunction, immune dysfunction, and chronic low-grade inflammation have been identified in the overweight obese patients.[6] Some studies have reported positive correlations between body fat mass and weight gain and plasma concentrations of inflammation-sensitive plasma proteins (ISP).[7] White adipose tissue serves as the largest endocrine organ to secrete ad-

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ipokines systemically.[8] Adipocytokines which are proinflammatory cytokines are thought to increase the hepatic synthesis of ISPs.[9] Ceruloplasmin is one of these ISPs. It is a copper-containing protein that is synthesized in the liver and is involved in copper transport, iron homeostasis, oxidant stress defense, angiogenesis and coagulation.[10] There are studies in the literature suggesting that serum ceruloplasmin level may be a biomarker associated with obesity and cardiovascular risk.[11,12] However, it is unclear whether losing weight will reduce serum ceruloplasmin levels in obese patients who are expected to have high serum ceruloplasmin levels.

Aim

The aim of our study was to investigate how fasting glucose, lipid profile, and serum ceruloplasmin levels were affected in obese patients who were enrolled in a low-calorie nutrition program, and whether ceruloplasmin is a biomarker of obesity status.

Material and methods

Ethical approval

Non-Interventional Clinical Research Ethics Committee of Istanbul Medipol University approved our study (date: 25.05.2022/Decision No: 477) and it was carried out in accordance with the Helsinki Declaration principles.

Study design

The study was designed as a single-center cross-sectional study. 157 patients (44 males and 113 females) who applied to internal medicine department of Istanbul Medipol University between June 2022 and August 2022, aged 18 and older with a BMI of 30 kg/m^2 or higher were included the study.

Patients with coronary artery disease and cerebrovascular disease, renal, hepatic, and thyroid dysfunction, diagnosed with diabetes and/or hyperlipidemia and taking medication for these reasons, acute or chronic inflammatory diseases, orthopedic limitations, pregnant women, breastfeeding women, smokers and those on medical treatment for obesity were excluded from the study.

Intervention

Patients were thoroughly informed about the content of the study and written informed consent forms were obtained. Detailed anamnesis and physical examinations were carried out and demographic data such as age, gender, height, weight, BMI and chronic diseases were obtained. Body weight and height were measured in the morning while fasting, naked and barefoot. Body mass index (BMI) was calculated as body weight in kilograms divided by height in square meters (kg/m2). After 12 hours of fasting, venous blood samples were collected. Total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), lipid profile including triglycerides (TG), fasting blood glucose (FBG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, creatinine, thyroid stimulating hormone (TSH), C-reactive protein (CRP), complete blood count and serum ceruloplasmin levels were all measured in the laboratory. The enzyme immunoassay was used to measure the level of ceruloplasmin in the blood (Assay-Max Human Ceruloplasmin Elisa Kit, AssayPro, MO, USA). Patients were enrolled in a 16-week balanced diet program (55% carbohydrates, 25% lipids, and 20% protein) with the goal of losing 10% or more of body weight while maintaining a daily energy deficit of 500–1000 kcal/day and advised to walk for 30–45 minutes a day, 3–4 days a week, at a pace of 5–6 kilometers per hour.

Patients were weighed every two weeks. The patients' weight measurements were re-measured at the end of the 16-week follow-up period, their BMI was calculated, blood tests were repeated, and the effect of weight loss on these values was examined. Due to noncompliance with the diet, failure to achieve weight loss, or study discontinuation, 13 male and 24 female patients were excluded from the study and the study was completed with a total of 120 patients, 31 males and 89 females.

Diabetes mellitus was defined as fasting blood glucose 126 mg/dl.

Dyslipidemia was defined as TG >150 mg/dl and/or LDL >130 mg/dl and/or HDL <40 mg/dl in men and <50 mg/dl in women.

Statistical analysis

In the reference "Plasma ceruloplasmin as a biomarker for obesity: A proteomic approach," the correlation between BMI and serum ceruloplasmin level was found to be 0.265.[11] In our study, Type 1 Error was calculated as α=0.05, the power of the study was calculated as 1-β=0.80 and the sample size was calculated as 109. The MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013) program was used for the sampling calculations.

Continuous variables were described using descriptive statistics (mean±standard deviation, minimum, median, and maximum).

The conformity of continuous variables with normal distribution was examined by the Shapiro-Wilk test.

The relationship between two continuously dependent variables that did not fit the normal distribution was examined using the Wilcoxon Signed Rank test.
The correlation between two continuous variables that did not fit the normal distribution was examined using the Spearman Rho Correlation Coefficient.

The statistical significance level was set at 0.05. The MedCalc® Statistical Software version 19.7.2 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021) program was used for the analyses.

Results

Demographic data including age, gender and comorbidities are given in Table 1.

Table 1. Demographic data

	n	%
Sex, n (%)		
Male	31	25.8
Female	89	71.8
Age	Mean±SD	Med (min–max)
	52.2±10.8	53.5 (23–71)
	n	%
Diabetes		
Yes	15	12.5
No	105	87.5
Dyslipidemia		
Yes	104	86.7
No	16	13.3
Dyslipidemia criterion 1 (HDL <40 in males, <50 for females, mg/dL)		
Yes	76	63.3
No	44	36.7
Dyslipidemia criterion 2 (Triglyceride >150 mg/dL)		
Yes	58	48.3
No	62	51.7
Dyslipidemia criterion 3 (LDL >130 mg/dL)		
Yes	32	26.7
No	88	73.3

When the patients' weight parameters (kilograms and BMI) were compared before and after the study, there was a significant difference. The patients' mean kilogram and BMI decreased statistically significantly (p<0.001). When the laboratory parameters (ceruloplasmin, total cholesterol, HDL, LDL, TG, and fasting blood glucose) were compared before and after the study, there was a significant difference. Mean ceruloplasmin, total cholesterol, LDL, fasting blood glucose decreased, and HDL increased statistically significantly (p<0.001) (Table 2).

There was a statistically significant and weak correlation between change in ceruloplasmin and change in BMI (p=0.016, R=0.233). There was a statistically significant and weak correlation between ceruloplasmin change and weight change (p=0.01, R=0.251, Table 3).

Discussion

In the present study fasting blood glucose, total cholesterol, LDL, TG, and ceruloplasmin levels were found to be lower in obese patients who lost weight with a 16-week balanced diet program, while HDL levels increased.

It is known that lifestyle change including diet and exercise is the first step and one of the most effective approaches in the treatment of obesity and related diseases.[13] Dietary therapy has been shown in studies to have a significant effect on lipid levels.[14] It has been reported that a 5-10% decrease in body weight results in a 20% decrease in triglycerides, a 15% decrease in LDL cholesterol, and an 8-10% increase in HDL cholesterol.[15] According to the results of this study, 86.7% of the obese patients examined had dyslipidemia, and the lipid profile improved significantly with weight loss, which is consistent with the findings of other studies in the literature.

Table 2. Weight parameters and laboratory parameters*

	Before	After	p
BMI (kg/m²)			
Mean±SD	35.8±5.6	31.9±4.9	<0.001
Med (min–max)	34.1 (24.2–54.4)	31.2 (21.5–45.8)	
Final weight (kg)			
Mean±SD	93.2±15.1	83.2±13.1	<0.001
Med (min–max)	90 (70–141)	83 (60–123)	
Ceruloplasmin (mg/dL)			
Mean±SD	25.2±4.7	23.6±4.9	<0.001
Med (min–max)	25.3 (7.2–35.7)	23.9 (8.1–32.5)	
Total cholesterol (mg/dL)			
Mean±SD	191.8±37.1	153.8±28.7	<0.001
Med (min–max)	188 (112–278)	148 (108–231)	
HDL (mg/dL)			
Mean±SD	43.7±10.7	47.7±12.3	<0.001
Med (min–max)	40 (27–68)	47 (26–74)	
LDL (mg/dL)			
Mean±SD	120.3±31.4	91.1±27.7	<0.001
Med (min–max)	119.4 (58–205.4)	85.8 (30.4–146)	
TG (mg/dL)			
Mean±SD	163.9± 84.7	104.9±55.7	<0.001
Med (min–max)	141 (35–427)	104 (34–285)	
Fasting blood glucose (mg/dL)			
Mean±SD	108.2±35	103.3±81.1	<0.001
Med (min–max)	98 (72–292)	90.5 (69–713)	

* Wilcoxon Signed Rank test

Table 3. Examination of the correlation between change in ceruloplasmin and BMI change/weight change*

	R	p
BMI x Ceruloplasmin	0.233	0.016
Weight x Ceruloplasmin	0.251	0.01

* Spearman's rho test

Similar to dyslipidemia, dysglycemia improves dramatically with weight loss. American Association of Clinical Endocrinologists has prioritized the fight against obesity in the treatment algorithm for type 2 diabetes. Excess weight loss improves blood glucose control and reduces the need for medication.[16,17] According to studies, a 7% reduction in body weight is enough to reduce the risk of type 2 diabetes by 58%. It has been emphasized that losing weight reduces the risk of diabetes even if it does not result in an ideal weight.[18,19] In this study, 12.5% of the patients had overt diabetes and there was a significant change in fasting blood glucose averages with diet.

The chronic low-grade inflammation associated with obesity and the subsequent altered metabolism has been termed "metaflammation."[20]

For many years, adipose tissue was thought to be static tissue; however, it is now known that it is an en-

docrine organ that secretes a large number of bioactive proteins known as adipocytokines.[21]

These adipocytokines stimulate the production of ISPs such as ceruloplasmin. The regression of ceruloplasmin levels with weight loss, as seen in our study, may be related to a decrease in adipocyte pool and cytokine synthesis.

There are some studies in literature examining the relationship between obesity and ceruloplasmin. Kim et al. looked into new biomarkers that could be used in obesity and found that C-reactive protein, fibrinogen and ceruloplasmin levels were higher in obese people than in non-obese people.[11] Böyük et al. evaluated the change in serum ceruloplasmin levels in obese patients who achieved weight loss after laparoscopic adjustable gastric banding operation. Weight loss was found to be 12.9±3.3 kg between the preoperative period and the 3rd month of the postoperative period, while the serum ceruloplasmin level decreased from 33.3±15.7 mg/dL to 23.9±8.8 mg/dL. A positive correlation was found between weight loss and a decrease in serum ceruloplasmin levels.[22]

Some studies have reported that high ceruloplasmin concentrations represent an elevated risk for cardiovascular diseases. Considering that conditions such as diabetes, hyperlipidemia, hypertension, and obesity are associated with inflammation and each of them is a factor contributing to cardiovascular risk, it is possible to explain this relationship.[23]

The relationship between obesity and ceruloplasmin has been investigated not only in adults but also in children. In a cross-sectional study that included 976 patients investigating the relationship between metabolic syndrome, systemic inflammation markers and ceruloplasmin in adolescents, adolescents with metabolic syndrome had higher TNF-α, IL-6, CRP and ceruloplasmin levels than those without metabolic syndrome, all values were associated with metabolic syndrome components and especially insulin resistance. However, it was determined that the marker with the highest correlation was ceruloplasmin. Serum ceruloplasmin level in adolescents has been identified as the most useful marker for inflammation and future cardiovascular disease risk.[24]

The reason for the increase of ceruloplasmin due to inflammation in obesity is because it is a powerful antioxidant. Myeloperoxidase is a neutrophil enzyme that increases oxidative stress in many inflammatory pathologies and catalyzes the production of free radicals via hydrogen peroxide. Ceruloplasmin is a potent inhibitor of myeloperoxidase. In other words, it rises to compensate for the oxidative stress caused by inflammation in obesity.[25]

Ceruloplasmin inhibits lipoperoxidation through iron metabolism. There is a similar mechanism not only in obesity but also in every disease that progresses with inflammation. For example Taysi et al. found that serum ceruloplasmin levels were higher in patients with rheumatoid arthritis than in those without a diagnosis in their study. Patients with rheumatoid arthritis have higher levels of free radicals in their blood. Peroxidation caused by free radicals damages lipid cell membranes, and ferrous (++) iron acts as a catalyst for lipoperoxidation. Ceruloplasmin is an antioxidant that prevents lipoperoxidation by converting ferrous iron to ferric iron (+++).[26]

Study limitations

Our study is a single-center study with 120 patients. Multicenter studies with more patients are needed. In addition, since it is a cross-sectional study, the causality relationship could not be established clearly. Cohort studies that give clearer cause-effect relationships are needed.

Conclusion

In this study, ceruloplasmin levels were found to be higher in obese patients in accordance with other studies in the literature and in addition to the results of these studies it was determined that ceruloplasmin levels decreased with weight loss. The results of the study suggest that ceruloplasmin may be a marker of obesity.

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Declarations

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Author contributions

Conceptualization, E.Y.; Methodology, E.Y. and I.S.; Software, E.Y. and I.S.; Validation, I.S.; Formal Analysis, E.Y.; Resources, E.Y. and I.S.; Data Curation, E.Y. and I.S.; Writing - Original Draft Preparation, E.Y.; Writing – Review & Editing, E.Y. and I.S.; Supervision, I.S.; Project Administration, E.Y.

Conflicts of interest

The authors declare no competing interests and no conflict of interests.

Data availability

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

Ethics approval

This study was approved by Non-Interventional Clinical Research Ethics Committee of Istanbul Medipol University approved our study (date: 25.05.2022/Decision No: 477) and it was carried out in accordance with the Helsinki Declaration principles.

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ORIGINAL PAPER

Biochemical and planimetric investigations of hydrophilic creams containing ceramides or dexpanthenol on the model of chemical burns

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ABSTRACT

Introduction and aim. Chemical burns of the skin are common type of injuries both in private life and in industries. Local treatment of chemical burns using wound healing creams and ointments is predominant. Hydrophobic wound healing medicinal products dominate the Ukrainian pharmaceutical market but their hydrophobic base disturbs the healing process of skin. The aim of this work was biochemical and planimetric investigation of treatment efficacy of chemical burns with hydrophilic creams containing ceramides and dexpanthenol.

Material and methods. The experiments were performed on 30 rats weighing 190–220 g. In a rat skin burn model, animals were exposed to 9% acetic acid solution. Treatment was initiated after wound appearance and included application of creams containing ceramides and dexpanthenol. The effectiveness of treatment was estimated using planimetric parameters, such as: surface area of necrotic tissue (S, mm^2) and cumulative reparative effect. Levels of the biochemical markers such as total protein, creatinine, C-reactive protein (CRP) and content of SH-groups were measured in the rats' blood serum.

Results. It was established that cream developed with ceramides and cream with dexpanthenol exhibits reparative properties at the level of 29 % and 4.5 %, respectively. Biochemical investigations demonstrated the treatment efficacy of creams containing ceramides and dexpanthenol. In terms of CRP level and content of SH-groups, the therapeutic action of cream with dexpanthenol was highly significant by a factor of 1.45 and 1.35, respectively in contrast to the cream with ceramides.

Conclusion. Using the chemical burn model and results of planimetric and biochemical research it was found that cream with ceramides and cream with dexpanthenol exhibit wound-healing properties. In-depth study on the wound-healing mechanism of investigated creams with the aim of creating effective hydrophilic creams for use in burn treatment is prospective.

Keywords. ceramides, chemical burns, dexpanthenol

Introduction

Currently, chemical burns are a very common trauma.[1-3] Many substances that are freely available in the community, either occupational or domestic items, have the potential to cause chemical burns. Every year up to 10000 cases of chemical burns are registered in Ukraine and 50-70% patients have no need for operative intervention.[4-6]

It should be noted that chemical burns commonly occur as a result of chemical ingestion that causes tissue-necrosis (for example, concentrated acids – acetic, sulfuric, hydrochloric and others). Depending on the concentration and exposure time to substance, deep lesions on skin can form as coagulation necrosis that caused by the coagulation of proteins due to quick elim-

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ination of water from the epidermis.[1-3] Significant intoxication is characteristic for deep burns due to the formation of toxic protein cleavage products and its absorption.[1,4] On the other hand, diluted acids result in edema and blisters on the skin because of migration of cellular water under epidermis. Such kinds of burns are interfacial and have a large area.

Local treatment of burn lesions using wound-healing agents is a beneficial. For now, dexpanthenol is a drug of choice because it is absorbed quickly by skin, converting to pantothenic acid that is a component of coenzyme acetyl-CoA that plays a significant role in cellular metabolism (synthesis of ATP, acetylglucosamines, mucopolysaccharides, elimination of waste products of deamination of amino acids, optimization of fatty acid and phospholipids metabolism).[6] It is known that pantothenic acid exhibits reparative properties that facilitate division of cells, make collagen fibers stronger, rejuvenates skin and also has anti-inflammatory action.[7] Nowadays, there are a lot of hydrophobic creams and ointments with dexpanthenol. This hydrophobic base promotes formation of a lipid layer on the skin and inhibits qualitative wound healing.[4] This is why development of hydrophilic topical medications is currently important.

Humidification of skin is considered to be one of the most prospective approaches to the optimization of regenerative processes of skin because it prevents excessive dryness of skin, deepening of necrosis and development of cicatricial malformations. One of the methods of skin correction is an application of ceramide-based cosmetic products because ceramides facilitate restoration of the epidermis and a decrease in skin dehydration.[8-10]

Aim

The aim of this research was a comparison of treatment efficacy of hydrophilic creams containing ceramide or dexpanthenol on the model of chemical burn.

Material and methods

Experiments were performed in 30 rats, weighing 190–220 g, which were divided in 5 groups of animals (n=6): 1 group – control group (healthy animals); 2 – peak of pathology (formation of chemical burn); 3 – control pathology (CP) – chemical burn was produced in animals and they were not treated in the experiment; 4 – animals with chemical burn treated with cream containing ceramides; 5 – animals with chemical burn treated with cream containing dexpanthenol. The technique for manufacturing creams with ceramides (composition: 0.5% of ceramides, hydrophilic base up to 100 g) and dexpanthenol (composition: 5 % of dexpanthenol, hydrophilic base up to 100 g) was developed in the State Scientific and Research Center of Medicinal products under supervision of Prof. M.O. Lyapunov.

Chemical burns of skin were induced by 0.5 ml subcutaneous injection of 9% acetic acid solution. On the third day of acetic acid injection coagulation necrosis appeared, leading to ulceration and skin inflammation.[4] When the wound occurred, cream-based treatment was started. The investigated creams were applied on the wound once a day to complete healing.

A wound area was measured as follows: transparent linear graph paper was applied on the wound outlines and area of wounds (cm^2) was measured at different time of observation (initial wound area, 5, 7, 9, 11, 13, 15, 17 day of treatment).

The effectiveness of treatment was estimated using planimetric parameters, such as: surface area of necrotic tissue (S, mm^2) and cumulative reparative effect.

Surface area of necrotic tissue (S, mm^2) was calculated according the following formula:

$$S = S_{initial} - S_{(t),} \text{ where}$$

$S_{initial}$ – initial wound area, mm^2;
$S_{(t)}$ – wound area at the day of measurement, mm^2.

Cumulative reparative effect was calculated using the statistical package «MedCalk, v. 9.3.7.0» and used as a value of integrated index of area under curve «surface area of healing – time».

Level of total protein (TP) and creatinine was determined in blood plasma by photometry using the kits made by "Filisist-Diagnosis", Ukraine. Level of C-reactive protein (CRP) was quantified by ELISA test («UkrMedService», Ukraine) using analyzer «Libline-90» (Austria); content of SH-groups was determined using the specific thiol reagent – 5,5′-Dithiobis (2-nitrobenzoic acid) (also called DTNB or Ellman's reagent).[4]

All laboratory animal experiments were performed according to the rules of humane treatment of laboratory animals and as per the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" and the Decree of the First National Congress on Bioethics.[11]

Laboratory animals got a nutritionally balanced diet (combined feed of PF "Vita" Kharkiv, Ukraine). During the experiments laboratory animals were kept in the plastic cages in an experimental animal room at temperatures between 18–24°C, relative humidity not more than 55% and a normal day/night cycle.

All interventions and euthanasia of animals was performed in accordance with animal bioethical standards.[11] Results of analysis were processed using the program "Statistica 8" with $p<0.5$ (StatSoft, Tulsa, USA).

Results

Penetration of acetic acid in the skin resulted in the formation of coagulation necrosis (ulceration) with an eschar area 259-290 mm^2. Analysis of planimetric parameters showed that in the group of untreated rats (CP) healing of wounds during five days was slow as evidenced by the small healing area of wounds (13.50 mm^2) (table 1). On the ninth day, the healing process in animals was more intensive as evidenced by the increase of healing area up to 94.83 mm^2. In the course of the treatment of animals with burns intensive healing takes place already on day 5 – healing area in the group of animals treated by the cream with ceramides was 33.17 mm^2, but in the group of animals treated by the cream with dexpanthenol the healing area was 35.33 mm^2, which is significantly higher by a factor of 2.45 and 2.62 in comparison with CP, respectively. On day 13 of treatment by a cream with dexpanthenol healing area (260 mm^2) was significantly higher than at the case of treatment by the cream with ceramides (233.33 mm^2).

Total reparative effect, calculated as area under curve «area of healing – time», for group of animals which were treated by the cream with ceramides was 2092.83 (difference is 29%), by the cream with dexpanthenol – 2295.82 (difference is 41.5 %) in comparison with group of control pathology – 1623.02 (Fig. 1).

Skin damage due to the burn can be caused by the direct loss of protein because of hemorrhaging and necrosis. This state is accompanied by an increased protein and carbohydrate supply resulting in a metabolism change. Major disturbances of protein metabolism results in arrested development of granulation tissue, and epithelialization. Also, it disturbs the healing process. That's why it makes sense to determine level of total protein (TP) and C-reactive protein (CRP) in the blood to estimate the treatment efficacy.

A statistically significant decrease of the total protein level (1.4×) took place after the simulation of burns caused by acetic acid in comparison with control group (Table 2). Increase of total protein level (1.1×) had been noticed after the application of cream with ceramides, but the level of TP did not get the intact values. The level of TP increased significantly (1.24×) in the group treated by the cream with dexpanthenol and it was close to the intact values as evidenced by the suppression of irritation in necrotic areas of skin.

Table 1. Dynamic pattern of healing area (mm^2) under the influence of investigated medicinal products on the chemical burn model (n=6) [a]

Days of treatment	Study groups		
	Control pathology	Cream with ceramides	Cream with dexpanthenol
Initial wound area	263.83±14.94	258.50±9.41	287.17±8.43
		Healing area	
5	13.50±6.34	33.17±2.02 *	35.33±2.65 *
7	36.00±6.47	96.67±6.4 *	100.67±7.27 *
9	94.83±5.97	123.33±4.95 *	129.33±5.73 *
11	135.17±10.98	174.67±6.52 *	192.83±8.56 *
13	197.83±10.98	233.33±8.99	260.00±6.56 */**
15	217.17±14.91	251.33±9.25	280.00±7.33 */**
17	233.83±13.03	258.50±9.41	287.17±8.43 *

[a] * – deviation is statistically significant in relation to the group of control pathology, p<0.05; ** – deviation is statistically significant in relation to the cream with ceramides, p<0.05 (Mann-Whitney test); n – number of animals in the group

Statistically significant increase of the CRP level was 23.4× in comparison with the intact group which provides evidence of the development of necrotic inflammatory process at the peak of pathology. After completion of the experiment, the level of CRP in all groups decreased significantly but in different manner – 4.2× in the group of control pathology; after the treatment by the cream with ceramides – 10.6×; after the treatment by the cream with dexpanthenol – 15.4×. Such changes are evidence of a reduction of adverse reactions.

A significant increase of creatinine level (2×) in rats' blood serum shows the intensity of destructive changes in the development of chemical burn (peak of pathology). This parameter in the group of CP changed little as evidenced by the stability of necrotic processes. After

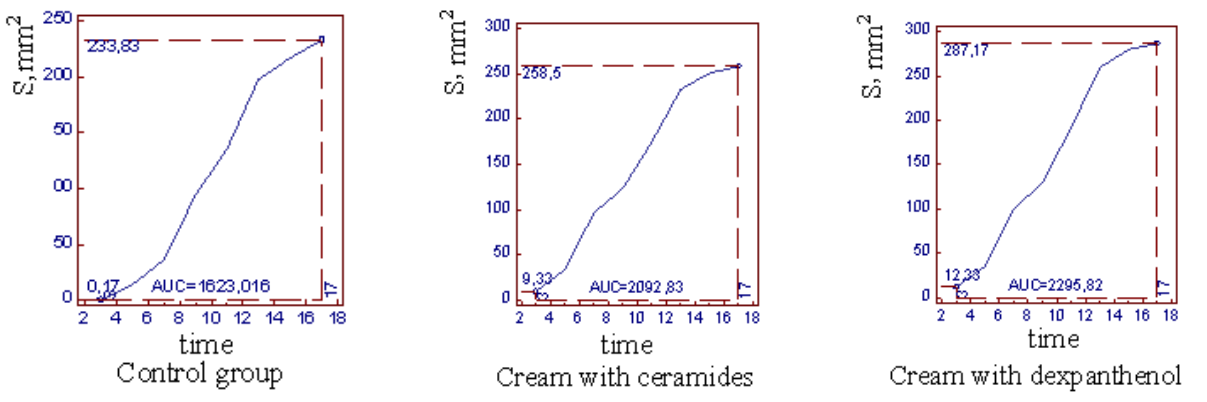

Fig. 1. Total reparative effect for the cream with dexpanthenol and cream with ceramides on the model of chemical burn

the treatment of animals, a lowering of the creatinine level occurred as evidenced by the completion of necrotic phase of wound process. Creatinine level decreased significantly in the group of animals treated by the cream with dexpanthenol – 1.3× compared to the group of maximum pathology and this was significantly less than in the group of intact animals. Normalization of creatinine level was observed after the treatment by the ceramide-based cream but the values were unreliable.

Table 2. Biochemical parameters after the treatment by investigated medicinal products using the model of chemical burn (n=6) [a]

Test group	Total protein (C, g/L)	CRP (C, mg/L)	Creatinine (C, μmol/L)	SH- (C, mmol/L)
Group 1 Intact group (healthy animals – basic data)	73.70±3.5	0.48±0.04	55.11±3.86	12.74±0.74
Group 2 Maximum pathology (necrosis occurs – third day after subcutaneous injection of 9% acetic acid solution)	51.11±1.76 *	11.24±0.31 *	111.34±9.01 *	22.46±1.22 *
Group 3 Control pathology (17th day of observation)	58.24±3.83 *	2.66± 0.08*/**	109.66±7.83 *	18.28±1.12 */**
Group 4 Cream with ceramides (17th day of observation)	58.98±3.76 *	1.06± 0.03 */**/***	96.16±6.33 *	15.70±0.89 */**
Group 5 Cream with dexpanthenol (17th day of observation)	63.52± 1.19 */**	0.73± 0.04 */**/***	87.73± 3.26 */***	11.67± 0.82 **/***

[a] * – significant difference in relation to intact animals, $p<0.05$; ** – in relation to maximum pathology, $p < 0.05$; *** – in relation to control pathology, $p<0.05$ (Mann–Whitney test); n – number of animals in the group.

Changes of the state of antioxidant system (AOS) evidence the intensity of the inflammatory necrotic processes in the course of the experiment. After the formation of burn (peak of pathology) statistically significant increase of the content of SH-group (1.76×) was observed in comparison with intact animals (Table 2). These changes provide evidence of the activation of lipid peroxidation and elevation of antioxidant defense during the development of inflammatory necrotic process.

After the completion of the experiment state of AOS was normalized as evidenced by the lowering of the content of SH-group in all test groups (Table 2). Thus, this parameter decreased 1.2× in the group of control pathology; 1.4× in the group treated by the cream with ceramides; 1.9× in the group treated by the cream with dexpanthenol.

Summing up what has been said according the results of the planimetric and biochemical investigations, it was found that developed hydrophilic medicinal products (cream with ceramides and cream with dexpanthenol) have evident reparative properties as evidenced by promoted healing compared to control group. The therapeutic action of cream with ceramides matches the action of cream with dexpanthenol for first 11 days of treatment course. On day 13 and 15 the reparative action of cream with dexpanthenol was significantly better than the action of cream with ceramides. Biochemical research demonstrated an efficacy of treatment with investigated creams. In accordance with the results of CRP level determination and SH-group content, the efficacy of cream with dexpanthenol vs. cream with ceramides was significantly better.

Discussion

The variation in the severity of certain pharmacological effects of hydrophilic creams containing dexpanthenol or ceramides can be explained by the difference in their pharmacological action.

When tissues are damaged, the necessity for structural (proteins, carbohydrates, lipids, water), energy (vitamins, particularly pantothenic acid) and other biomaterials increases sharply. This is due to the fact that these materials take part in different biochemical processes, accelerating them and, thereby, restoring damaged tissues.[4] It is known that when applied topically, dexpanthenol is readily absorbed and rapidly converted enzymatically to pantothenic acid, a constituent of coenzyme A, which plays an important role in cellular metabolism (synthesis of ATP, acetylglucosamines and mucopolysaccharides, disposing of products of amino acid deamination, optimization of fatty acids and phospholipid metabolism).

Apart from reparative properties, pantothenic acid exhibits anti-inflammatory (it takes part in the synthesis of anti-inflammatory hormones) and immunomodulatory (it stimulates antibody production) effects.[5] The main components of ceramides are glycoceramides, cholesterol, and phospholipids. Glycoceramides decrease metabolic cost and loss of structural material for synthesis, renewing the content of endogenous glycoceramides, as well as work within lipid lamellar systems of the intercellular space facilitating restoration of epidermis and reducing dehydration. Phospholipids and cholesterol play an important role in the regeneration of the lipid bilayer. Pantothenic acid and cholesterol are necessary for synthesis of steroid hormones. It should be noted that hydration of skin is considered to be one of the most promising approaches for optimization of regeneration processes as it prevents extremely dry skin and enhancement of necrosis, preventing the development of cicatricial deformities.[9]

Conclusion

In planimetric studies on the model of chemical burn it was shown that application of cream with dexpanthenol and cream with ceramides facilitate healing necrotic ul-

cers on the skin. Cumulative reparative effect of cream with ceramides was 29%, but the reparative effect of cream with dexpanthenol was 41.5%. We consider the advantages of cream containing dexpanthenol is associated with its penetration in all skin layers and its ability to accelerate cell fission. This active substance effects the strength of collagen and restores the skin structure while ceramide-cream acts in the surface layers of epidermis and promotes cell repair.

Biochemical research proved an efficacy of treatment with investigated creams. In terms of CRP level and content of SH-groups therapeutic action of cream with dexpanthenol was highly significant by a factor of 1.45 and 1.35, respectively, compared to the cream with ceramides.

Application of drugs containing dexpanthenol and ceramide, that are wound-healing agents with different reparative action, is a promising and reasonable approach for the treatment of burn wounds.

Declarations

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Authors have no commercial interest and financial interest. The costs of the research were covered by the researchers.

Author contributions

Conceptualization, Y.B. and T.T.; Methodology, Y.B.; Software, T.T.; Validation, Y.B.; Formal Analysis, YB; Investigation, Y.B.; Resources, T.T.; Data Curation, Y.B.; Writing – Original Draft Preparation, Y.B. and T.T.; Writing – Y.B. and T.T.; Visualization, Y.B. and T.T.; Supervision, Y.B.; Project Administration, Y.B.; Funding Acquisition, Y.B.

Conflicts of interest

The authors have no conflict of interest.

Data availability

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

Ethics approval

The ethical approval was obtained from Ethics Committee of Clinical and Diagnostics Center of National University of Pharmacy (NUPh), Kharkiv (protocol No. 2 dated February 19, 2019).

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ORIGINAL PAPER

Care dependency in radiation oncology patients and related factors – a descriptive study

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ABSTRACT

Introduction and aim. The incidence of cancer is increasing on a daily basis. One of the methods used for treatment is radiotherapy. Owing to interventions during the radiotherapy process, the patient may experience care dependency. In this study, the aim was to investigate care dependence and related factors in radiation oncology patients.

Material and methods. This was a descriptive and cross-sectional study. Data were collected between September 2020 and September 2021. In the collection of data, a sociodemographic information form and a Care Dependency Scale were used. The sample consisted of 52 people.

Results. Number of participants was 52, mean age was 60.25±11.715, mean care dependency score (initial) 66.19±18.966, mean care addiction score (final) 66.27±22.795.

Conclusion. The care dependency of patients hospitalized in the radiation oncology clinic is moderate. The care dependency of these patients decreased partially during their stay in the clinic. The patient's inability to walk, speak and the presence of a companion affected the patient's condition. By evaluating the care dependency levels of the patients, the awareness of the nurses about their patients can be increased. In addition, it may be appropriate to consider the care dependency levels of the patients for the nurse workforce planning to work in the oncology clinic.

Keywords. care, care dependency, nursing, radiation oncology

Introduction

Cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths, or one in six deaths in 2018.[1] More people in Turkey are diagnosed with, and die from cancer each year. In Turkey, the incidence of cancer is 223.1 per hundred thousand, and the number of newly diagnosed individuals is 180,288.[2] Cancer also causes dependence, workforce loss, care needs, and treatment costs. Many cancer patients depend on family and friends, lose their jobs, and require support for their fundamental care needs and treatment costs.[3]

The best way to beat cancer is not to have cancer. However, if one is diagnosed with cancer, one undergoes the best treatment (surgery, chemotherapy, or radiotherapy) according to clinical and radiological evaluations.[1] Radiotherapy is a cancer treatment that uses high doses of radiation to kill cancer cells.[4] However, it is pretty challenging for both patients and caregivers because patients have more care needs depending on the progression of the disease.[5]

Cancer patients have more care needs because they depend heavily on others. Care dependency is defined as a need for assistance in at least one care domain to make

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up for a self-care deficit. Care dependency is a critical component for patients, family members, and nurses.[6] The goal of care is to promote patient independence in self-care. Nurses are responsible for helping patients gain independence.[7] Studies have reported that patients have some needs and patients expect nurses to communicate accurately and provide effective care and treatment.[8-10] Oncology inpatients need more nursing care and support from caregivers.[5,11]

It is critical to identify and meet care needs. In order to identify care needs, we should evaluate patient independence in terms of "Activities of Daily Living," such as taking a bath, eating, getting dressed and undressed, getting in and out of bed, and deferring evacuation (continence).[12] Nursing is based on assistance and care, and therefore, nurses plan and deliver individualized care.[4] Nurses who evaluate care dependency are likely to provide patients with better care tailored to their needs.[13]

Nurses who implement the nursing process properly are more likely to provide high-quality care. The nursing process consists of five steps: (1) evaluating the patient, (2) identifying problems, (3) planning care, (4) providing care, and (5) assessing health outcomes.[14] Oncology patients expect nurses to have a sound grasp of key nursing concepts and communicate effectively. Therefore, nurses should know how to provide high-quality and individualized care, which depends on their number and awareness.[15-17] However, there is no research on care dependency in radiation oncology clinics.

Aim

In this study, it was aimed to investigate care dependence and related factors in radiation oncology patients.

Material and methods

Ethical approval

Ethics committee approval and application permission were obtained for the study (Bursa City Hospital Ethic Committee/13012450-514.10). Authorization was received via email from the author, who established the Turkish validity and reliability of the Care Dependency Scale. Patients were informed about the research purpose and procedure, and written consent was obtained from those who agreed to participate. Strobe rules and Helsinki Declaration were compiled with at all stages of the research.

Population and sample

This was a descriptive and cross-sectional study.

The study was conducted between September 1, 2020, and September 1, 2021, in the radiation oncology clinic of a Ministry of Health hospital. The sample size was determined based on the logistic regression conducted by Bilgin et al., who reported that hearing and walking problems had an odds ratio of 4.547 and 20.133, respectively.[18] The power analysis (G*power, v. 3.1) showed that a sample size of 48 would be large enough to detect significant differences (power =0.85, alpha margin of error=0.05, and effect size=0.85). The sample consisted of 52 oncology inpatients with a post hoc power of 0.88. The inclusion criteria were (1) being over 18 years of age, (2) being conscious (who can give accurate and meaningful answers to questions about himself/herself), and (3) being able to communicate (speaking, hearing, having no problem in verbal communication). Those who had difficulty expressing themselves were excluded. In the inclusion/exclusion criteria; detailing the type/location of the tumor and the type/location of radiotherapy of the participants.

Research questions

1. How care-dependent are radiation oncology inpatients?
2. Is there a difference in radiation oncology inpatients' care dependency levels between admission and discharge?
3. What factors affect radiation oncology inpatients' care dependency?

Data collection tools

Data were collected using a sociodemographic characteristics questionnaire (SCQ) and the Care Dependency Scale (CDS).

The questionnaire was based on a literature review.[6,7,12,13] It consisted of items on age, gender, marital status, education, economic status, living arrangement, using eyeglasses, prosthesis, a walking stick, hearing aids, and vision, hearing, speech, and walking problems.

The Care Dependency Scale (CDS) was developed by Dijkstra and revised by Dijkstra et al.[19,20] The scale was adapted to Turkish by Yönt et al. The instrument consists of 17 items scored on a five-point Likert-type scale ("1= completely care-dependent" to "5=almost independent"), with the total score ranging from 17 to 85. The instrument has no cut-off point or subscales. Higher scores indicate higher care dependency.[21] The original scale has a Cronbach's alpha of 0.96.[19,20] The Turkish version of the scale has a Cronbach's alpha of 0.91, which was 0.93 in the present study.[21]

Data collection

The researcher collected the data face-to-face through participant observation. Interviews were held in the patient's room. During the interview, the privacy of the patient was ensured, with the patient and the nurse alone in the room. Necessary protective measures have been taken. The person collecting the data was a clinical nurse. Data were collected between September 2020 and September 2021. She received informed consent from

all participants. Afterward, the participants filled out the SCQ and CDS. The research did not interfere with the routine treatment and follow-up. The patient received routine care in the clinic. Before discharge, the participants filled out the CDS again.

Statistical analysis

The data were analyzed using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM, Armonk, NY, USA). The Kolmogorov–Smirnov test was used for normality testing (n>30). The results showed that the data were nonnormally distributed (p<0.05). Mean, standard deviation, number, and percentage were used for descriptive data. The Mann-Whitney U test was used for data comparison between two groups. The Kruskal-Wallis H was used for data comparison between three or more independent groups. The Wilcoxon signed-rank test was used for pretest and posttest comparison within the groups. Spearman's rank correlation coefficient was used to determine the relationship between scale scores. A logistic regression analysis was performed to determine the factors affecting care dependency.

Results

Sociodemographic data of the participants; calculated as mean, standard deviation, number and percentage. Number of participants was 52, mean age was 60.25±11.715, mean care dependency score (initial) 66.19±18.966 (Median: 14.18), mean care addiction score (final) 66.27±22.795 (Median: 18.88). Most of the group was male (73.1%), married (85.5%), living with spouse/children (92.3%), not using glasses (82.7%), not using a cane (576.9), not using hearing aids (75.0%, no speech problem (58.3%), walking problem (61.5%), accompanying person (76.9%), diagnosed with lung cancer (59.6%). Half the participants had metastasis (50%).

The Mann-Whitney U and Kruskal Wallis tests were used to determine the effect of independent variables on participants' pretest and posttest CDS scores. According to the Kruskal Wallis test results, education, economic status, and cancer type did not affect participants' pretest and posttest CDS scores (p>0.05). According to the Mann-Whitney U test results, gender, age, marital status, living arrangement, wearing eyeglasses, using walking sticks and hearing aids, having chronic diseases, having metastasis, and radiotherapy duration had no effect on participants' pretest and posttest CDS scores (p>0.05). Those who had speech problems, walking problems, and had a companion had a higher level of care dependency (CDS score was lower) and the difference was significant (p<0.05) (Table 1).

The CDS score obtained when the participants came to the hospital was lower, but the difference was statistically insignificant (p>0.05) (Table 2).

Table 1. The distribution of CDS scores by variables (n=52)*

Variables		Mean	SD	Min- max			
Age		60.25	11.715	28–78			
CDS Pretest		66.19	18.966	19–85			
CDS Posttest		66.27	22.795	17–85			
		n	%	Pretest CDS		Posttest CDS	
				Mean Rank	Sum of Ranks	Mean Rank	Sum of Ranks
Gender	Female	14	26.9	28	392	27.14	380
	Male	38	73.1	25.95	986	26.26	998
				U=254		U=257	
				p=0.659		p=0.849	
Marital status	Married	45	86.5	27.74	1248.5	67.62±3.39	77
	Single	7	13.5	18.5	129.5	57.57±8.635	
				U=101.5		U=101.5	
				p=0.126		p=0.125	
Living arrangement	Alone	4	7.7	27.13	108.5	24.88	99.5
	With a spouse/child	48	92.3	26.45	1269.5	26.64	1278.5
				U=93.5		U=89.5	
				p=0.93		p=0.819	
Wearing eyeglasses	Yes	9	17.3	24.11	217	30.44	274
	No	43	82.7	27	1161	25.67	1104
				U=172		U=158	
				p=0.596		p=0.38	
Using a walking-stick	Yes	12	23.1	24.25	291	21.3	256.5
	No	40	76.9	27.18	1087	28.04	11211.5
				U=213		U=178.5	
				p=0.55		p=0.172	
Using hearing aids	Yes	13	25.0	24.54	293	23.35	303.5
	No	39	75.0	27.82	1085	27.55	1074.5
				U=202		U=212.5	
				p=0.268		p=0.376	
Speech problems	Yes	24	46.2	15.31	367.5	17.17	412
	No	28	53.8	36.09	1010.5	34.5	966
				U=67.5		U=112	
				p<0.001		p<0.001	
Walking problems	Yes	32	61.5	17.05	545.5	18.16	581
	No	20	38.5	41.63	832.5	39.85	797
				U=17.500		U=53	
				p<0.001		p<0.001	
A next of kin as a caregiver	Yes	40	76.9	24.21	968.5	24.23	969
	No	12	23.1	34.13	409.5	34.08	409
				U=148.500		U=149.000	
				p=0.043		p=0.043	
Diagnosis time (month)	1-12	45	86.5	28.32	1274.5	27.86	1253.5
	≥13	7	13.5	14.79	103.	17.79	124.5
				U=75.5		U=96.5	
				p=0.025		p=0.095	
Chronic diseases	Yes	31	59.6	25.76	798.5	25.29	784
	No	21	40.4	27.60	579.5	28.29	594
				U=302.50		U=288	
				p=0.662		p=0.475	
Metastasis	Yes	26	50.0	25.62	666	26.83	697.5
	No	26	50.0	27.38	712	26.17	680.5
				U=315		U=329.5	
				p=0.668		p=0.874	
				Mean Rank		Mean Rank	
Education (degree)	Primary school	34	65.4	27.18		25.96	
	Middle school	11	21.2	28.5		30.14	
	Bachelor's	7	13.5	20.07		23.43	
				KW=1.575		KW=1.008	
				p=0.455		p=0.604	
Economic status	Income<expense	7	13.5	17.86		18.64	
	Income= expense	24	46.2	30.4		29.52	
	Income> expense	21	40.4	24.93		25.67	
				KW=4.239		KW=3.03	
				p=0.12		p=0.22	
Diagnosis	Lung cancer	31	59.6	27.47		26.11	
	Cervical cancer	4	7.7	23.59		27.03	
	Others	17	32.7	24.25		21	
				KW=0.802		KW=0.553	
				p=0.67		p=0.759	

* Mann-Whitney U test *p<0.05

Table 2. Difference between pretest CDS and posttest DCS scores*

	Median	Min	Max	Test statistic	p
Pretest CDS	14.18	19	85	-0.421	0.673
Posttest CDS	18.88	17	85		

*Wilcoxon Signed Ranks Test

A Spearman's correlation coefficient was used to determine the relationship between age, and CDS scores. When the coefficient values are statistically significant, the magnitudes of the correlations are classified as follows: ≤0.25 very low; 0.26–0.49 low; 0.50–0.69 moderate; 0.7–0.89 high; 0.9–1 very high.[22,23] There is a positive and strong correlation between the Pretest CDS and posttest CDS. The correlation between age and both CDS scores is negative and weak ($p<0.05$) (Table 3).

Table 3. The correlation between pretest CDS, posttest CDS scores and age variables*

	Pretest CDS		Age	
	R	p	R	p
Posttest CDS	0.826	<0.0001	-0.324	0.018
Age	-0.245	<0.0001		

*Spearman's correlation coefficient

Discussion

In this study, the care dependency of individuals, whether there is a difference between the dependence levels on the first day of hospitalization and the day they are discharged, and the factors affecting care dependency are discussed in the light of the literature.

Being a cancer patient is a factor that increases care dependency.[24,25] Bilgin et al. reported that the CDS score of inpatients in the oncology clinic was 60.1±17.34, and 37.83±21.42 in another study.[18,26] In the study of Koyuncu, the CDS score was similar in patients who received chemotherapy treatment (68.98±15.89).[27] In general, it can be said that care dependence in patients in the radiation oncology clinic is at a moderate level. Radiation therapy shrinks some types of tumors.[28] Our participants had lower care dependency levels during discharge. It can be thought that radiotherapy treatment contributes to the regression of the disease.

There are many factors that affect care dependency in oncology patients. One of them is to be in the hospital and the other is to be in the terminal period.[25] Prolongation of hospitalized patients increases care dependency of patients.[25,26,29] Being older supports this situation.[25,30] In the study of Bilgin et al., it was stated that 60.2% of the group aged >65 years were care dependent.[18] Schuttengruber et al. reported that 72% of the participants were care dependent in their study in the geriatric age group.[25] This is supported by low education, living alone, and physical disabilities.[30] The difference in the study of Bilgin et al. and Schuttengruber et al. may be due to the different age groups. Similarly, in this study, age seems to negatively affect care dependency. It can be said that care dependency increases as age increases.

Another factor affecting care dependency is the presence of the individual's physical disability. According to the study of Güler et al., the mean BDI score of the physically disabled group is 56.53±14.46 and they need more care.[31] As the physical disability of the patient increases, the dependency on the caregiver increases.[32] 96.6% of individuals with speech problems and 91.5% of individuals with walking problems constitute the group with high care dependency.[18] Having a walking and speaking disability may be a factor that increases care dependency for the individual.

The care dependency of the patient with oncological diagnosis can decrease with the contribution of the treatment during the treatment process.[27] It has been stated that individuals who continue radiotherapy treatment need emotional support, physical care and education.[33] In this process, patients need the support of both health personnel and their relatives.[34] In patients diagnosed with cancer, the radiotherapy process increases their anxiety and individuals expect support from their environment.[34-36] During this period, patients can sometimes positively perceive being dependent on someone else. These individuals especially think that care has a healing effect on them.[37] It can be said that patients expect their companions to take care of them in this process.

Study limitations

The study had two limitations. First, it was conducted during the COVID-19 pandemic. Second, the sample included patients from only one hospital.

Recommendations

By evaluating the care dependency levels of the patients, the awareness of the nurses about their patients can be increased. In addition, it may be appropriate to consider the care dependency levels of the patients in the nurse workforce planning to work in the oncology clinic. New studies may be planned in which the relationship of patients' care addictions to the type of tumor and radiotherapy is evaluated.

Conclusion

The care dependency of patients hospitalized in the radiation oncology clinic is moderate. The care dependency of these patients decreased partially during their stay in the clinic. The patient's inability to walk, speak and the presence of a companion affect his or her condition.

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ORIGINAL PAPER

The relationship of biochemical parameters and radiological parameters in the evaluation of the clinical severity of acute pancreatitis in the emergency department – a retrospective analysis

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ABSTRACT

Introduction and aim. Computed tomography severity index (CTSI) and Balthazar score are among the most frequently used scorings in the determination of severe acute pancreatitis. The primary purpose of this study is evaluation of the effects of biochemical parameters, Balthazar score and CTSI on mortality in acute pancreatitis. At the same time, correlations with biochemical parameters, CTSI and Balthazar score were evaluated in patients with AP.

Material and methods. In this study, the amylase, lipase, CRP, and procalcitonin values of patients diagnosed with acute pancreatitis were retrospectively recorded. Contrast-enhanced computed tomography (CECT) images obtained at the time of presentation to the emergency department or within seven days of admission were re-evaluated by two radiologists. The CTSI scores and Balthazar scores of the patients were calculated.

Results. The study included 240 patients. The amylase level of the patients was positively correlated with the Balthazar score at a statistically significant level (R=0.189, p=0.003). In addition,, the relationship between pancreatic scoring systems and mortality, the AUC value for CTSI was 0.9 (95% CI: 0.826-0.973) and was higher than other scoring systems.

Conclusion. CTSI had better performance in the prediction of mortality in patients with acute pancreatitis.

Keywords. acute pancreatitis, amylase, computed tomography severity index, emergency department, lipase

Introduction

Acute pancreatitis (AP) is an inflammatory condition of the pancreas, most commonly caused by gallstones or excessive alcohol use. AP mostly has a mild course with rapid clinical improvement following fluid resuscitation, management of pain and nausea, and early oral feeding. However, 20-30% of AP cases are severe. The mortality rate reaches 15% in patients with severe AP.[1] Therefore, these patients should be recognized early, and their treatment should be initiated in a timely and more aggressive manner.

Many algorithms, such as the Ranson criteria, Atlanta scoring, acute physiology and chronic health evaluation (APACHE) scoring, and computed tomography severity index (CTSI), Balthazar Score have been developed for the classification of AP according to clinical severity. Balthazar evaluated the CECTs of AP patients in his study in 1985 and defined a relationship between the clinical severity of AP and the images in CECT. According to this study, images in CECT were divided into five classes from A to E according to the clinical severity of AP. Then, Balthazar included pancreatic necrosis on

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CECT images of AP patients in his initial scoring and defined CTSI.[2] CTSI is a scoring system based on the grading of peripancreatic inflammation, pancreatic necrosis, and phlegmon formation within a week after the onset of AP, and it helps determine the severity of the disease.[1,3] Procalcitonin and C-reactive protein (CRP) are other parameters used to determine the severity of AP in clinical follow-up[1]. CRP and procalcitonin are elevated in the presence of inflammation. However, since both biomarkers increase in many inflammatory conditions, neither is specific to AP.[4] In addition, the use of CRP in the emergency department is limited due to the late peak (48-72 hours) of this parameter. However, considering that organ failure, sepsis, and pancreatic necrosis determine the severity of AP, increased CRP and procalcitonin can be useful in the evaluation of clinical severity in patients with AP.[5-7]

One of the necessary criteria for the diagnosis of AP is a high amylase or lipase value. It has been shown that serum lipase is preferred over serum amylase due to the limited sensitivity, specificity, and positive and negative predictive values of the latter.[8] In addition, lipase rises within three to six hours after the onset of AP, has a half-life of 6-15 hours, and is reabsorbed by renal tubules; therefore, it tends to remain elevated longer than amylase in patients with AP.[9] Amylase and lipase levels can also provide an idea concerning the etiology of AP.[10]

Aim

In the literature, there are studies comparing amylase, lipase, CRP, and procalcitonin parameters separately with CTSI.[11-13] However, we found no study that evaluated all these four parameters together with CTSI. Therefore, in the current study, we aimed to evaluate the correlation of amylase, lipase, CRP, and procalcitonin parameters with CTSI in AP.

Material and methods

This study was conducted retrospectively in the emergency department of a tertiary hospital. Approximately two hundred twenty-three thousand five hundred patients apply to the emergency department of the tertiary hospital where the study was conducted per year. Patients who presented to the emergency department of our hospital from January 1, 2017, through September 1, 2022, and were diagnosed with AP (ICD diagnosis code: K85, K85.8, and K85.9) were included in the study. Data were obtained by screening the electronic patient files from the hospital information management system. Approval for the study was obtained from the local ethics committee (decision number: 22, session: 8, date: 27.10.2022). The study was performed in accordance with the tenets of the Declaration of Helsinki.

Study population

Using the hospital management system, a total of 1,029 patients were identified to have presented to the emergency department and received a diagnosis of AP during the study period. Patients aged over 18 years without any other inflammatory disease, whose data and patient files were available in the electronic system, were included in the study. Cases where >48 hours had passed from the onset of symptoms were excluded from the study. Patients with malignancies, pregnant women, and patients with immunodeficiency, known kidney or liver dysfunction, inflammatory bowel diseases, or other inflammatory conditions were also excluded from the sample. In addition, since the CTSI values were to be calculated within the scope of the study, patients that did not undergo contrast-enhanced computed tomography (CECT) at the time of presentation to the emergency department or within 48 hours of admission, as well as those with poor image quality that was not suitable for the calculation of CTSI, were also excluded. Of the patients planned to be included in the study, those with missing data and those that wanted to be discharged from the hospital during clinical follow-up were also excluded. After applying all the inclusion and exclusion criteria, 240 patients were included in the sample. The patients included in the study were divided into two groups according to their clinical outcomes as those that died and those that were discharged (Fig. 1).

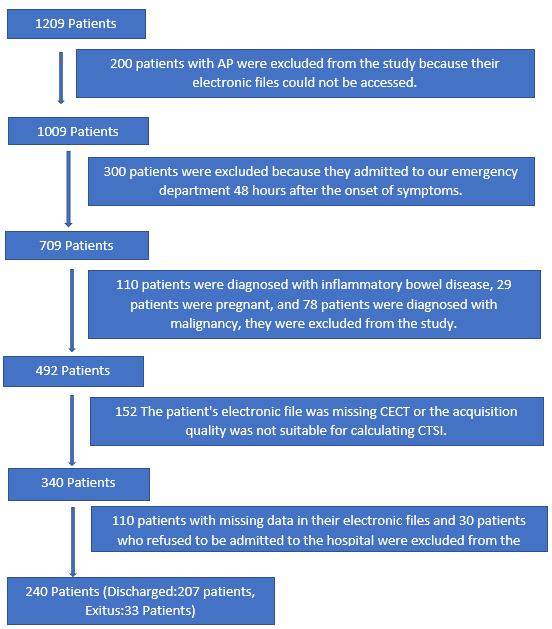

Fig. 1. Patient selection flow chart

Data collection

The patients' age, gender, laboratory results, clinical outcomes (mortality or discharge), and CECT images were obtained from the electronic files. Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), aspar-

tate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, direct bilirubin, glucose, and blood urea nitrogen (BUN), creatinine, amylase, lipase, procalcitonin, CRP, white blood cell (WBC), hemoglobin, and hematocrit values measured from blood samples taken at the time of presentation to the emergency department were recorded.

CECT images obtained at the time of presentation to the emergency department or within seven days of admission, were re-evaluated by two radiologists with five to 10 years of professional experience. The CTSI scores of the patients were calculated using the Balthazar score and pancreatic necrosis degree. The Balthazar score was evaluated from A, B, C, D, and E according to the severity of pancreatitis, and the corresponding score was calculated as 0, 1, 2, 3, and 4, respectively. The presence of pancreatic necrosis was calculated by assigning a score of 0 for no necrosis, 2 for <30% necrosis, 4 for <30-50% necrosis, and 6 for >50% necrosis. The overall CTSI score was grouped as mild (0-3), moderate (4-6), and severe (7-10)[2] (Table 1).

Table 1. Balthazar Sore and CTSI[1]

Grade	CT Finding			
A	Normal pancreas			
B	Pancreatic enlargement			
C	Pancreatic inflammation and/or peripancreatic fat			
D	Single peripancreatic fluid collection			
E	Two or more fluid collections and/or retroperitoneal air			
CTSI [1]	Necrosis			
CT Grade	Points	Percentage	Additional points	Severity index*
A	0	0	0	0
B	1	0	0	1
C	2	<30	2	4
D	3	30-50	4	7
E	4	>50	6	10
* CT grade points are added to points assigned for percentage of necrosis				

Statistical analysis

Statistical analyses were performed using IBM SPSS v. 23.0 software package (IBM, Armonk, NY, USA). Categorical data were presented as frequency and percentages, and numerical data as mean and standard deviation if normally distributed and median and interquartile range (IQR) values otherwise. The Kolmogorov-Smirnov test was used to check the normality of data distribution. For the comparison of two groups in terms of non-normally distributed data, the Mann-Whitney U test was used. The Spearman correlation test was conducted for the correlation analysis of the data that did not show a normal distribution. The receiver operating characteristic (ROC) analysis was performed to explore the relationship of the Balthazar score, pancreatic necrosis grade, and CTSI with patient outcome. Multivariate logistic regression analysis was performed to determine risk factors on death. Enter model was used in multivariate logistic regression analysis. Statistical significance was taken as $p<0.05$.

Results

The study included 240 patients, of whom 67.5% (n=162) were female, and the median age was 59 (47-74) years. When the distribution of the patients according to the groups was examined, it was determined that the median age of the mortality group (n=33) was 56 (44.5–72) years, and that of discharged group (n=207) was 60.0 (48–74) years, indicating no statistically significant difference (p=0.272). The GGT, AST, and ALT values were determined to be 83.5 (36.3–200.8), 56.0 (24.5–243.5), and 34.5 (17.3–125) respectively in the mortality group and 180.0 (42.5–388.5), 141.5 (50.3–295.5), and 122.5 (31.3–263), respectively in the discharged group. All three laboratory findings statistically significantly differed between the two groups ($p<0.05$). Table 2 presents the demographic characteristics and laboratory findings of the patients according to the groups.

Table 2. Demographic characteristics and laboratory findings of the patients according to the groups

Variable, median (IQ)	Mortality (n = 33)	Discharged (n = 207)	p
Age, years	56.0 (44.5–72)	60.0 (48–74)	0.272
Gander, n (%)			
Male	12 (5%)	66 (27.5%)	0.611
Female	21 (8.75%)	141 (58.75%)	
Alkaline phosphatase (U/L)	108.0 (68.5–172)	127.0 (91–208.3)	0.114
Gama glutamyl transferase (U/L)	83.5 (36.3–200.8)	180.0 (42.5–388.5)	0.015
Aspartate aminotransferase (U/L)	56.0 (24.5–243.5)	141.5 (50.3–295.5)	0.014
Alanine aminotransferase (U/L)	34.5 (17.3–125)	122.5 (31.3–263)	0.008
Total bilirubin (mg/dL)	0.88 (0.55–1.92)	1.07 (0.6–2.)	0.295
Direct bilirubin (mg/dL)	0.25 (0.12–0.82)	0.41 (0.18–1.09)	0.119
Glucose (mg/dL)	129.0 (107.5–151)	130.0 (108–161.5)	0.779
Blood urea nitrogen (mg/dL)	15.0 (10.3–18.7)	14.55 (11.68–18.57)	0.968
Creatinine (mg/dL)	0.69 (0.59–0.89)	0.75 (0.6–0.97)	0.297
Amylase (U/L)	1043 (465–1780)	1342 (689–2616)	0.118
Lipase (U/L)	2400 (1111.3–2985.9)	2657 (1500–4230)	0.084
C-reactive protein (mg/dl)	40.5 (10.2–48.2)	40.5 (19.8–56)	0.407
Procalcitonin (ng/mL)	0.19 (0.07–1.04)	0.14 (0.07–0.89)	0.877
White blood cell (10^3/µL)	12.3 (9.7–16.8)	10.9 (8.6–13.5)	0.071
Hematocrit (%)	42.2 (38.3–45.2)	42.8 (39.2–45.7)	0.312
Hemoglobin (g/dL)	14 (12.4–15.1)	14.2 (13.0–15.4)	0.442

The comparison of the Balthazar score, pancreatic necrosis grade, and CTSI between the groups is given in Table 3. Accordingly, these three parameters were found to be statistically significantly affect mortality ($p<0.001$).

Table 4 shows the correlation of the patients' amylase, lipase, CRP, and procalcitonin values with the Balthazar score, pancreatic necrosis grade, and CTSI. Accordingly, the amylase level was positively correlated with the Balthazar score at a statistically significant level (R=0.189, p=0.003). However, the remaining parameters did not have a statistically significant correlation with the Balthazar score, pancreatic necrosis grade, and CTSI ($p>0.05$).

The relationship of the Balthazar score, pancreatic necrosis grade, and CTSI with patient outcome is shown in Figure 2 and Table 5. The area under the ROC curve

value was determined as 0.861 [95% confidence interval (CI): 0.776–0.947] for the Balthazar score, 0.648 (95% CI: 0.531–0.764) for pancreatic necrosis degree, and 0.900 (95% CI: 0.826–0.973) for CTSI, and all these values were statistically significant (p<0.05).

Table 3. Comparison of the Balthazar score, pancreatic necrosis grade, and CTSI between the groups

Variable, n (%)	Mortality (n=33)	Discharged (n=207)	p
Balthazar score			<0.001
Normal pancreas	2 (6.1%)	58 (28%)	
Pancreatic enlargement	1 (3%)	17 (8.2%)	
Pancreatic inflammation and/or peripancreatic	3 (9.1%)	86 (41.5%)	
Single peripancreatic fluid collection	1 (3%)	34 (16.4%)	
Two or more fluid collections and/or retroperitoneal air	26 (78.8%)	12 (5.8%)	
Percentage of pancreatic necrosis			<0.001
0	23 (69.7%)	205 (99%)	
<30	4 (12.1%)	2 (1%)	
30-50	4 (12.1%)	–	
>50	2 (6.1%)	–	
Computed tomography severity index			<0.001
Low degree	5 (15.2%)	195 (94.2%)	
Middle degree	23 (69.7%)	12 (5.8%)	
High degree	5 (15.2%)	–	

Table 4. Correlation of amylase, lipase, C-reactive protein, and procalcitonin values with the Balthazar score, pancreatic necrosis grade, and CTSI*

Variables		Balthazar score	Percentage of pancreatic necrosis	CTSI
Amylase	R	0.189	0.022	0.071
	p	0.003	0.738	0.274
Lipase	R	0.135	0.019	0.042
	p	0.05	0.656	0.548
C-reactive protein	R	0.012	-0.051	-0.034
	p	0.850	0.432	0.595
Procalcitonin	R	-0.109	-0.037	-0.067
	p	0.09	0.576	0.317

* CTSI – computed tomography severity index

Table 5. Receiver operating characteristic curve for the predictors of cases with acute pancreatitis*

Parameter	Cut off	AUC	% 95 CI	p	Sensitivity (%)	Specificity (%)	PPD	NPD
Balthazar score	3.5	0.861	0.776–0.947	<0.001	0.818	0.778	68%	97%
Percentage of pancreatic necrosis	1	0.648	0.531–0.764	0.006	0.303	0.99	83%	90%
Computed tomography severity index	1.5	0.9	0.826–0.973	<0.001	0.848	0.942	70%	98%

* CI – confidence interval; AUC – area under curve; PPD – positive predictive value; NPD – negative predictive value

Multivariate logistic regression was performed using the Enter model. As a result of logistic regression analysis, it was determined that mortality and CTSI were statistically significant (p <0.001) (Table 6). A 1-degree increase in CTSI increases a person's risk of dying 270 times. No statistical significance was found in other variables (p>0.05).

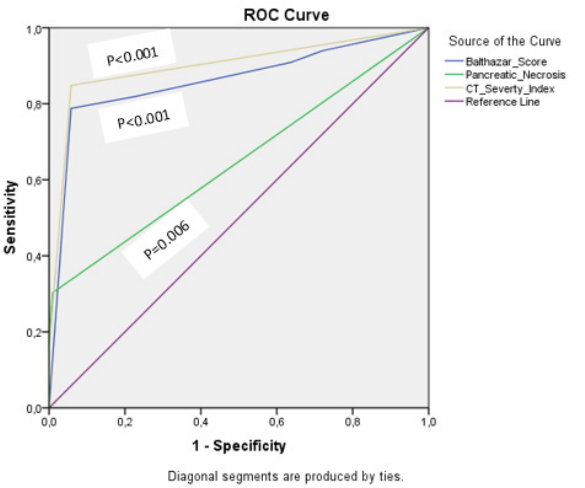

Fig. 2. Relationship between pancreatic scoring systems and mortality

Table 6. Multivariate logistic regression analysis results for acute pancreatitis

Variables	B	SE	OR	95% CI for OR	p
Age	0.028	0.021	1.029	0.988–1.072	0.174
Gender	0.437	0.315	1.548	0.834–2.872	0.315
Alanine aminotransferase	0.002	0.002	0.998	0.994–1.002	0.338
Balthazar score	-0.404	0.395	0.668	0.308–1.448	0.306
Percentage of pancreatic necrosis	0.237	0.432	1.267	0.543–2.954	0.584

Discussion

In this study, the correlation of the amylase, lipase, CRP, and procalcitonin values with the Balthazar score, pancreatic necrosis grade, and CTSI was investigated in patients with AP. Among these parameters, a weak positive correlation was found between the amylase value and the Balthazar score. In addition, the Balthazar score, pancreatic necrosis grade, and CTSI had a strong correlation with patient outcome. When the relationship between mortality and the Balthazar score, pancreatic necrosis grade, and CTSI was examined, it was determined that mortality had the strongest correlation with CTSI (area under the ROC curve: 0.9, p<0.01). This finding is consistent with previous studies in the literature.[1] In light of these results, we consider that the use of amylase and lipase values in the emergency department would not be appropriate to determine mortality, but CTSI can be used as a mortality indicator.

Amylase and lipase are the most commonly used biomarkers in the diagnosis of AP.[14] The amylase level may also be elevated in gastrointestinal pathologies other than AP and diseases of the salivary glands. Therefore, although amylase is also widely increased in AP, it is less sensitive and

specific than lipase.[15] Amylase and lipase values in patients with AP are important not only for diagnosis but also in elucidating the etiology of the disease. The lipase/amylase ratio has been proposed as a possible new index that can differentiate AP attacks with or without alcohol use.[16] In line with all these data, amylase and lipase values are measured in emergency departments to diagnose AP. Some studies have also evaluated the use of amylase and lipase levels in determining the clinical severity of AP, but these parameters were not found useful for this purpose.[17,18] In the literature, there are also studies in which CECT tests and scoring systems have been used in combination with amylase or lipase or both parameters to determine the severity of AP. In a study by Hamer et al., the lipase value was found to be positively correlated with modified CTSI.[19] In contrast, Thakur et al. observed no correlation between the serum lipase value and CTSI.[20] In the current study, there was no correlation between the lipase value and CTSI, Balthazar score, and pancreatic necrosis grade. In another study, the severity of AP was determined using CECT, and the correlation between the amylase level and AP severity was evaluated. No correlation was reported between the severity of AP determined by CECT and the amylase level.[13] Contrary to that study, we found a weak positive correlation between amylase and CTSI.

CRP and procalcitonin, which are other biomarkers associated with clinical severity and mortality in AP, are more valuable than amylase and lipase in evaluating the severity of the disease.[21] However, since these parameters are non-specific, they are not sufficient to determine clinical severity. In a previous study, a weak correlation was found between the CRP value and CTSI in patients diagnosed with AP. In the same study, a weak negative correlation was reported between CRP and the Balthazar score.[22] In our study, however, the CRP value had no correlation with the Balthazar score, pancreatic necrosis grade, and CTSI, which is also supported by the study of Ganesh et al.[23] In addition, we detected no correlation between the procalcitonin value and the Balthazar score, pancreatic necrosis grade, and CTSI.

Predicting the severity of AP and administering adequate treatment can reduce mortality rates. Therefore, APACHE II, the bedside index for severity in AP, and the Ranson score are widely used to estimate the severity of AP in clinical practice. However, these scoring systems are complex and difficult to apply in the emergency department. For this reason, CECT is used as a reference standard for both the diagnosis of AP and the assessment of its severity.[24] In CECT, the Balthazar score is defined primarily. Later, CTSI was defined by adding pancreatic necrosis to this score.[2,25] CTSI was found to be more effective than the Balthazar score in predicting mortality.[2] In the current study, consistent with the literature, the success of CTSI in predicting mortality was higher than that of pancreatic necrosis grade or the Balthazar score alone.

Study limitations

This study had a single-center and retrospective design, resulting in a small number of participants. In addition, the etiology of AP was not available in many cases included in the sample. Therefore, we were not able to classify the cases according to their etiology.

Another limitation is that the CECTs evaluated in our study were performed in the early period. In the emergency department where the study was conducted, CECT is performed in the evaluation of patients with a diagnosis of AP to rule out complications or intra-abdominal conditions that require urgent treatment.

Conclusion

In this study, no correlation was found between the procalcitonin and CRP values, which are the most common parameters in predicting the clinical severity of AP, and the amylase and lipase values, which are valuable in diagnosing AP. There was also no correlation between the radiological methods used to predict the clinical severity of AP (the Balthazar score, pancreatic necrosis grade, and CTSI) and the lipase, procalcitonin, and CRP values. However, a weak positive correlation was observed between the amylase value and CTSI. Therefore, we consider that the use of amylase or lipase level alone is not appropriate in predicting the clinical severity of AP. In addition, as a result of our study, it can be concluded that CTSI, which includes both the pancreatic necrosis level and the Balthazar score, is more successful than either parameter alone in the prediction of mortality in patients with AP who have undergone CECT.

Declarations

Funding

None declared by the authors.

Author contributions

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

Conflicts of interest

None declared by the authors.

Data availability

All data used in the study are available.

Ethics approval

Approval for the study was obtained from the local ethics committee (decision number: 22, session: 8, date: 27.10.2022).

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ORIGINAL PAPER

Antiviral drug resistance rates among patients with chronic hepatitis B infection

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ABSTRACT

Introduction and aim. Chronic hepatitis B infection (CHB) affects millions of people around the world. Many clinicians find it challenging to choose therapeutic agents due to the mutations that occur in the hepatitis B virus (HBV) that cause drug resistance. Thus, the aim of this study was to determine the HBV resistance rates against the currently recommended first-line therapies in the region of our country where HBV prevalence is high.

Material and methods. A total of 96 patients (56 men and 40 women) with HBV infection were enrolled in the study. The serum samples collected from those were analyzed with real-time polymerase chain reaction analysis followed by pyrosequencing (PyroStar HBV Drug Resistance Test, Altona Diagnostics, Germany) for drug resistance mutations associated with lamivudine, adefovir, telbivudine, entecavir, and tenofovir.

Results. HBV drug-resistance mutations were investigated in 80 treatment-naïve and 16 treatment-experienced patients (6 entecavir, 4 PEGylated-interferon, 4 tenofovir, 2 lamivudine). None of the HBV-DNA samples had mutations cause to drug resistance were detected in any codons regions that were analyzed.

Conclusion. Antiviral resistance poses serious obstacles for clinicians in the treatment of CHB. Determining whether antiviral resistance exists in HBV is critical to choose the appropriate treatment agent.

Keywords. antiviral agents, antiviral drug resistance, chronic hepatitis B, hepatitis B

Introduction

Hepatitis B virus (HBV) infection is still considered a significant global health problem. Even though the hepatitis vaccine has been used for approximately thirty years, the global prevalence of chronic HBV infection has declined slightly.[1,2] According to the World Health Organization (WHO), approximately 296 million people had chronic HBV infection (CHB) in 2019 and it is estimated that 63 million new cases and 17 million HBV-related deaths will occur between 2015 and 2030.[3] Acute HBV infection leads to CHB in 5%-10% of adults, which may lead to cirrhosis, hepatic decompensation, hepatocellular carcinoma, and thus death.[2,4] The main goal of the treatment in CHB is to reduce the development of those complications. Currently, international guidelines recommend PEGylated-interferon (PEG-IFN) and nucleos(t)ide analogs (NAs) as first-line therapies. NAs include lamivudine (LAM), adefovir (ADV), telbivudine (TBV), entecavir (ETV), and tenofovir (TDF).[5,6] Those treatment options aim to stop the progression of the disease by suppressing the replication of HBV.[5]

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However, CHB requires long-term treatment which leads to mutations thus emerging drug resistance.[2]

Reverse transcriptase (RT) is the major enzyme that is required for viral replication in HBV. However, RT promotes errors during replication generated at a rate of approximately 3×10^{-5} mutations per nucleotide per year which is 10-fold greater than that of other DNA viruses. These mutations change the conformational structure of RT and consequently lead to drug resistance in NAs.[7] Furthermore, those mutations may become severe obstacles for patients with CHB and mostly force clinicians to change the drug, prolonging the treatment duration and thus increasing the costs.

Our country is located in the middle endemic region for HBV with a prevalence of 2-8%, similar to Southern Europe and the Middle East countries.[8] The prevalence is also higher in the eastern region including the city where we conducted this current study.[8]

Aim

Thus, the aim of this study was to determine the resistance rates of the currently recommended first-line therapies against HBV.

Materials and methods

Ethical approval

All of the patients enrolled in this study provided written informed consent. The research was conducted ethically in accordance with the Declaration of Helsinki (2014). This study was approved by the local ethics committee (Van Yuzuncu Yil University Faculty of Medicine Invasive Clinical Trials Ethics Committee - Van. Date: 13.03.2014 decision number: 06).

Study design

A total of 96 patients (56 men and 40 women) were admitted to the gastroenterology clinic in a referral university hospital in Eastern Anatolia for one year period and were diagnosed with CHB (hepatitis B surface antigen (HBsAg) positive by blood test and HBV-DNA positive by polymerase chain reaction (PCR) for more than six months) were enrolled in the study.

Patients' HBV-DNA, HBsAg, hepatitis B "e" antigen (HBeAg), alanine transaminase (ALT), and aspartate transaminase (AST) results were recorded. Those with ALT or AST levels above 40IU/L in the serum samples of the patients were considered elevated. HBeAg and HBsAg levels were detected by the enzyme-linked immunosorbent assay (ELISA) technique (Cobas 401, Roche, Germany), and HBV-DNA was detected by a real-time quantitative PCR kit (QIAsymphony, Qiagen, Germany) with a lower detection limit of 100 IU/mL, ALT and AST levels were detected by Architect c8000 device (Abbott, ABD) in patients' plasma samples according to the manufacturer's instructions.

The Pyrosequencing method of DNA sequencing was used to analyze antiviral resistance in HBV-DNA-positive patients. EZ1 Virus Mini Kit v2 (Qiagen, Hilden, Germany) was used to isolate viral DNA from the serum samples. DNA was extracted according to the manufacturer's instructions using an EZ1 Advanced Instrument (Qiagen, Hilden, Germany). The final elution volume of 50 µL containing viral DNA from each sample was stored at -20°C for long-term usage.

Patients were categorized into three groups according to their HBV-DNA levels; 10^3 to 10^5 IU/ml in group 1, 10^5 to 10^6 IU/ml in group 2, and more than 10^6 IU/ml in group 3. Patients were categorized according to age groups as 16-24 years, 25-49 years and, 50 years and older.

The PyroStar HBV Drug Resistance Test consists of two steps: HBV real-time PCR analysis and pyrosequencing. Two HBV real-time PCR analyses were performed, the first (PCR-1) to detect mutations in codons 169, 173, 180, 181, 184, and 194, and the second (PCR-2) for mutations in codons 202, 204, 236, and 250 of the HBV polymerase gene. The PCR products were then sequenced with pyrosequencing primers to detect HBV drug resistance mutations. The forward primers used in both PCR reactions (PCR-1 and PCR-2) were biotinylated, resulting in a biotinylated amplification product that binds to streptavidin-coated Sepharose beads to isolate single-stranded DNA (ssDNA), and pyrosequencing was carried out with sequence primers. After amplification of ten codon regions containing potential mutations by real-time PCR, ssDNA templates of positive samples hybridized to six different sequence primers were incubated with the enzymes, substrate, and dNTPs. The enzyme mixture includes DNA polymerase, ATP-sulfuryl, luciferase, and apyrase, substrate mixture comprising adenosine 5'-phosphosulphate and luciferin, and each dNTP (dATPαS, dTTP, dCTP ve dGTP) were added to the wells of a Pyromark Q24 cartridge and placed in the Pyromark Q24 (Qiagen) workstation. The DNA sequences of the ten codon regions of each positive sample were analyzed by comparing them with the sequences of the wild-type and the mutant type-with HBV drug-resistance mutation. Mutations that cause antiviral drug resistance, mutant and wild type codons and which antiviral resistance develops as a result are given in Table 1.

The demographic data of the patients and the determined hepatitis B antiviral drug resistance mutations were recorded.

Statistical analysis was performed using Minitab ver.14 (Minitab Statistical Software LLC, Chicago IL USA). The events of interest were reported with mean and standard deviation. To compare proportions between groups, the Chi-square test was used. In all statistical tests, a significance level of 5% ($p<0.05$) was used.

Table 1. Mutations that cause antiviral drug resistance, mutant and wild type codons and which antiviral resistance develops as a result

Mutation	Wild Type Codon	Mutant Codon	Antiviral Resistance
I169T	ATA, ATT	ACA, ACT	Entecavir
V173L	GTG	CTG	Lamivudine
L180M	TTG, CTG	ATG	Lamivudine, Entecavir, Telbivudine
A181V	GCT	GTT	Lamivudine, Adefovir, Tenofovir
T184S	ACT	AGT, TCT	Lamivudine, Entecavir
A194T	GCT	ACT	Telbivudine
S202I	AGC, AGT	ATC, ATT	Entecavir
M204V/I	ATG	GTG, ATC/T/A	Lamivudine, Tenofovir, Telbivudine, Entecavir
N236T	AAC, AAT	ACC, ACT	Adefovir
M250V	ATG	GTG	Entecavir

Results

A total of 96 patients (56 men and 40 women) who were diagnosed with CHB infection were enrolled in the study. The patients' mean age was 37.3±16.7 years (minimum:16; maximum:90). The time elapsed after CHB diagnosis was from one month up to sixteen years (mean: 4 years). HBV drug-resistance mutations were investigated in 80 treatment-naïve and 16 treatment-experienced patients (6 ETV, 4 PEG-IFN, 4 TDF, 2 LAM) (Table 2). No significant difference was found between genders among HBV-DNA levels groups (p=0.175).

Table 2. Antivirals that treatment-experienced patients used and their treatment durations

Patient	Antiviral	Treatment duration
Patient 1	PEGylated-Interferon	2 months
Patient 2	PEGylated-Interferon	3 months
Patient 3	PEGylated-Interferon	5 months
Patient 4	PEGylated-Interferon	4 months
Patient 5	Lamivudine	1 year
Patient 6	Lamivudine	1 year
Patient 7	Entecavir	2 months
Patient 8	Entecavir	5 years
Patient 9	Entecavir	1 year
Patient 10	Entecavir	3 months
Patient 11	Entecavir	6 months
Patient 12	Entecavir	1 year
Patient 13	Tenofovir	3 months
Patient 14	Tenofovir	3 months
Patient 15	Tenofovir	2 years
Patient 16	Tenofovir	1 year

HBeAg was detected positive in 41 (42.8%) patients (19 men and 22 women). HBeAg positivity was found to be more frequent among women (22/40 (55%) vs. 19/56 (34%); p=0.040). Also, it was found that HBeAg positivity was significantly lower in patients older than 50 years compared to younger counterparts (7/24 (29.2%); 34/72 (47%)) and it was highest in patients younger than 25 (19/27; p<0.019). AST levels were high in 49 patients (33 men, 16 women) and ALT levels were high in 47 patients (31 men, 16 women) (Table 3). No significant differences were found between genders and AST or ALT levels.

Table 3. ALT and AST levels and their distribution by gender[a]

	AST levels			ALT levels		
Gender	High n (%)	Low n (%)	p	High n (%)	Low n (%)	p
Men	33 (67%)	23 (49%)	0.067	31 (66%)	25 (51%)	0.65
Women	16 (33%)	24 (51%)		16 (34%)	24 (49%)	
Total	49	47		47	49	

[a] ALT or AST levels above 40 IU/L in the serum samples of the patients were considered elevated; AST – aspartate transaminase; ALT – alanine transaminase

In terms of the relationship between HBV-DNA levels and HBeAg positivity; the difference between the 1st and the 3rd groups and the 2nd and the 3rd groups was statistically significant (p<0.001). And in terms of HBV-DNA levels and elevated levels of liver enzymes, between the 1st and 3rd groups for both ALT and AST, it was found to be a statistically significant difference (p=0.030 and p=0.048 respectively). Patients' HBV-DNA levels and their correlation between HBeAg, AST, and ALT levels are shown in Table 4.

Table 4. Patients HBV-DNA levels and the correlation between HBeAg, AST, and ALT levels [a]

	HBeAg			AST			ALT		
HBV-DNA (IU/ml)	Positive n (%)	Negative n (%)	p	High n (%)	Normal n (%)	p	High n (%)	Normal n (%)	p
10^3–10^5 (n=26)	4 (15.4%)	22 (84.6%)	<0.001	8 (30.8%)	18 (69.2%)	0.049	7 (26.9%)	19 (73.1%)	0.03
10^5–10^6 (n=13)	3 (23.1%)	10 (76.9%)		8 (61.5%)	5 (38.5%)		7 (53.8%)	6 (46.2%)	
>10^6 (n=57)	34 (59.7%)	23 (40.3%)		33 (57.9%)	24 (42.1%)		33 (57.9%)	24 (42.1%)	
Total (n=96)	41 (42.7%)	55 (57.3%)		49 (51%)	47 (49%)		47 (49%)	49 (51%)	

[a] HBeAg – hepatitis B "e" antigen; AST – aspartate transaminase; ALT – alanine transaminase

All the HBV-DNA samples were genotype D and no mutations caused to drug resistance were detected in any codons regions that were analyzed.

Discussion

The ideal target for HBV therapy, defined as a functional cure, is the sustained loss of detectable HBsAg and HBV DNA in serum after a finite course of treatment.[9] However, it may take years to reach that goal, such that only 3% to 11% of the patients treated with PEG-IFN and only 1% to 12% were treated with NAs for 5 to 7 years achieve it.[10] Besides, prolonged treatment time with those agents may increase the likelihood of side effects and thus decreases the treatment success. Nevertheless, the greatest danger of long-term treatment is that it increases the risk of developing resistance to these drugs.

Drug-resistant HBV variants have been compelling for clinicians through the years. As a result, although many antiviral agents have been used for CHB treatment, current international guidelines recommend only TDF and ETV, those associated with high barriers against HBV resistance.[5]

PEG-IFN has limited utilization in CHB treatment due to its side effects (i.e. bone marrow suppression and exacerbation of existing neuropsychiatric symptoms such as depression), and as well as administration way.[11] On the other hand, PEG-IFN has a finite treatment duration and no drug resistance was reported until this time.[10] Four of the patients in the current study received PEG-IFN and no PEG-IFN resistance was detected in any patients, consistent with previous data.

Although current treatment guidelines recommend either PEG-IFN or NAs, because of the narrow usage area of PEG-IFN, NAs are the preferred treatment option in most patients.[11] NAs do not affect covalently on closed circular DNA of HBV. Therefore, the treatment requires long-term administration which could cause drug-resistant mutations in the viral enzyme RT.[12] This is especially evident in LAM, which is safe, well-tolerated, and the first NA approved for the treatment of HBV infection. However, LAM resistance rates were found to be extremely high around the world. Since the LAM resistance rate could be 23% after one year of treatment and could reach up to 70–80% after 4–5 years.[13,14] In a multicenter study conducted on 1568 patients in 18 European countries, the LAM resistance rate was found to be 60.1%.[15] Another study conducted in China was performed on 1223 HBV-infected patients and the drug resistance rate was found to be 46.5% for LAM.[16] In our country, LAM resistance was also found to be high. In a recent study performed by Alacam et al., drug resistance mutations were found 46.86% in patients with HBV infection and the resistance rate for LAM was found 36.79%.[17] Due to those high resistance rates, LAM use has decreased over the years.[13,14] Nevertheless, none of our patients had mutations related to LAM resistance.

ADV is one of the other options that can be used for CHB infection. ADV resistance is lower than the LAM resistance. A previous study conducted by Hadziyannis et al. found ADV resistance rate of 5.9%, with more than 144 weeks of ADV monotherapy.[18] Another comprehensive study conducted in China found that 11.4% of the patients had ADV-resistant HBV variants.[16] ADV is both used against treatment-naïve and LAM-resistant HBV variants.[19,20] Unfortunately, previous studies have shown that LAM resistance could facilitate ADV resistance. A recent study conducted in LAM-resistant and treatment-naïve CHB patients found that after two years of ADV treatment ADV resistance emerged considerably higher compared to treatment-naïve CHB patients.[21] LAM, ADV, and TBV are considered agents with low genetic barriers due to the number of mutations required to confer resistance are low. A recent study conducted a 1-year trial of TBV found that 4.5% of patients who received TBV treatment for 48 weeks developed resistance.[22] Resistance rates against TBV are lower compared to LAM and ADV, however, after 2 years of treatment, these rates reach 17 percent.[6] Therefore, it is recommended that patients resistant to TBV should be immediately switched to TDF monotherapy.[6,23] Although many CHB patients are still being treated with other NAs, current guidelines recommend using NAs with a high barrier to resistance such as ETV or TDF because of their high efficacy in virological suppression and the lowest risk of the emergence of resistance.[5,6] Moreover, to date, there has been no confirmed resistance to TDF was detected, even after long treatment durations. Suzuki et al. followed up with 40 patients that received TDF monotherapy or combination for a median of 45 months and found no resistance against it.[12] Snow-Lampart et al. also reached the same conclusion in their study conducted with 641 patients after receiving 144 weeks of TDF monotherapy.[24] In contrast to TDF, development of resistance to ETV has been reported, but this resistance is significantly lower compared to other NAs resistances.[25] None of our patients had resistance to ETV. In support of this, a recent study found only 1.2% of the treatment-naïve patients enrolled to their study had ETV resistance.[26] However, similar to ADV, the rate of resistance to ETV is higher in LAM-resistant patients than in patients who have not used drugs before.[27,28] Zoulim et al. found that although ETV resistance rate is very low in treatment-naïve patients, that rate increases to 51% of the patients.[28]

This study has also several limitations to be considered. Although, it was conducted in the largest referral hospital in the region with the highest prevalence of HBV infection in our country, it was a single-center study, so our findings cannot be generalized. Another potential limitation relates to our investigation was our relative small sample size. Particularly in light of increasing resistance rates among HBV being a worldwide concern, we believe that this issue clearly requires further investigation, and new and comprehensive studies in this field are increasingly important.

Conclusion

In this study, we assessed the genetic variability of HBV in Eastern Anatolia and HBV genotype D was the determined genotype in all patients and this finding is in line with the general data of our country.[29] CHB treatment has been posing an obstacle for clinicians and patients due to increasing antiviral resistance rates. Our findings on drug resistance mutations showed that either treatment-naïve or treatment-experienced CHB patients have no HBV polymerase resistance mutation rate which means resistance while initiating treatment was

not seem to be a major problem in our region. However, further monitoring of the newly diagnosed HBV-infected patients should be continued, in order to evaluate the presence of transmitted drug resistance and its influence on the response to treatment.

Declarations

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Author contributions

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

Conflicts of interest

No conflict of interest was declared by the authors.

Ethics approval

This study was approved by the local ethics committee (Van Yuzuncu Yil University Faculty of Medicine Invasive Clinical Trials Ethics Committee – Van. Date: 13.03.2014 decision number: 06).

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ORIGINAL PAPER

Blood pressure profile and nutritional status of pupils benefitting from the National Home-Grown School Feeding Programme in southwest Nigeria

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ABSTRACT

Introduction and aim. Childhood hypertension is an important precursor to adult hypertension. This study was used to investigate blood pressure level and nutritional status of pupils in public primary schools that were benefitting from the National Home Grown School Feeding Programme in southwest Nigeria.

Material and methods. A cross-sectional study conducted among randomly selected 40 public primary schools where feeding programme was on-going in Oyo and Ogun States. Pretested semi-structured questionnaire was used to obtain information from the pupils. Anthropometric measurements and blood pressure readings were assessed using relevant tools.

Results. Some of the pupils (129; 41.6%) aged 10-15 years ($p<0.0001$) were stunted compared to those aged 5-9years (60; 11.3%). Undernutrition among pupils aged 10-15 years was 47.7%, which was significantly higher than ($p<0.0001$) among pupils aged 5-9 years, 18.5%. Overall, prevalence of hypertension among the pupils was 6.0%. No significant difference between male and female groups with regards to MUAC ($p=0.115$), blood pressure ($p=0.302$) and BMI-for-age ($p=0.100$). A significant association found between blood pressure and BMI-for-age ($p=0.004$).

Conclusion. Prevalence of blood pressure among the pupils assessed suggests more presence of high blood pressure in the population of primary school pupils. School feeding programme could be an avenue to improve nutritional indices among the pupils.

Keywords. anthropometric measurements, blood pressure, nutritional status, public primary school pupils, school feeding

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Introduction

Child malnutrition, especially undernutrition has profound health consequences in later life of the children including their future economic potentials.[1] School-age children are growing fast physiologically and depend mainly on the nutrients supply from the family foods. If the level of nutrients intake is below the requirements, the nutritional status will be negatively affected and this could be reflected in the level of stunting, wasting and under-weight.[2] Nutritional status of school-age children, especially stunting, which reliably indicates past nutritional insults due to poor dietary intake, infection and low socio-economic indices over long period of time is being used as a gauge to measure child's health and development potentials.[3] Nigeria has the second highest burden of stunted children in the world with a national prevalence rate of 32% of children under five.[4] Stunting (height-for-age z-score of <-2SD) is a major public health problem in low and middle-income countries because of its association with increased risk of mortality during childhood.[5,6] Apart from causing significant childhood mortality, stunting also leads to significant physical and functional deficits among survivors.[5-7] The prevalence of stunting among school children and adolescents 5-19 years in Ogun State was reported to be 17.4%.[8] The prevalence of underweight for school age children was 24.4%.[9] This indicates a problem of child undernutrition in southwest Nigeria.

The Federal Government of Nigeria with the assistance of the New Partnership for Africa's Development (NEPAD) launched the School Feeding Programme in 2016 named- The National Home-Grown School Feeding Programme (NHGSFP). This programme is a federal government-led ₦70 (0.167US dollars) per day school feeding programme that aims to improve primary school attendance and possibly, the nutritional status of public primary school pupils.[10]

The design was to provide one meal per school day to primary school pupils in Nigeria, so as to increase enrolment, improved health and nutrition of pupils in schools as an added advantage.[11] Healthy school meal contributes to the nutritional status, cognitive development and the general well-being of school children.[12] Adverse health consequences during childhood have been reported to be significantly associated with childhood malnutrition which is responsible for half of the in-hospital mortalities and morbidity.[13] Double burden of malnutrition, which is the co-existence of undernutrition and over-nutrition among young children, overweight and obesity among older children, adolescents, and adults have been reported.[14-16] The International Obesity Task Force estimated the global prevalence of overweight in children and adolescents to be 10%.[17] In Nigeria, the prevalence of obesity and overweight among school-age children was reported as 8.3% and 10.3% for male and 10.3% and 16.8% for female, respectively.[18] More than 60% of overweight children generally have at least one additional risk factor for cardiovascular disease, such as raised blood pressure, hyperlipidaemia or hyperinsulinaemia.[19] Higher blood pressure in childhood together with other risk factors causes target organs and anatomical changes that are associated with cardiovascular risk.[19]

Risk of chronic diseases such as diabetes, hypertension, renal disease and cardiovascular disease that may appear later in life are increased by early malnutrition, which will in turn lead to high adult health care costs.[20] Childhood malnutrition leads to poor school readiness and performance, resulting in fewer years of schooling and reduced productivity. It also diminishes adult intellectual ability and work capacity, causing economic hardship for individuals and their families.[20] Childhood hypertension is an important precursor to adult hypertension, it begins early in childhood and progresses into adulthood.[21,22] Children with high blood pressure tends to maintain such level of blood pressure even as they grow into adulthood. In Nigeria, prevalence of hypertension among children aged 5-18 years ranged from 1.6-17.5% between year 2000 and 2016.[22] To reduce the burden of hypertension in adulthood, timely intervention against childhood hypertension is urgently required.[23] Children with good nutritional and health status tend to learn better and become healthier and more productive adults in the future.[24]

Aim

Few studies exist, focusing on blood pressure of pupils under the national home-grown school feeding programme in south west Nigeria. Hence, the purpose of this study was to investigate the blood pressure level and nutritional status of pupils in public primary schools benefitting from the NHGSF programme in southwest Nigeria.

Material and methods

Ethical approval

Ethical approval for the study was obtained from the Health Research Ethics Review Committee of the Oyo State Ministries of Health (Ref. No. AD 13/479/1645[B]) and Ogun State (Ref No. HPRS/381/332) respectively. Permission was obtained from the Ministry of Special Duties and Ministry of Education in Ogun and Oyo States, respectively and was complemented by consent from parents and guardians of the pupils.

Study design and setting

This was a cross-sectional design study that analyzed data of variables collected using a quantitative instrument at one given point in time across samples of pupils from randomly selected public primary schools in Oyo and Ogun States.

This study was carried out in public primary schools where NHGSF programme is currently on-going in two States in southwestern Nigeria (Oyo and Ogun States). Currently, only pupils in primaries one to three were being fed school meals under the NHGSF programme.

Study population

Pupils attending public primary schools where NHGSF programme was currently on-going in Oyo and Ogun States were studied. These included children with no obvious health challenges in primary 3 classes, the highest class included in feeding programme and judged to be able to respond to the interview. However, where the sample size could not be achieved among primary 3 pupils in a school, pupils who could respond to the questionnaire were selected from primary 2 classes. Pupils who did not participate in the school meals programme (by parental preference) were excluded from the study. Rural and urban primary schools representing each of the three senatorial districts in the two States were included. Pupils without obvious health challenges were recruited.

Sample size and sampling procedure

The sample size required for this study was the higher of the two calculated samples using reported prevalence of hypertension among children aged 5-18 years in Nigeria (17.5%) and stunting among primary school pupils in Ogun State (17.4%), at 5% tolerable error, 95% confidence and adjusting for 10% non-response.[5,19] A minimum sample size of 407 and 410 was calculated for Oyo and Ogun State, respectively.

A multistage sampling technique was used to select 20 schools from each of the States.

In Oyo State, the three Senatorial Districts (SD) were stratified into two based on the size and cosmopolitan indices. The largest SD was classified into one stratum while the remaining two constituted another stratum. The LGAs in each of the stratum were grouped into urban and rural LGAs, using the State parameters to designate a local government area as rural or urban. Two LGAs were randomly selected from the largest SD where one was an urban settlement and the other a rural settlement. One LGA each was randomly selected from each of the two remaining SD; and each of the LGAs consisted of both the urban and rural settlements, making a total of four LGAs selected in Oyo State. This made up the two smaller SDs forming another sub-total of 12 public primary schools. The overall number of selected public primary school was 20.

In Ogun State, 2 LGAs (one rural, one urban) were selected from each of the three senatorial districts in the State to make a total of 6 LGAs. The six LGAs were stratified into 2 based on the size and population. One stratum had four LGAs while the other stratum had 2 LGAs. Three public primary schools were randomly selected through balloting from each of the 4 LGAs in one stratum (making a sub-total of 12 public primary schools) while 4 public primary schools were randomly selected from each of the remaining 2 LGAs in the other stratum (making 8 public primary schools). This also summed up to 20 public schools in Ogun State.

The sample size was allocated equally to the schools and 20 pupils were selected randomly among the primary 3 pupils of each school through balloting in the two States. For schools that had more than one arm for the primary 3 pupils, the research assistants divided the sample size for each school among the arms and randomly selected equal number of pupils across the arms and for schools where the sample size could not be achieved among the primary 3 pupils, primary 2 pupils who could respond to the questionnaire were randomly selected by balloting.

Data collection

Ten interviewers who were graduates of tertiary institutions with experience in conducting community-based surveys in each of the two States were trained to take anthropometric measurements - weight, height, mid upper-arm circumference, and blood pressure as well as collect information on the socio-demographic characteristics of the pupils. All interviewers were fluent in English and Yoruba. Nutritional status is assessed using anthropometric, biochemical tests, clinical and dietary methods, among others. Anthropometric measurements used to determine nutritional status include the assessment of the body physiological parameters based on the height and weight. Body Mass Index (BMI) is the most popular and common method for nutritional status assessment.[25] It is defined as the ratio of weight (kg) to squared height (m^2). BMI is dependent on age and gender for children and referred to as BMI-for-age. Mid upper arm circumference (MUAC) is also another method for measuring nutritional status of children and adults. It measures in millimeters/centimeters on the arm using MUAC tape or Shakir stripe. It is cheap and very easy to compute with different colours to suggest the nutritional status of the child e.g. green colour indicates good nutritional status, yellow is a warning sign of poor nutritional status and red confirms undernutrition.[26] MUAC has been reported as the best case-detection method for severe malnutrition.[27] Weights were measured to the nearest 0.1 kg with newly purchased portable bathroom scales (Harson Emperor™) which were validated with a known weight object. They were also regularly checked and adjusted to zero point after every 10th measurement. The pupils were weighed wearing light clothing and without shoes. Heights were measured with a mobile stadiometer placed on a level ground, with pupils standing erect without shoes and

with the eyes looking horizontally and the feet together on a horizontal level. Heights were recorded to the nearest 0.1 cm. Standardization checks on the height boards were done periodically during the study period. The height and weight were measured according to the international procedure.[28] Systolic and diastolic blood pressure were measured using an automated digital blood pressure monitor (OMRON M3) after each child had rested for at least 5 minutes, well seated with the back rested on the chair and feet on the ground. Blood pressure was measured twice, with an interval of two minutes, in the left arm and with an appropriate size cuff for the child's arm. Talking was not allowed during the processes of taking measurements.

Statistical analysis

Data were analyzed using the IBM SPSS 25.0 (Armonk, NY, USA) software statistical package for descriptive statistics, such as means and standard deviation to summarise continuous variables while categorical variables were presented using frequency and proportions. Proportional differences between the rural and urban location were explored and tested for significance using Chi-square test with the p-value set at ≤ 0.05 considered statistically significant.

The body mass index for age (BMI-for-age) was classified based on the World Health Organization reference tables (BMI percentile-for-age from five to 19 years) as normal weight, overweight and obese.

Blood pressure (BP) was classified using the standard BP charts developed by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents, United States of America[29] as normal, prehypertension and hypertension.

Mid upper arm circumference (MUAC) was classified as ≥13.50cm as normal, 12.50-13.49cm as mild malnutrition and ≤12.49cm as moderate to severe malnutrition.

The z-scores < - 2.0 was used to classified stunted and undernourished children based on their height-for-age and weight-for-age, respectively according to WHO child growth standards.[29]

Results

A total of 847 pupils were studied in the two States (381 in Oyo State; 466 in Ogun State). Out of the 847 pupils, 426 (50.3%) were males and the others females, 365 (43.1%) lived in the rural area and 708 (84.0%) were in primary 3. The mean age of the pupils was 9.0±1.5 years. The mean age of the male pupils was 9.1±1.4 years while female was 8.9±1.5 years. Table 3 shows that the majority of the pupils (331; 68.7%) aged 5-9 years were living in the urban area than in rural area 203 (55.6%), which was statistically significant (p<0.000). Five hundred and thirty (63.0%) lived with both parents (Table 1).

Table 1. Frequency distribution of socio-demographic characteristics of respondents by location of school

Variable	Rural		Urban		Total		p
	n	%	n	%	n	%	
Age (in years)							<0.001*
5-9	203	55.6	331	68.7	534	63.2	
10-15	161	44.2	149	30.9	310	36.7	
Sex							0.525
Male	179	49	247	51.2	426	50.3	
Female	186	51	235	48.8	421	49.7	
Class							<0.001*
Pry 2	20	5.5	119	24.7	139	16.4	
Pry 3	345	94.5	363	75.3	708	83.6	
Who respondents lived with							0.039*
Both Parents	244	66.8	286	59.3	530	62.6	
Mother	47	12.9	88	18.3	135	15.9	
Grandparent	45	12.3	58	12.0	103	12.2	
Father	13	3.6	21	4.4	34	4	
Auntie	8	2.2	15	3.1	23	2.7	
Uncle	1	0.3	8	1.7	9	1.1	
Siblings	1	0.3	4	0.8	5	0.6	

* Chi-square test with the level of significance set at p≤0.05

Table 2. Distribution of anthropometric parameters and blood pressure by location of school

Variables	Rural		Urban		Total		p
	n	%	N	%	n	%	
MUAC							0.324
Normal	360	98.6	468	97.1	828	97.8	
Mild malnutrition	3	0.8	9	1.9	12	1.4	
Severe malnutrition	2	0.5	5	1	7	0.8	
Height for age classifications							0.306
Normal	276	75.8	379	78.8	655	77.5	
Stunted	88	24.2	102	21.2	190	22.5	
Weight for age classifications							0.058
Normal	246	67.4	353	73.4	599	70.8	
Undernourished	119	32.6	128	26.6	247	29.2	
BMI-for-age Classifications							0.925
Normal	355	97.3	469	97.5	824	97.4	
Overweight	6	1.6	8	1.7	14	1.7	
Obese	4	1.1	4	0.8	8	0.9	
Blood pressure classifications							0.244
Normal	339	92.9	456	94.8	795	94	
Hypertension	26	7.1	25	5.2	51	6	

* Chi-square test with the level of significance set at p≤0.05

The overall prevalence of hypertension was 6.0%. In the rural area, the prevalence of high BP was (7.1%) but this was not significantly higher than that of the urban area (5.2%), (p=0.398). The majority (97.4%) of the pupils studied were in the normal BMI-for-age category. The prevalence of overweight and obesity group in the study population were 1.7% and 0.9%, respectively. There was no significant difference between the rural and urban groups in relation to BMI-for-age (p=0.925) and MUAC (p=0.324). The prevalence of stunting

among the pupils in the rural area was 88 (24.2%) and this was not statistically different from those in the urban area 102 (21.2%). (p=0.306). The overall prevalence of undernutrition among the school pupils was 29.2%. Twelve (1.4%) and 7(0.8%) of the pupils had mild and severe malnutrition, respectively as shown in Table 2.

Table 3 shows that there was no significant difference between the male and female group as regards MUAC (p=0.115), blood pressure (p=0.302), BMI-for-age (p=0.1) and height-for-age (p=0.129). However, more females were wasted 148 (35.2%) than their male counterpart 99 (23.2%), which was statistically significant (p<0.0001).

Table 3. Distribution of anthropometric and blood pressure measurements by sex

Variables	Male		Female		Total		p
	n	%	N	%	n	%	
MUAC							0.115
Normal	418	98.1	410	97.4	828	97.8	
Mild malnutrition	3	0.7	9	2.1	12	1.4	
Severe malnutrition	5	1.2	2	0.5	7	0.8	
Blood pressure classifications							0.844
Normal	401	94.1	394	93.8	795	94	
Hypertension	25	5.9	26	6.2	51	6	
BMI-for-age Classifications							0.1
Normal	416	97.7	408	97.1	824	97.4	
Overweight	4	0.9	10	2.4	14	1.7	
Obese	6	1.6	2	0.5	8	0.9	
Height for age classifications							0.129
Normal	321	75.4	334	79.7	655	77.5	
Stunted	105	24.6	85	20.3	190	22.5	
Weight for age classifications							<0.001*
Normal	327	76.8	272	64.8	599	70.8	
Undernourished	99	23.2	148	35.2	247	29.2	

* Chi-square test with the level of significance set at p≤0.05

Almost all the pupils in the age category 10-15 years had normal BMI 309 (99.7%), which was significantly different (p=0.008) from those in the 5-9years age category 514 (96.3%). More pupils (129; 41.6%) aged 10-15 years (p<0.0001) were stunted compared to those aged 5-9years 60 (11.3%). The prevalence of wasting among pupils aged 10-15 years was 47.7%, which was significantly higher than (p<0.0001) among pupils aged 5-9 years 18.5%.

Fourteen (10.1%) and 37 (5.2%) pupils in primary 2 and primary 3, respectively had high blood pressure (p=0.028). There was a significant association between blood pressure and BMI-for-age (p=0.004). Forty-six (5.6%) of the pupils who were of normal weight had hypertension; 3 (21.4%) of overweight and 2 (25.0%) of the obese also had high blood pressure. The results of MUAC measurement also showed a significant difference in blood pressure assessments (p=0.012). However, age, sex, location, height-for-age and weight-for-age did not show any significant association with blood pressure as shown in Table 4

Table 4. Relationship between the pupils' characteristics and blood pressure status

Variable	Normal		Hypertension		P
	n	%	N	%	
Age (in Years)					0.474
5-9	500	93.6	34	6.4	
10-15	294	94.8	16	5.2	
Sex					0.844
Male	401	94.1	25	5.9	
Female	394	93.8	26	6.2	
Class					0.028*
Pry 2	125	89.9	14	10.1	
Pry 3	670	94.8	37	5.2	
Location					0.244
Rural	339	92.9	26	7.1	
Urban	456	94.8	25	5.2	
BMI-for-age Classification					0.004*
Normal	778	94.4	46	5.6	
Overweight	11	78.6	3	21.4	
Obese	6	75.0	2	25	
Height for age classifications					0.059
Normal	610	93.1	45	6.9	
Stunted	184	96.8	6	3.2	
Weight for age classifications					0.724
Normal	564	94.2	35	5.8	
Undernourished	231	93.5	16	6.5	
MUAC					0.012*
Normal	780	94.3	47	5.7	
Mild malnutrition	10	83.3	2	16.7	
Several malnutrition	5	71.4	2	28.6	

* Chi-square test with the level of significance set at p≤0.05

Discussion

The prevalence of overweight and obesity reported in this study was lower than that reported in some similar studies among children in Nigeria. Adegoke et al. reported prevalence of 2.8% overweight and 0.3% obesity among school children in Ile-Ife.[30] Adebimpe reported prevalence of overweight and obesity among children aged 6-10 years to be 2.1% and 1.7%, respectively and Ajayi et al., in a population based study reported 4.7% and 15.0%, respectively among children less than 10 years residing in an urban city in Ibadan.[31,32] The differences in prevalence may be due to location of the study as affluence of the city may be responsible for the higher figure. In this study, BMI-for-age in boys was not significantly different from girls, which was contrary to a study that reported a higher prevalence of overweight in girls than in boys while the prevalence of obesity was higher in boys than in girls.[33]

Stunting and undernourishment reported in this study were higher than that reported in other similar studies in Nigeria. Akor et al. reported prevalence of stunting and wasting among primary school pupils in Jos, Nigeria as 11.1% and 2.4%, respectively.[34] Another study carried out to assess the physical growth and nutritional status among pupils in Enugu, Nigeria also reported the

prevalence of 0.4% stunting and 9.3% wasting. The lower prevalence reported in these other studies could be due to the difference in location in that the studies were carried out in another region in the Country. In Abeokuta, Ogun State, one of the States where this study was carried out, Senbanjo et al. reported stunting prevalence of 17%, which is also high though not as high as in this study.[8] However, a higher prevalence of stunting and wasting was reported in a study carried out in Makurdi, Nigeria as 52.7% and 43.4%, respectively.[35] It was reported that children attending primary school in that region were from relatively low socio-economic backgrounds, which could affect the quality of food they eat.[35] This study found that more female pupils were wasted compared to the male pupils, which was contrary to some studies where more male were wasted than female.[36,37] However, no significant difference in stunting was found by gender and this was contrary to finding in the study carried in Abeokuta, where the prevalence of stunting was higher among young female children aged 5-9 years while the reverse was the case among children aged 15-19 years.[8] This was adduced to possible increased access to food at the older age when the females are culturally involved in the cooking of family-food, and hence, their better nutritional state compared to the male counterparts. Explanation proffered for more boys aged 15-19 years stunted than girls was that poor, stunted girls dropped out of school leaving behind better-nourished girls.[8] The higher prevalence of stunting among younger female children and high prevalence of wasting among females could be due to the effect of extension of cultural preference for boys at birth.[38,39] In another study among primary school pupils in Ethiopia, older age and male sex were significantly associated with stunting.[8] This could be due to older children being in the transition life stage to adolescence when several unique challenges, including an increased body requirement for nutritional need are observed.[40] Another plausible reason for males being more at risk might be that males' growth and development is more influenced by environmental and nutritional stress than females and thus, making males more likely to be affected by stunting.[41]

The high prevalence of malnutrition generally reported in Sub-Saharan Africa has also been ascribed to poverty, poor environmental conditions, and overpopulation which might predispose children to inadequate food intake or intake of foods of poor nutritional quantity and quality.[42] Only a few of the pupils were severely malnourished according to their MUAC, which was slightly lower than that reported in a study carried out in Jos, Nigeria among primary school children partaking in the school feeding programme.[43] This could be due to differences in study locations, feeding and child care practices and probably differences in the socio-economic parameters. Seasonal variations in food availability and utilization could also be another possible reason for the differences observed.

The overall prevalence of high BP found in this study was higher than some other studies carried out with populations of the same age group in Nigeria. A study carried out among primary school pupils in Port Harcourt Nigeria reported the prevalence of hypertension as 4.7%.[44] Similarly, Sadoh et al. reported prevalence of hypertension as 2.6% among pupils aged 5-15 years in midwestern Nigeria.[45] A report from a study among children and adolescents in Uyo metropolis stated the prevalence of hypertension as 2.5%.[46] The lower prevalence reported in these studies could be due to difference in location as the reported studies were carried out in South-South region of Nigeria where the prevalence of overweight and obesity has been reported to be higher than in this study. This could also be a reason why the prevalence of hypertension recorded in this study is higher. However, a population based study carried out in urban part of Ibadan, Southwest Nigeria reported the prevalence of hypertension to be 12.8% among children less than 18 years, 16.9% among those ≤ 10 years and 8.0% among 11-17 years, which were higher than that reported in this study.[47] The high prevalence reported in this study calls for intervention because hypertension in children is an indicator to morbidity and mortality in adulthood, which can place enormous burden on the healthcare system. Change in lifestyle has been reported in studies as the cause of the increasing prevalence of hypertension in sub-Saharan Africa. This could also be responsible for the high blood pressure reported in the urban area; although this study did not show a significant association between location of schools and blood pressure, which was contrary to findings from a study carried out among school children in India where hypertension was reported to be higher in children from urban area than those in the rural area.[20]

Findings from this study, which showed increase trend of hypertension with increasing BMI-for-age was in agreement with report from Ajayi et al., Okoh et al. and Thangjam et al.[20,44] This reaffirms the positive correlation of overweight and obesity with hypertension. The effect of BMI on high blood pressure has been demonstrated in other studies.[44,46] Ledwaba et al. reported that MUAC was significantly associated with blood pressure in a study among children aged 6-13 years, which was in agreement with findings in this study.[48] This implies that blood pressure of the pupils can be controlled if much attention is paid to their nutritional status. Gender was not significantly associated with blood pressure as shown by the Mokola initiative study (Ajayi et al.), but which is contrary to a study which reported that girls were more hypertensive than boys because of the puberty-induced growth spurt and psychosocial stress in females.[20]

Study strength and limitations

One main strength of this study is that it was conducted among children in an age group that has the potential for catch-up growth and therefore could benefit from targeted interventions.[49] One key limitation, was that we could not establish the cause-and-effect relationships; because of the cross-sectional nature of the study design. The findings from this study may not be generalizable to other States implementing the NHGSF programme but it has highlighted key nutritional and blood pressure issues to be attended to in improving the health of the pupils and forestall development of diseases such as high blood pressure in the future. A larger multistate study is recommended.

Conclusion

High blood pressure was observed among pupils benefitting from the National Home-grown School Feeding programme. Nutritional status of the pupils was found to be related to high blood pressure. This suggests the need to monitor the nutritional status of the pupils and educate them on the health implications of their nutritional status. Intervention should be focused on the nutritional status of the pupils as it plays a major role in determining their blood pressure. The high prevalence of stunting underscores the fact that school feeding should be a primary target of programmes aiming at preventing stunting. The continuation of the school feeding programme should be encouraged as the programme can be used as a platform to promote good nutritional status and reduce morbidity and mortality in older age.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declared no potential conflicts of interest.

Data availability

Data are available upon request through the corresponding author.

Ethics approval

Participation in the study was voluntary, assent was obtained from the pupils and consent from their parents or guardians. The study posed no risk to the pupils. Ethical approval for the study was obtained from the Health Research Ethics Review Committee of the Oyo State Ministries of Health (Ref. No. AD 13/479/1645[B]) and Ogun State (Ref No. HPRS/381/332), respectively.

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ORIGINAL PAPER

p-Coumaric acid as a potent additive in blood storage solution

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ABSTRACT

Introduction and aim. Stored erythrocytes develop lesions involving changes in their structure and function reducing their efficacy. Oxidative Stress (OS) being one of the main causes of storage lesion, can be attenuated by antioxidants as additives in the storage solution. This study aims to evaluate the effect of *p*-Coumaric acid (CA) on erythrocytes during whole blood storage.

Material and methods. Blood collected from Male Wistar rats was stored at 4°C in CPDA-1 solution for 21 days. Blood samples were stored with and without 1mM CA (CA 1) and 10 mM CA (CA 10). The erythrocytes were isolated every week during storage and the biomarkers for OS and antioxidant status were analysed.

Results. Superoxide dismutase and catalase elevated on day 14. Conjugate dienes decreased in CA 10 on day 14. Thiobarbituric acid reactive substances increased on day 7 and decreased on day 14 in CA groups. Protein sulfhydryls decreased in controls and CA 1 on day 14 whereas, it was maintained in CA 10.

Conclusion. Coumaric acid upregulated the antioxidant enzymes and protected the cells from oxidative damage. Thus, coumaric acid can be employed as a potent additive during storage and opens new avenues of employing it in similar OS situations in erythrocytes.

Keywords. antioxidants, blood storage, oxidative stress, *p*-coumaric acid

Introduction

Erythrocytes or red blood cells (RBCs) undergo several changes during storage, collectively referred to as the storage lesion, which reduce their effectiveness following transfusion.[1] The rheological properties undergo changes due to the RBC membrane loss with storage. Hemolysis and formation of microparticles occur due to loss of RBC integrity, which further contribute to the complications associated with transfusion.[2]

Erythrocytes undergo changes like morphologic and metabolic alterations during storage which include loss of biconcave disc shape, depletion of potassium, 2, 3-diphosphoglycerate, adenosine triphosphate, and lipids, increased erythrocyte rigidity and impaired oxygen delivery.[3] Erythrocytes are subjected to oxidative stress due to exposure to oxygen during storage[4] resulting in diminished antioxidants and increase in reactive species. Erythrocytes are well-equipped with endogenous antioxidant systems (superoxide dismutase, catalase, glutathione peroxidase, glutathione, ascorbic acid).[5] Changes in ion permeability on the erythrocyte membrane, increase in lipid peroxidation, oxidation of protein sulfhydryl groups, inactivation of membrane-bound receptors and enzymes, degradation of proteins, and activation of proteolysis have been reported following the challenge of erythrocytes with different oxygen radical-generating

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systems.[6,7] Erythrocytes are prone to storage lesions due to oxidative stress (OS). A mixed population of young and old erythrocytes are present in circulation. OS influences the aging of erythrocytes during the storage, thereby diminishing the quality of stored blood.[8] Young erythrocytes can endure OS efficiently than the old erythrocytes.[9] Antioxidant intervention has proven to reduce the effect of aging on erythrocytes.[10]

Antioxidants as additives such as Trolox, curcumin, carnosine, spermine, phloretin and ascorbic acid have proven to attenuate the oxidative insult during erythrocyte storage.[11-14]

Studies have reported the preventive effects of *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid; CA), a phenolic acid which is found widely distributed in plants and as a part of human diet.[15-18] Coumaric acid has antiproliferative, antiapoptotic, antimicrobial, anti-inflammatory effects and free radical scavenging capacity.[17-22] The effects of CA on erythrocytes during storage have not been explored.

Aim

Hence, this study was carried out to study the effect of CA as an additive during storage.

Material and methods

Animals

Male Wistar rats were obtained from Shri Raghavendra Enterprises. Animal care and maintenance were in accordance with the ethical committee regulations (841/b/04/CPCSEA).

Chemicals

Hemoglobin reagent was obtained from Coral Clinical Systems, Goa, India. Acrylamide, bis thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), and bovine serum albumin (BSA) stock were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were of reagent grade and organic solvents of spectral grade obtained from HiMedia, Mumbai, India.

Blood sampling

Animals were lightly anaesthetized and restrained in dorsal recumbency as described earlier.[23] In brief, the syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. Blood was carefully aspirated from the heart into plastic collecting tubes with citrate-phosphate-dextrose-adenine-1 (CPDA-1) solution.

Experimental design

Blood was drawn from male adult Wistar rats (4 months old) and stored in CPDA-1 solution. Blood was collected from 6 animals and stored at 4°C for a period of 21 days. Sample from each animal was divided into the following three groups 1) control group; 2) CA 1-samples with CA as additive at a concentration of 1 mM; 3) CA 10-samples with CA as additive at a concentration of 10 mM. RBCs were isolated from stored blood at regular intervals (every seventh day) and the biomarkers of oxidative stress (OS) were studied.

RBCs separation

RBCs were isolated by centrifugation for 20 min at 1000 *g* at 4°C. Plasma and buffy coat were removed using a micropipette. The cell pellet was washed three times with isotonic phosphate buffer, pH 7.4, centrifuged at 1000 *g* for 10 min, and finally suspended in an equal volume of isotonic phosphate buffer to a final hematocrit of 50%.[24] This constituted of the erythrocyte suspension.

Hemoglobin (Hb)

Hb was measured using Hemocor-D Kit (Coral Clinical Systems), which utilizes the cyanmethemoglobin method.[25] Erythrocytes were incubated with Hb reagent for 3 minutes at room temperature and absorbance was measured at 540 nm (PC-based Double Beam Spectrophotometer 2202, Systronics, Gujarat, India). Hb concentration was represented in terms of g dL^{-1}.

Superoxide dismutase (SOD)

Carbonate buffer was added to the samples (0.05 M, pH 10.2, 0.1 mM ethylenediaminetetraacetic acid (EDTA)) followed by which Epinephrine (30 mM in 0.05 % acetic acid) was added to the mixture. The absorbance was measured at 480 nm for 4 min.[26] SOD activity was expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50% which is equal to 1 unit.

Catalase (CAT)

Absolute ethanol was added to the samples and incubated in ice bath for 30 min. After incubation, phosphate buffer was added to the above mixture. 6 mM hydrogen peroxide (H_2O_2) was added just before reading the absorbance at 240 nm.[27] The molar extinction coefficient of 43.6 Mcm^{-1} was used to determine the catalase activity.

Conjugate dienes

Samples were transferred to ether/ethanol, 1:3 (v/v) mixture and vortexed. The mixture was centrifuged and the level of conjugate dienes was measured spectrophotometrically at 235 nm.[28] Conjugate dienes produced was calculated using the molar extinction coefficient of 29,500 M^{-1} cm^{-1}.

Thiobarbituric acid reactive substances (TBARS)

Sample with 0.9% NaCl was incubated at 37°C for 20 min. 0.8 M hydrochloric acid (HCl) containing 12.5% trichloroacetic acid and 1% thiobarbituric acid was added and kept in boiling water bath for 20 min and cooled at 4°C. Centrifugation was carried out at 1500g and absorbance

was measured at 532 nm. TBARS was calculated by using the extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.[29]

Protein sulfhydryls (P-SH)

In brief, 0.08 mol/L sodium phosphate buffer (pH 8.0) containing Na_2-EDTA, and SDS was added to each assay tube containing the membrane protein. 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was added and the solution was vortexed. Color was allowed to develop for 15 min at room temperature and absorbance was measured at 412 nm, using an equivalent concentration of protein as the blank.[30] Sulfhydryl concentration was calculated from the net absorbance and molar absorptivity, 13,600 $mol/L^{-1}\ cm^{-1}$.

Hemolysis

A 5% suspension of packed erythrocytes in buffered saline was mixed with an equal volume of 1% H_2O_2 solution. The mixtures were incubated at 37°C for 1 hour. Hemolysis was determined by measuring released Hb into the supernatant of the induced samples at 540 nm and expressed on the basis of the maximum absorbance [100 %] in the aliquots of erythrocytes completely hemolyzed in distilled water.[31]

Statistical analysis

Results are represented as mean ± SE. Values between the groups were analyzed by two-way ANOVA and $P < 0.05$ was considered significant. Bonferroni's post-test was performed for all the assays using GraphPad Prism 8 software (GraphPad Software, Inc., Dotmatics, San Diego, California).

Results

Result analysis have been depicted as changes during storage i.e., with respect to day 0 and also changes on a particular day of storage between the sub groups (control, CA 1 and CA 10).

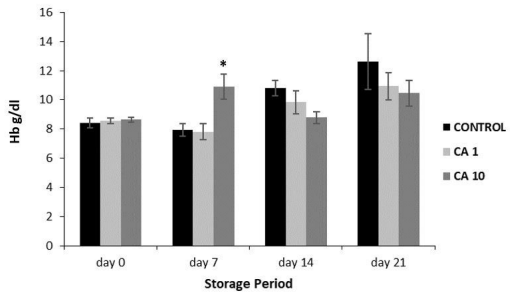

Fig. 1. Hemoglobin in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant at p<0.001; * represents significance between the subgroups (CA 1 and CA 10) against control)

Hemoglobin

Changes in Hb were significant (p<0.001) during the storage period. Controls showed an increase of 30% on day 14 against day 0. Significant changes were observed with sub groups (CA 1 and CA 10). Hb increased by 38% in CA 10 on day 7 against control (Fig. 1).

Superoxide dismutase

Significant changes in SOD activity (p<0.0001) were observed in different groups with storage. SOD increased by 15% in control on day 21 compared to day 0. SOD also increased in CA groups (CA 1 and CA 10) on day 14 and day 21 by 17% compared to day 0. SOD activity increased by 10% in CA 10 against control on day 14 (Fig. 2).

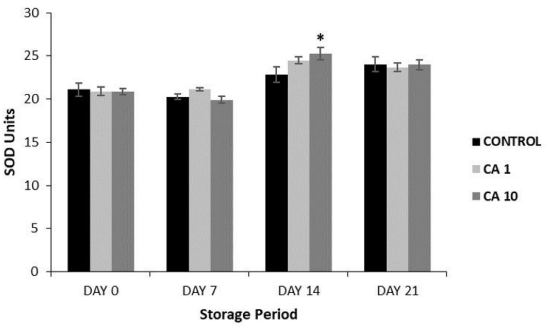

Fig. 2. Superoxide dismutase activity in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); p<0.05 was considered significant. Changes between groups (storage) are significant at p<0.0001; * represents significance between the subgroups (CA 1 and CA 10)

Catalase

CAT activity varied significantly with storage (p<0.001). CAT increased in CA 1 by 35% on day 14 against day 0. CAT activity increased by 93% in CA 1 and 54% in CA 10 on day 14 (p<0.0001) against the control (Fig. 3).

Conjugate dienes

Variations in conjugate dienes were significant during storage (p<0.0001). Conjugate dienes decreased in controls by 27% on day 7, 35% on day 14 & day 21; while in CA 10 it decreased by 23% on day 14 with respect to day 0. Conjugate dienes decreased against the control by 35% in CA 1 and 26% in CA 10 on day 0 (Table 1).

Thiobarbituric acid reactive substances

Variations in TBARS were significant (p<0.01) during storage. TBARS increased in CA 10 by 200% on day 7 and declined to day 0 levels on day 14. TBARS increased by 98% in CA 10 on day 7 against control (Table 1).

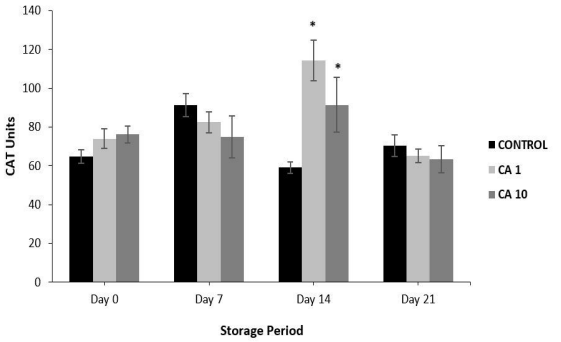

Fig. 3. Catalase activity in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between the groups (storage) are significant at p<0.0001; *represents significance between the sub groups (CA 1 and CA 10)

Table 1. Conjugate dienes, TBARS and protein sulfhydryls in erythrocytes of stored blood [a]

Storage days	Groups	Conjugate dienes (mM/mg protein)	TBARS (µmol/mg protein)	Protein sulfhydryls (Umol/mg protein)
Day 0	Control	0.77±0.08	8.39±1.84	218.52±30.72
	CA 1	0.49±0.03*	2.57±0.31	211.91±18.24
	CA 10	0.57±0.02*	2.70±0.31	81.76±30.18*
Day 7	Control	0.56±0.05	4.91±1.06	105.88±39.66
	CA 1	0.49±0.02	3.85±1.45	103.67±21.76
	CA 10	0.45±0.02	9.5±0.55*	163.52±37.12
Day 14	Control	0.48±0.03	4.67±1.05	78.92±30.07
	CA 1	0.44±0.02	3.70±0.53	80.51±11.82
	CA 10	0.44±0.02	3.54±0.78	70.15±13.45
Day 21	Control	0.52±0.02	2.43±1.12	155.00±41.00
	CA 1	0.48±0.01	3.44±1.21	119.85±33.97
	CA 10	0.47±0.01	7.25±1.96	114.12±39.61

[a] values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant at p<0.01; * represents significance between the subgroups (CA 1 and CA 10).

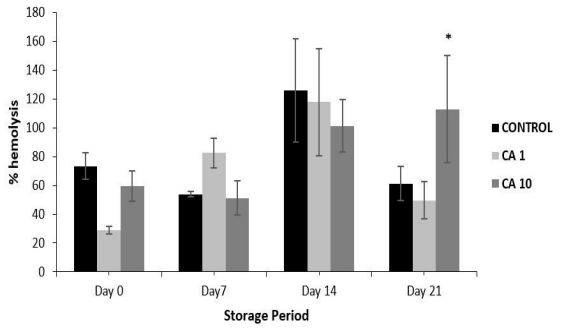

Fig. 4. Hemolysis in erythrocytes of stored blood (values are expressed as mean ±SE from 6 samples. CA 1 –*p*-coumaric acid (1mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant p<0.0001; * represents significance between the sub groups (CA 1 and CA 10)

Protein sulfhydryls

P-SH varied significantly (p<0.01) in different groups with storage. Control and CA 1 showed decrements of 63% on day 14 with day 0 whereas, in CA 10 it was maintained. CA 10 showed decrements of 63% and 54% against control and CA 1 on day 0 (Table 1).

Hemolysis

Significant changes (p<0.0001) in hemolysis were observed in all groups with storage. Hemolysis increased in CA 1 by 85% on day 7 and 90% in CA 10 on day 21 when compared to day 0. Hemolysis increased on day 21 by 84% in CA 10 against control (Fig. 4).

Discussion

The continual changes in erythrocytes during storage and their response to CA as an additive has been assessed to gain insights into the interaction between reactive species and antioxidants. Hb undergoes autoxidation to form MetHb which can be restored by the antioxidants to its reduced state.[32,33] Hb increased on day 7 as OS usually sets in from the second week of storage, resulting in hemolysis.[34] An increase in the hemolysis on day 21 demonstrates the elevations in OS levels towards the end of storage. CA maintained Hb up to day 21, signifying the antioxidant property of coumaric acid through direct scavenging of reactive oxygen species.[35]

SOD catalyzes superoxide radicals to H_2O_2 and O_2.[36] CAT degrades H_2O_2 to H_2O and O_2.[5] SOD reduced in controls during early storage due to ROS levels being lower than the threshold to activate SOD. Urfalioglu et al. reported an increase in the SOD activity in the lung tissue treated with CA.[37] Shen et al. have reported the ability of CA to increase the response of antioxidant genes to ROS.[15] SOD levels elevated on days 14 and 21 in CA samples, suggesting that coumaric acid upregulated SOD which was in accordance with the earlier studies. CAT elevations on day 14 was in accordance with SOD levels as ROS is at its peak during the second week of storage.[23] CAT elevations in CA groups are also in response to coumaric acid's ability to upregulate antioxidant enzyme activity.[15]

The results of the primary products of lipid peroxidation, i.e., conjugate dienes, are indicative of the protective effect of coumaric acid on lipids. CA inhibits low-density lipoprotein thereby, reducing the production of malondialdehyde, a product of lipid peroxidation.[38]

Conjugate dienes showed decrements from day 7 indicating the efficient scavenging activities of antioxidant enzymes. Conjugate dienes also reduced in CA 10 reflecting the antioxidant activity of coumaric acid. CA can prevent excessive lipid peroxidation with a specific scavenging activity for OH^- radicals as observed in the results of conjugate dienes.[36] TBARS were significant on day 7 and normalised to day 0 levels on day 14 due to the activation of antioxidant enzymes on day 14.

P-SH can be reversibly or irreversibly modified based on the extent of oxidative modifications.[39] P-SH variations correlated with the oxidative insult as observed in the lipid peroxidation. Higher concentrations of reactive oxygen species (ROS) oxidizes sulfhydryls (SH) to disulphides. Since there is high ROS on day 14, CA10 has increased SOD activity which is also reflected in the results of conjugate dienes.[34] CA scavenges hydroxyl radical, superoxide anion and H_2O_2 thereby, normalizing oxidative stress on day 21.[40] Though the changes are insignificant, the variations are due to CA which is reflected in SOD levels. The activation of antioxidant defenses has led to homeostasis and thereby, maintained sulfhydryls in reduced state.

Hemolysis is a result of lipid peroxidation and protein oxidations. Hemolysis increased on day 7 and day 21. A significant increase on day 7 is due to the production of free radicals as reflected in TBARS results. Hemolysis increased on day 21 leading to the evidence that antioxidants and coumaric acid could not scavenge the free radicals completely towards the end of storage, although it could attenuate lipid peroxidation and protein oxidation.

CA at 10 mM concentration was more beneficial than Coumaric acid at 1 mM concentration. CA10 augmented antioxidant defenses such as SOD and CAT, retained sulfhydryls in reduced state and hemolysis was observed only towards the end of the storage period whereas, hemolysis was evident on day 7 in CA1.

Conclusion

Antioxidant defenses of erythrocytes play a major role in attenuating OS during storage. Coumaric acid upregulated the antioxidant enzymes and thereby protected the cells from lipid peroxidation and protein oxidation. Coumaric acid at 10 mM concentration was more beneficial than Coumaric acid at 1 mM concentration. Thus, these results substantiate the potential of coumaric acid as an antioxidant additive during storage and opens new avenues of employing it in similar oxidative stress situations in erythrocytes.

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Author contributions

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

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

Ethical approval

Animal care and maintenance were in accordance with the ethical committee regulations (841/b/04/CPCSEA).

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ORIGINAL PAPER

A promising approach to wound healing – *in-vivo* study of carbon nanodots infused PVA hydrogel with Kamias extract as antibacterial wound dressing

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ABSTRACT

Introduction and aim. The use of carbon nanodots (C-nanodots) synthesized from Kamias leaves for developing antibacterial wound dressings has gained attention due to their potential in promoting wound healing and contraction. To extract Kamias leaves, synthesize C-nanodots through microwave-assisted pyrolysis, characterize the synthesized C-nanodots, and test the polyvinyl alcohol (PVA) hydrogel infused with C-nanodots for antibacterial activity and wound contraction in Sprague Dawley rats.

Material and methods. Kamias leaves extract was used to synthesize C-nanodots with varying amounts of monoethanolamine. The C-nanodots were characterized using UV-Vis spectrophotometer, electron microscope, and the paper disk method. The PVA hydrogel infused with C-nanodots was tested for antibacterial activity and wound contraction in Sprague Dawley rats.

Results. The synthesized C-nanodots exhibited antibacterial properties against Staphylococcus aureus and Subtilis bacillus, with a zone of inhibition ranging from 15 mm to 23.6 mm at different concentrations. The carbon nanodots-PVA hydrogel patch showed potential wound healing ability, with significant differences in wound contraction compared to the positive and negative controls.

Conclusion. C-nanodots synthesized from Kamias extract have potential applications in antibacterial and wound healing fields. However, further studies are required to investigate the mechanism of action and potential side effects of using carbon nanodots in these applications.

Keywords. antibacterial, carbon nanodots, Kamias leaves, PVA hydrogel, wound dressing, wound healing

Introduction

A wound is a breakdown in the protective function of the skin and the loss of continuity of epithelium, with or without loss of underlying connective tissue following injury to the skin or underlying tissues. When it happens, bacteria will invade easily and start to form colonies thereby leading to severe wound infection and delay wound healing.[1,2] Therefore, substantial efforts are being made to develop new materials for protecting damaged skin from infections and dehydration. For this purpose, traditional dry dressings are used during the initial stages of wound healing, because they are dry and cannot provide a moist environment, however, they are also often liable to adhere to desiccated wound surfaces and finally induce trauma upon removal.[2] To overcome these drawbacks, inspired by the concept of moist wound healing, various wet dressings have been developed. Among them, special attentions have been paid to hydrogels because they can maintain a moist environment at the wound interface, allow gaseous exchange, act as a barrier to microorganisms, remove excess exudates, have excellent biocompatibility, promote a rapid heal-

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ing of wound, and be easily removed without trauma.[2]

Hydrogels are able to store a lot of water due to their three-dimensional hydrophilic network.[3] The demand for efficient, biodegradable hydrogels is increasing as a result of synthetic polymers' non-biodegradability. Polyvinyl alcohol (PVA) is a well-liked polymer for hydrogel applications because of its water solubility, biocompatibility, non-toxicity, biodegradability, and film-forming properties.[4] PVA can be chemically modified and crosslinked with other polymers, such starch, for use in biomedical applications. Since 10 years ago, carbon nanodots (C-nanodots) have gained popularity because to their antimicrobial, wound-healing, and disinfectant properties as well as their biocompatibility, stability, low toxicity, and environmental friendliness. When utilised in dressings, their optical characteristics can be changed by varying pH levels, making them appropriate for monitoring wound pH.[5] In this study, C-nanodots were obtained from Kamias (*Averrhoa bilimbi*). The Oxalidaceae family includes the medicinal plant known as bilimbi, which is widely cultivated and has many different names. It is the perfect option for the synthesis of C-nanodots for use in treatments for wound healing because of its medicinal qualities and accessibility.[6] *A. bilimbi* is a small tree which grows up to 15 m high with sparsely arranged branches. It has compound leaves with twenty–forty leaflets each and 5–10 cm long. The leaves are hairy with pinnate shapes and form clusters at the end of branches. The tree is cauliflorous with 18–68 flowers in panicles that form on the trunk and other branches. The flowers are heterotricycles with petal 10–30 m long, yellowish green to reddish purple. The fruits are produced on the bare stem and trunk. The fruits are greenish in color with a firm and juicy flesh which becomes soft on ripening. The fruit juice is sour and extremely acidic. *A. bilimbi* holds great value in complementary medicine as evidenced by the substantial amount of research on it.[6]

The leaves ethanol extract of *A. bilimbi* was reported to exhibit appreciable antimicrobial activity against six pathogenic microorganisms, namely two Gram-positive bacteria (*Bacillus cereus* and *Bacillus megaterium*), two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and two fungi (*Aspergillus ochraceous* and *Cryptococcus neoformans*). The aqueous and chloroform extracts of *A. bilimbi*'s leaves and fruits (100 mg/ml) showed a positive antibacterial activity against *S. aureus*, *Staphylococcus epidermis*, *B. cereus*, *Salmonella typhi*, *Citrobacter freundii*, *Aeromonas hydrophila*, *Proteus vulgaris*, and *Kocuria rhizophila*. Whole bilimbi fruit and blended bilimbi juice (not filtered) at a concentration of 1:2 and 1:4 w/v, respectively, displayed a significant activity against *Listeria monocytogenes* Scott A and *S. typhimurium* in an *in vitro* antibacterial assay. The fruit preparations were also found to reduce the microbial load of *L. monocytogenes* Scott A and *S. typhimurium* on raw shrimps after washing and during storage (4°C). This demonstrated the potential of *A. bilimbi* fruits to be adopted as a natural method of decontaminating shrimps just before preparation and consumption. In another study, fruits and roots extracts of *A. bilimbi* were also found to exhibit the positive activity against *Mycobacterium tuberculosis* with MIC of 1600 µg/ml. The leaves extracts have also been reported to display moderate antifungal activity against *Blastomyces dermatitidis*, *Candida albicans*, *Cryptococcus neoformans*, *Pityrosporum ovale*, and *Trichophyton* spp. with MIC values ranging from 15.65 to 62.50 µg/ml. Kamias is considered as antibacterial, astringent, antiscorbutic, febrifuge, antidiabetic, stomachic, refrigerant. Its Fruits are considered as astringent, refrigerant, and stomachic.[6]

During the purification of single-walled carbon nanotubes, carbon nanodots (C-nanodots), a family of carbon nanomaterials smaller than 15 nm, were initially identified. They are useful for a variety of nanobiotechnology applications, including biosensors, due to their bright fluorescence, high aqueous solubility, chemical inertness, ease of functionalization, resistance to photobleaching, low toxicity, and biocompatibility.[7-10] One study investigated C-nanodots' optical properties for potential use in optical biosensors.[11] Carbon nanodots can be prepared from organic matter or small organic molecules and typically possess numerous functional groups on their surface.[12] Two approaches to prepare carbon nanodots are the top-down and bottom-up approaches. Top-down preparations include arc-discharge method, electrochemical oxidation, and laser ablation, while bottom-up approaches involve polymerization reactions for small molecules to form nanoscale C-nanodots, including hydrothermal method, microwave-assisted pyrolysis method, ultrasonic method, acid dehydration method, and pyrolysis method.[13] The most widely used methods are the hydrothermal method and microwave-assisted pyrolysis method, which can prepare fluorescent C-nanodots in a single step.[14-16] Some synthesis methods use passivating agents to improve the quantum yield and photoluminescence emission of synthesized C-nanodots. These agents are usually amino-containing molecules or polymers, including TTDDA, 1-hexadecylamine, octadecylamine, PEG, and N-(β-aminoethyl)-γ-aminopropyl methyldimethoxy silane.[11,17] The need for passivating agents results in a rich surface-functional group presence on C-dots, making them highly hydrophilic and easily functionalized with various organic, polymeric, inorganic, or biological species. Surface passivation forms a thin insulating capping layer that protects C-nanodots from impurities, since they are vulnerable to contamination due to the endogenous nature of carbon and oxygen to react with organic molecules.[18] After synthesizing C-nanodots, their pho-

toluminescence emission can be measured using a UV-Vis spectrophotometer. High-resolution transmission electron microscopy (HR-TEM) images can be obtained using a JEOL JEM-2100 Electron Microscope, operating at 80kV. Samples are prepared by evaporating droplets placed on Formvar/Carbon coated TEM grids and allowing the solvent to evaporate under atmospheric conditions. Scanning electron microscopy (SEM) can also be used to investigate the surface morphology and size distribution of C-nanodots.[11]

A wound is a disruption in the skin or mucosa's continuity caused by physical or thermal damage. Wounds are categorized as acute or chronic based on the healing process duration and nature. Acute wounds result from accidents or surgery and heal predictably within 8-12 weeks. Chronic wounds fail to progress through normal healing stages and often stem from decubitis ulcers, leg ulcers, or burns. Wound healing is a complex, dynamic process involving four overlapping phases: coagulation and hemostasis, inflammation, proliferation, and maturation. The kind of the wound, any accompanying pathological problems, and the material being used as a dressing all affect how quickly it heals. Ideal wound dressings depend on wound type and should be selected based on their ability to: a) maintain a moist environment, b) enhance epidermal migration, c) promote angiogenesis and connective tissue synthesis, d) allow gas exchange, e) maintain appropriate tissue temperature, f) protect against bacterial infection, g) be non-adherent and easy to remove, h) provide debridement action, and i) be sterile, non-toxic, and non-allergic.[19]

Hydrogels consist of three-dimensional hydrophilic network, which is responsible for the water holding capacity of the gel and usually used for scaffolding and wound healing management.[3] Hydrocolloid dressings are among the most widely used hydrogel wound dressings. The role of hydrocolloid dressings, their properties, mechanism of action and the range of wounds for which they are useful have been reviewed. The term 'hydrocolloid' describes the family of wound management products obtained from colloidal (gel forming agents) materials combined with other materials such as elastomers and adhesives. Typical gel forming agents include carboxymethylcellulose (CMC), gelatin and pectin. Examples of hydro- colloid dressings include Granuflex™ and Aqua- cel™ (Conva Tec, Hounslow, UK), Comfeel™ (Coloplast, Peterborough, UK) and Tegasorb™ (3M Healthcare, Loughborough, UK). Hydrocolloid dressings occur in the form of thin films and sheets or as composite dressings in combination with other materials such as alginates.[20] The dressing combines moisture vapour permeability with absorbency and conformability, and its transparency allows for wound observation. Hydrocolloid dressings are useful clinically because unlike other dressings, they adhere to both moist and dry sites. Hydrocolloid dressings are used for light to moderately exuding wounds such as pressure sores, minor burns and traumatic injuries. In their intact state, hydrocolloid dressings are impermeable to water vapour but on absorption of wound exudate, a change in physical state occurs with the formation of a gel covering the wound. They become progressively more permeable to water and air as the gel forms. As they do not cause pain on removal, they are particularly useful in pediatric wound care for management of both acute and chronic wounds.[21]

Duoderm dressing is a modern hydrocolloid dressing for the management of light to moderately exuding wounds. It is versatile, easy to use and are suitable for managing different stages of wound healing and multiple wound types in a protocol of care.

The Kirby-Bauer test for antibiotic susceptibility (also called the *disc diffusion test*) is a standard that has been used for years. First developed in the 1950s, it was refined and by W. Kirby and A. Bauer, then standardized by the World Health Organization in 1961. However, the K-B is still used in some labs, or used with certain bacteria that automation does not work well with. This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals, which can then be used by the clinician for treatment of patients with bacterial infections. Table 1 shows the summary of SIR table for target organisms in this study.

Table 1. Summary of SIR table for test microorganisms (Clutterbuck, Cochrane, Dolman, Percival, 2007)

Microorganisms	Zone of inhibition in mm		
	Susceptible	Intermediate	Resistance
Subtilis bacillus	>/=22	>/=12	</=11
Staphylococcus aureus	>/=21	>/=15	</=14

Aim

Hence, this study aimed to produce an alternative way to promote and enhance wound healing and produce hydrogels with C-nanodots that can be applied as an antibacterial agent. The study gave knowledge and ideas in the release behaviour of the wound patch that will help in wound management and in fulfilling the conditions such as maintaining a local moist environment, protecting the wound from side infection, absorbing the wound fluids and exudates, minimizing the wound surface necrosis, preventing the wound dryness, depressing the bacterial growth rate, and provide a non-toxic, non-antigenic, biocompatible and biodegradable dressing materials.

This study contributes to the community by developing a wound dressing that can prevent bacterial infections and promote wound healing, potentially serving as an alternative to expensive, synthetically prepared wound dressings on the market. Furthermore, the research provides additional knowledge on C-nanodots

applications, which may serve as a reference for future researchers interested in these nanoparticles. The study involved the extraction of Kamias leaves, synthesis of C-nanodots, preparation of hydrogels, antibacterial and *in-vivo* assays of the C-nanodots infused hydrogel at various institutions in the Philippines. The study was limited to synthesizing C-nanodots from Averrhoa bilimbi using microwave-assisted pyrolysis with varying amounts of monoethanolamine as a passivating agent. Characterization of the synthesized C-nanodots was limited to SEM for determining nanostructure and particle size distribution, and UV-vis for photoluminescence emission measurement. The antibacterial activity was tested only against *Staphylococcus aureus* and *Bacillus subtilis*. The application of C-nanodots was limited to their infusion in PVA hydrogels for antibacterial wound dressing that promotes healing and contraction on Sprague Dawley rats over a 10-day treatment period.

The main focus of this study was to analyze in-vivo the effectiveness of PVA hydrogel infused with C-nanodots, which were synthesized from Kamias leaves through microwave-assisted pyrolysis, as an antibacterial wound dressing. The study aimed to extract Kamias leaves using 80% ethanol, synthesize C-nanodots through microwave-assisted pyrolysis using Averrhoa bilimbi or Kamias leaves extract at varying amounts of monoethanolamine, and characterize the synthesized C-nanodots in terms of photoluminescence emission using UV-Vis spectrophotometer, nanostructure and size distribution of C-nanodots using SEM, and antibacterial activity using paper disk method. Furthermore, the study aimed to prepare PVA hydrogel infused with C-nanodots, test the antibacterial activity at different concentrations of PVA hydrogel infused with C-nanodots using Agar disk-diffusion method, measure wound contraction in Sprague Dawley Rats applied with C-nanodots-PVA hydrogel at 3rd, 7th, and 10th day after application, and compare the contraction of wound applied with C-nanodots-PVA hydrogel and duoderm at 3rd, 7th, and 10th day after application.'

Material and methods

Ethical approval

This study was conducted per the ethical guidelines and was approved by the College of Arts and Sciences at Nueva Ecija University of Science and Technology (2018-008). All procedures involving the use of animals were performed in compliance with the institution's guidelines for the care and use of laboratory animals.

Sample collection and preparation

The leaves of Kamias was collected from Cabanatuan City local market and served as the raw material in this study. The sample was washed and air-dried. A 40g of the powdered plant material was soaked in 400 mL of 85% ethanol for 72hr. The resultant extracts was filtered through Whatman filter paper No. 1. The filtrate was concentrated under reduced pressure using rotary evaporator at 50°C. The crude extracts was collected and allowed to dry at room temperature. Kamias extract solution with concentration of 100 µL/ml (crude extract:water) was prepared.

Synthesis of carbon nanodots

The methods used in the synthesis of C-Nanodots is the Bottom-up Approach's microwave assisted pyrolysis adopted from the previous study.[22] C-nanodots was synthesized using 630W power condition of microwave. Three (3) mL of Kamias extract solution with varying amount of 1.5 ml, 3 ml, 4.5 ml, and 6 ml of monoethanolamine was poured in a 50 ml erlenmeyer flask separately and heated in 2 minutes and repeated until reddish brown solutions are obtained. Four treatments were observed in the synthesis of C-nanodots, considering the varying amount of monoethanolamine as passivating agent:

$$T_1 = 1.5ml;\ T_2 = 3ml;\ T_3 = 4.5ml;\ \text{and}\ T_4 = 6ml.$$

Characterization of synthesized carbon nanodots from Kamias

The synthesized carbon nanodots was characterized by determining its nanostructure and size distribution using SEM. The SEM that was used is a Hitachi SU8230 Field-Emission Scanning Electron Microscope at 500nm px. Also, the photoluminescence emission synthesized C-nanodots was measured by UV-Vis (U-2900UV/VIS spectrophotometer) at 200V and 630nm wavelength.

Antibacterial assay

The synthesized C-nanodots from Kamias was subjected to individual microbiological tests to ascertain its antibacterial activity against gram negative bacteria and gram positive bacteria at different concentrations (1%, 0.75%, 0.50%, 0.25% and 0%). The antibacterial activity of the C-nanodots was determined by measuring the diameter of the zone of inhibition (ZI). Antibacterial activity was determined against *S. aureus* and *S. bacillus* using the paper disk assay method.[23,24] Whatman No. 1 filter paper disk of 6-mm diameter was sterilized by autoclaving for 15 min at 121°C. The sterile disks were impregnated with the different concentrations (1%, 0.75%, 0.50%, 0.25% and 0%) of C-nanodots. Agar plates were surface-inoculated uniformly from the broth culture of the tested microorganisms. The impregnated disks were placed on the medium suitably spaced apart and the plates were incubated at 37°C for 24 h.

Preparation of antibacterial C-dots-PVA hydrogel

Analytical Grade of PVA produced by Sigma-Aldrich was used to form hydrogel. Five percent (5% w/v) solu-

tion of PVA was prepared using 100 ml distilled water.[25] The mixture was heated with continues stirring until all powder polymers were dissolved. Ten ml of PVA solution was combined with 10 ml distilled water and was heated. One and a half gram of agar was dissolved into the heated solution with continuous stirring. Three ml of C-nanodots solution was added drop by drop with continuous stirring. Subsequently, the mixture was injected into a square mould for the formation of hydrogel patches. The moulds were freezed and thawed to complete the moulding process.

Analgesia injection and wound excision

Table 2 presents the key aspects of the major components of the methods used to induce and excision wounds in a laboratory rat model. Sharp mayo scissors and a scalpel were used to make 1x1 cm full-thickness cuts on the dorsal region of the rats' backs for the study. Lidocaine was used as an anesthetic for pain alleviation and to ensure the animals were treated humanely throughout the procedure. This information is presented in a clear and ordered way in the table, which can be helpful in comprehending the methodology as well as analyzing the findings of the study.

Table 2. Analgesia injection and inducing excision wounds

Aspect	Details
Species	Laboratory rats
Location	Dorsal area (back)
Anesthesia	Lidocaine (23mg/kg subcutaneously)
Wound size	1x1 cm
Wound depth	Full-thickness
Instruments	Sharp mayo scissors and scalpel
Humane treatment	Method carried out in the most humane way

Evaluation of wound healing

The laboratory rats were equally distributed into three treatment groups: $T_{1=}$ Daily Topical application of antibacterial C-dots-PVA Hydrogel at 3 hours after wound creation and daily; T_2= Commercial wound patch changed daily, first application at 3 hours after wound creation and daily; and T_3= group that did not receive treatment after wound creation. All the laboratory rats were housed in a clean and well-ventilated area to observe the healing process of wound. Wound surface area on days 3, 7, and 10 after wound creation was evaluated (in cm^2 unit) and the percentage of healing was normalized by the following formula:

$$\text{Percentage wound contraction (\%WC)} = \\ = \frac{\textit{wound surface on day 1- wound surface on day}}{\textit{wound surface on day 1}} \times 100$$

where *x* is the day when the wound surface is evaluated.

Statistical analysis

The research design used in this study was completely randomized design (CRD) for the antibacterial assay of the C-nanodots and PVA-C-nanodots hydrogel while Randomized complete randomized block design was used in the evaluation of wound healing in Sprague Dawley rats. One-way ANOVA was used to determine the significant differences in the zone of inhibition and in the wound contraction of Sprague Dawley Rats. Comparison of means using Duncan's multiple range test or DMRT was also used in treatments that was found to be significantly different using ANOVA.

Results

Synthesis of carbon nanodots from Kamias

Varying amount of monoethanolamine were used as a passivating agent in synthesizing C-nanodots from Kamias extract solution through microwave assisted pyrolysis in 2-minute repetitive heating time as shown in Figure 1. A total of 18 minutes of heating time was needed before a reddish brown solution of C-nanodots was acquired. The acquired solution was slightly viscous.[22]

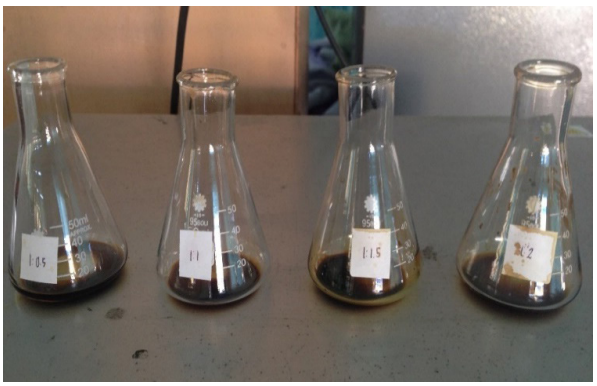

Fig. 1. Synthesized C-Nanodots from Kamias at varying amount of monoethanolamine

Characterization of synthesized carbon nanodo

The synthesized carbon nanodots from Kamias extract at different amount of passivating agent (monoethanolamine) was characterized by observing its photomuniscence (PL) absorption using UV-Vis Spectrophotometer at a wavelength of 630 nm. The absorbance of the C-nanodots at different amount of passivating agent (T1, T2, T3, T4) is presented in Table 3.

Table 3. PL emission of synthesized carbon nanodots from Kamias at varying monoethanolamide

Treatment	Monoethanolamide (ml)	Absorbance at 630nm
1	1.5	0.477
2	3	0.480
3	4.5	0.670
4	6	0.325

Shown in Table 3, photoluminiscence (PL) intensity illustrated by the absorbance at 630 nm increases with increasing amount of monoethanolamine and peaked at T3, with 4.5 ml of monoethanolamine and then decreased with the next increment of amines. This indicates that T3 was the optimum concentration in the synthesis of C-nanodots. PL intensity was improved upon surface passivation using monoethanolamine. Adding proper amount of amine into the reaction system was beneficial in improving and increasing the PL intensity resulting to a rich surface-functional groups of C-nanodots which are responsible for its high hydrophilicity and readiness for functionalization with a polymer and other inorganic and biological species. Surface passivation formed a thin insulating capping layer that protects C-nanodots from impurities and results for a stable and long life usage of C-nanodots. It has been shown that diverse oxygen functionalities may shift the PL emission wavelength. Thus, experimental and theoretical analyses associate the sp^2-hybridized carbon network with the increasing PL emission at short wavelength. Even though it was used worldwide, the exact mechanism of photoluminescence property of C-nanodots is not yet revealed because of the complicity of its structure.[18] The synthesized C-nanodots was also viewed under UV-light where its photoluminescence property were observed as shown in Figure 2. Blue photoluminescent C-nanodots with increasing intensity were observed in where T3 has the highest intensity but decreases with further addition of monoethanolamine at T4, which corresponds with most of the C-nanodots that has been synthesized that emit blue luminescence under UV irradiation. Surface passivation forms a thin insulating capping layer that protects C-nanodots from impurities and ensures stable, long-lasting usage. It has been shown that diverse oxygen functionalities may shift the PL emission wavelength.[26] Experimental and theoretical analyses associate the sp^2-hybridized carbon network with increasing PL emission at short wavelengths.[27] Although widely used, the exact mechanism of C-nanodots' photoluminescence property has not yet been revealed due to the complexity of their structure.[26] The synthesized C-nanodots were also viewed under UV-light, where their photoluminescence properties were observed (Fig. 2). Blue photoluminescent C-nanodots with increasing intensity were observed, with T3 having the highest intensity. However, the intensity decreases with further addition of monoethanolamine at T4, corresponding to most synthesized C-nanodots that emit blue luminescence under UV irradiation.[28]

The nanostructure and size distribution of the synthesized C-nanodots from Kamias was characterized using SEM and the micrograph is shown in Figure 3. The scanning electron micrograph of C-nanodots shows 14.96 nm as the computed particle size. The nanostructure form of the synthesized C-nanodots from Kamias was found to be spherical.[17]

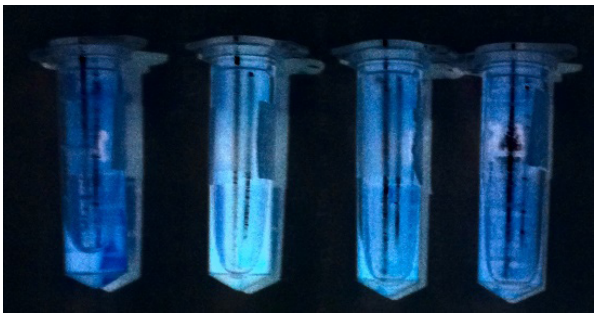

Fig. 2. Synthesized C-nanodots under UV light

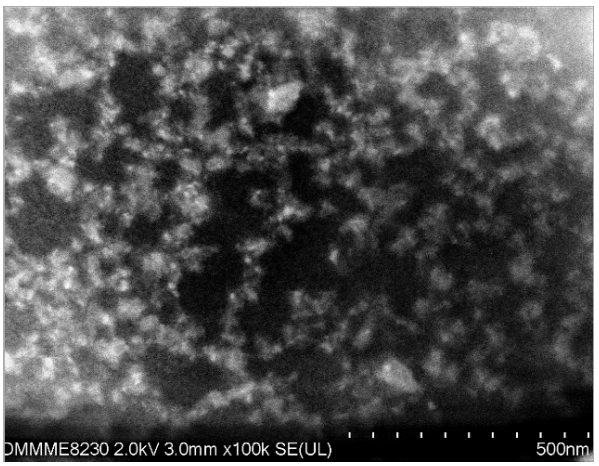

Fig. 3. Scanning electron micrograph of CD from Kamias

Antibacterial assay

The antibacterial activities of C-nanodots solutions were investigated against *Staphylococcus aureus* and *Subtilis bacillus* using paper disc assay method. In Table 4, it shows the average zone of inhibition (ZI) in millimeter exhibited by C-nanodots at different concentrations of 0.25%, 0.50%, 0.75%, and 1%.

Table 4. Antibacterial zone of inhibition of different concentrations of C-nanodots from Kamias against *Subtilis bacillus*

Treatment	0.25%	0.50%	0.75%	1%
1	8	7	10	8
2	8	8	9	8
3	9	8	8	7
4	10	9	8	7

The zone of inhibition ranges from 7 mm to 10 mm. According to the SIR Table for test microorganisms, zone of inhibition with less than or equal to 11 is described as "resistance".[29] *S. bacillus* is therefore resistance to C-nanodots. Table 5. Antibacterial zone of inhibition of different concentrations of C-nanodots from Kamias against *S. aureus.*

Table 5. Antibacterial zone of inhibition of different concentrations of C-nanodots from Kamias against *S. aureus*

Treatment	0.25%	0.50%	0.75%	1%
1	12	17.5	18	15
2	16	18	18	17.9
3	16	23.6	21	18
4	15	22	16	16.5

Table 5, it shows the average zone of inhibition in millimeter exhibited by different concentrations of C-nanodots at 0.25%, 0.50%, 0.75%, and 1%. The average zone of inhibition ranges from 15 mm to 23.6 mm. According to the SIR Table for test microorganisms, the zone of inhibition for *S. aureus* with greater than or equal to 15 is described as intermediate.[29] If the measurement is greater than or equal to 21, then the result is susceptible. *S. aureus* is therefore susceptible to 0.50% and 0.75% C-nanodots. The antibacterial properties of the C-nanodots against *S. aureus* were confirmed.[5] The average zone of inhibition ranges from 15 mm to 23.6 mm. According to the SIR Table for test microorganisms, a zone of inhibition greater than or equal to 15 is described as intermediate.[30] If the measurement is greater than or equal to 21, then the result is susceptible. Staphylococcus aureus is therefore susceptible to 0.50% and 0.75% C-nanodots.[31] This confirms the antibacterial properties of the C-nanodots against *S. aureus.*

Wound healing

In order to investigate the percentage wound size reduction using the formulated C-nanodots-PVA hydrogel patch, and duoderm, wound excisional on rat model was used. The mean percentage of the wound contraction at days 3, 7, and 10 is shown in Table 6.

Table 6. Mean Percentage of the wound contraction on 3rd, 7th, and 10th day*

Treatment	3rd day	7th day	10th day
T_A - C-dots-PVA	27.2 [a]	69.4 [a]	90.8 [a]
T_B - Duoderm	16.8 [b]	59.4 [b]	80.2 [b]
T_C - Untreated	9.0 [c]	29.0 [c]	51.4 [c]

* a – wound contraction percentage for TA- C-dots-PVA at specific time points, with the highest rate compared to other treatments; b – wound contraction percentage for TB- Duoderm at specific time points, lower than TA- C-dots-PVA but higher than TC- Untreated; c – wound contraction percentage for TC- Untreated at specific time points, with the lowest rate among all groups.

Table 6 presents the mean percentage of wound contraction for three treatment groups at specific time points (3rd day, 7th day, and 10th day). The letters a, b, and c are used to indicate significant differences between these groups, as determined by statistical tests such as ANOVA and a post hoc test like Duncan Multiple Range Test. For the TA- C-dots-PVA treatment group, the letter 'a' denotes that this group had the highest wound contraction rate at each time point when compared to the other two treatments. The TB- Duoderm treatment group, represented by the letter 'b,' had a lower wound contraction rate than the TA- C-dots-PVA group but higher than the TC- Untreated group. Finally, the letter 'c' signifies the TC- Untreated (negative control) group, which had the lowest wound contraction rate among all three groups at each time point. The presence of different letters (a, b, and c) for each treatment group at a specific time point indicates that there are statistically significant differences between the mean wound contraction rates of these groups. If two or more groups had shared the same letter, this would have implied no statistically significant difference between their mean wound contraction rates at that time point.

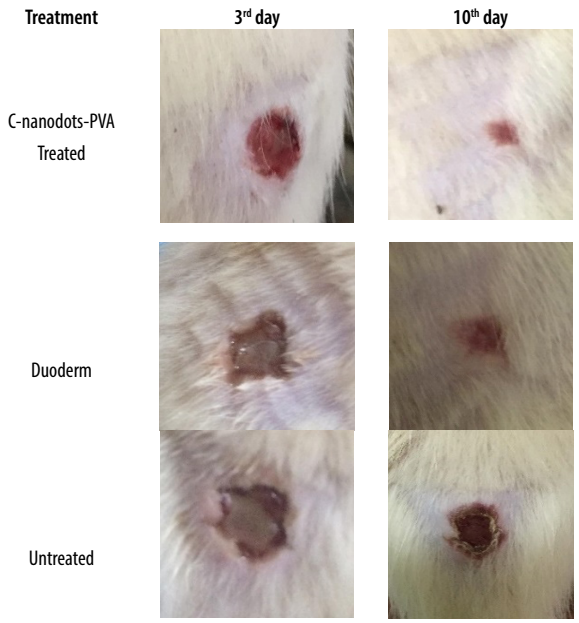

Fig. 4. Visual effect of C-nanodots-PVA Hydrogel and Duoderm vs. natural wound healing on 3rd and 10th day of evaluation

Analysis of variance at α=0.05 showed significant differences in the observed wound contraction applied with C-nanodots-PVA hydrogel, positive control (duoderm), and negative control in all days of observation. In the 3rd day, the wound contraction was reduced by 27.2% for T_A, 16.8% for T_B, 9.0% for T_c. In the 7th day, the wound contraction was reduced by 69.4% for T_A, 59.4% for T_B, 29.0% for T_c. In the 10th day, the wound contraction was reduced by 90.8% for T_A, 80.2% for T_B, 51.4% for T_c. Comparison of means using Duncan Multiple Range Test (Table 5) showed that the mean percentage of wound applied with C-nanodots-PVA hydrogel was higher compared to that of positive control and negative control at day 3. Similar trend was observed at day

7 and day 10. The effective performance of the C-nanodots-PVA hydrogel was shown visually in Figure 4. Due to its strong antibacterial and nontoxicity properties, C-nanodots can be applied as an effective wound-dressing material for enhancing the healing and pH monitoring of wounds at the same time.[5,30]

Discussion

The successful synthesis of C-nanodots from Kamias extract using microwave-assisted pyrolysis and the optimization of monoethanolamine as a passivating agent contribute to the growing body of research on carbon nanodots. C-nanodots have drawn interest in a number of sectors, including optoelectronics, bioimaging, and drug administration, as was previously highlighted in the literature review.[18] This is because of their distinctive features, such as photoluminescence, biocompatibility, and low toxicity. In comparison to current procedures, the findings of this study suggest that Kamias extract may be used as an economical and environmentally friendly carbon nanodot synthesis alternative.[22]

Characterizing the synthesized C-nanodots revealed their spherical shape and a particle size of 14.96 nm.[17] The photoluminescence intensity was found to be optimal at a specific concentration of the passivating agent, monoethanolamine.[26] This supports the hypothesis that surface passivation plays a critical role in the stability and photoluminescence properties of C-nanodots.[27] Further studies on the surface passivation and the exact mechanism of photoluminescence properties of C-nanodots are essential, considering the widespread use of C-nanodots and the complexity of their structure.[18,26]

S. aureus and *S. bacillus* were used as test organisms to see if the synthesised C-nanodots had any antibacterial characteristics. The findings demonstrated that C-nanodots had moderate sensitivity to *S. while S. aureus, S. bacillus* demonstrated C-nanodot resistance.[5, 29,30] According to these results, C-nanodots may be used as an antibacterial agent, notably against *S. aureus*, a bacteria that is known to infect wounds.[5]

The wound-healing potential of the synthesized C-nanodots was also evaluated using a rat model, where C-nanodots were incorporated into a polyvinyl alcohol (PVA) hydrogel patch. The results demonstrated that the C-nanodots-PVA hydrogel patch significantly improved the wound-healing process compared to the positive control (Duoderm) and the negative control (untreated) groups.[5,30] This finding indicates that C-nanodots, due to their strong antibacterial and non-toxic properties, can be effectively used as a wound-dressing material for enhancing wound healing and pH monitoring simultaneously.[5,30]

This study contributes to the existing knowledge on carbon nanodot synthesis, characterization, and potential applications. The findings show that Kamias extract may be used as an inexpensive and environmentally acceptable substitute for making C-nanodots, and that using monoethanolamine at the right dosage as a passivating agent enhances the photoluminescence capabilities. Furthermore, C-nanodots' antibacterial qualities suggest that they might be used as an antibacterial agent, notably against *S. aureus*. Additionally, the addition of C-nanodots to a PVA hydrogel patch accelerates the healing of the lesion, indicating a prospective use for the substance in wound-dressing materials.

Although the study's findings seem promising, more investigation is required to pinpoint the precise process underlying C-nanodots' photoluminescence capabilities and to examine their potential for use in other fields. Investigating C-nanodots' effectiveness against different bacterial strains and their potential as antifungal and antiviral medicines, for instance, might be advantageous. Furthermore, a more thorough investigation of the interactions between C-nanodots and different biological systems may provide important details about their biocompatibility and potential negative consequences. Exploring other approaches to include C-nanodots into wound dressings, such as putting them into various kinds of hydrogels, fibrous materials, or films, might also be beneficial. These studies could help optimize the wound-dressing materials and maximize the benefits of C-nanodots in promoting wound healing. It would also be crucial to carry out research on C-nanodots' long-term stability in various settings and storage circumstances, as well as how well they function when put through various sterilisation processes. Investigating C-nanodots' possible uses in other industries, such as optoelectronics, bioimaging, and drug delivery systems, would be another interesting topic. For instance, C-nanodots' photoluminescent characteristics might be used to create sensors and imaging tools, and their biocompatibility and surface functionalization could make them appropriate for customised drug delivery systems. Finally, to assess the safety and effectiveness of C-nanodots-based wound dressings in people, it would be crucial to carry out more in vivo research and eventually clinical trials. These studies would help determine the ideal concentration of C-nanodots and the optimal formulation for wound dressings, ensuring their safety and effectiveness in promoting wound healing in clinical settings. This study has demonstrated the potential of C-nanodots synthesized from Kamias extract in enhancing wound healing and their antibacterial properties. To explore the full potential of C-nanodots in diverse applications and to get a deeper comprehension of their characteristics and mechanisms of action, more study is essential. Researchers may contribute to the creation of novel and efficient solutions for a variety of applications by continuing to look into the prospective applications of C-nanodots, which will eventually enhance human health and wellbeing.

Conclusion

The research findings provide valuable insights into the potential use of C-nanodots for antibacterial and wound healing applications. The first conclusion highlights the high antibacterial activity of the synthesized C-nanodots against gram (+) bacteria. This finding is significant as gram (+) bacteria are known to cause various infections, including skin infections, pneumonia, and sepsis. C-nanodot's high antibacterial activity indicates their potential as an effective treatment option for such infections. The second conclusion reveals that the formulated C-nanodots-PVA hydrogel patch is more efficient than the standard Duoderm regarding wound healing and contraction. This finding suggests that the C-nanodots-PVA hydrogel patch could be a more effective and faster-acting wound healing option than current treatments. The third conclusion highlights the potential of the C-nanodots-PVA hydrogel patch as an alternative patch with wound healing activity against gram (+) bacteria. This is significant as it suggests that the patch could treat various infections caused by gram (+) bacteria, including skin infections, surgical site infections, and wound infections. Moving forward, there are several recommendations for further research to enhance the potential use of C-nanodots for antibacterial and wound healing applications. Firstly, using other amines as passivating agents for synthesizing C-nanodots could improve surface morphology and potentially enhance antibacterial activity. Secondly, increasing the concentration of C-nanodots in UV-vis analysis could provide more accurate and detailed information about the physicochemical properties of the C-nanodots. Thirdly, further characterization of synthesized CD using TEM and XRD could provide additional information about the morphology and crystal structure of C-nanodots. Finally, analysis of the C-nanodots-PVA hydrogel patch's tensile strength and bio adhesive strength could provide valuable insights into its physical properties and potential use as a wound healing treatment. Overall, the findings and recommendations provide promising avenues for further research into using C-nanodots for antibacterial and wound healing applications. With further investigation and development, C-nanodots could be a more effective and efficient treatment option for various infections caused by gram (+) bacteria.

Declarations

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Author contributions

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

Conflicts of interest

The author of this work, hereby declare that we have no competing interests to disclose.

Data availability

Data is available on request of the author.

Ethics approval

Permission for the study was obtained from the College Ars and Sciences Ethics and Research Committee of Nueva Ecija University of Science and Technology (2018-008).

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ORIGINAL PAPER

The interaction of synbiotic of the environment and the endoecosystem as one of the mechanisms of action of balneotherapy

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ABSTRACT

Introduction and aim. Drinking mineral waters are one of the environmental factors that affect the condition of the human body. Of particular interest are therapeutic waters of the Naftussya type, which contain autochthonous microbes and organic oil-like substances and can be considered as a kind of ecosystem. On the other hand, gastrointestinal tract also is an ecosystem that associates a resident microbiota and cells of various phenotypes lining the epithelial wall. We assumed that one of the mechanisms of the therapeutic effect of Naftussya water is the interaction of external and internal ecosystems. This article is the first in a series in support of the hypothesis.

Material and methods. The object of clinical-physiological observation were residents of the city of Truskavets' (21 men aged 24-67 years and 8 women 33-76 years) with chronic pyelonephritis in remission. The objects of study: leukocyturia, bacteriuria, components of microbiota, phagocytosis function of neutrophils, leukocytary adaptation index, plasma and urine electrolytes and nitrogenous metabolites.

Results. The weekly use of bioactive Naftussya water from the Opaka deposit causes a favorable normalizing effect on the stool microbiota: it increases the reduced content of *Bifidobacteria* and *Lactobacilli*, instead it reduces the increased content of pathogenic strains of *Escherichia coli*, as well as *Klebsiela* and *Proteus*. Reduction of dysbacteriosis is accompanied by an increase in the reduced bactericidal capacity of blood neutrophils against *E. coli* and *Staphylococcus aureus*, and reduction of bacteriuria and leukocyturia. At the same time, the elevated level of creatinine in the plasma decreases, instead, the decreased levels of sodium and chloride increase. As expected, the daily diuresis and excretion of urea, creatinine, phosphates, calcium, magnesium and chloride increases, but not sodium and uric acid, the concentrations of which in the urine decrease. The described physiologically beneficial effects are interpreted as adaptogenic, which is confirmed by an increase in the reduced leukocyte adaptation index.

Conclusion. The healing effect of Naftussya bioactive water is the result of the interaction of external and internal ecosystems. The next article will consider the role of the nervous and endocrine systems in this interaction.

Keywords. bacteriuria, metabolites, microbiota, Naftussya water, phagocytosis, pyelonephritis

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Introduction

Drinking mineral water, along with fresh water, is one of the environmental factors that affect the condition of the human body. Back in 1975, with the chemical analysis of over 300 mineral waters of the then USSR, organic matter was discovered in all of them without exception. It is shown that for water of one type, the presence of bitumen, naphthenic acids and phenols is typical, while for other types of humic, carboxylic acids and again phenols are characteristic.[1]

Despite this, it is still assumed that the physiological activity of drinking mineral waters is due to their electrolytes, the concentration of which is from 2 to 30 g/L, as well as the trace elements, while the role of organic substances is ignored, apparently because of their relatively insignificant concentration (5-40 mg/L). And only regarding therapeutic waters of the Naftussya type of Ukrainian Carpathians and Podolia as well as similar to them Berezivs'ka, Kala-Alta, Kurgazak, Volzhanka, Serebryanyi klyuch, Munoc waters, which are not formally mineral, because they contain less than 1 g/L of electrolytes, organic substances are considered as active principles.[2-8]

Another important component of the composition of waters this type is autochthonous microflora. In Naftussya water of the Truskavets' field 900 bacterial and yeast cultures were found. Identified bacteria are classified as genera: *Pseudomonas, Bacillus, Nocardia, Corinebacterium, Micrococcus, Brevibacterium*. By type of food bacteria are ammonifying, denitrifying, iron bacteria, oligonitrophilic, hydrocarbon oxidizing, sulfate-reducing, thionic acid bacteria.[3] However, only the last three groups are considered specific, which together with aquifer and filtration water are an attribute of the Naftussya "water-forming triad".[9]

We adduce data by Dats'ko et al., 2008[10] about organic compounds (in mg/L) Naftussya water obtained by Solid Phase Extraction method and mass-spectroscopy by using as Sorbents Tenacle GC 60/80 and Polysorb-2. Paraffins 4.10 and 4.20; monoolefins 1.67 and 1.75; dienes and monocycloolefins 0.84 and 0.85; alkylbenzene 1.55 and 1.54; alkenylbenzene 0.47 and 0.46; esters of aromatic acids 1.32 and 1.33; alkyl phenols 1.14 and 1.14; polyaromatic hydrocarbons 0.077 and 0.059; sulfur-containing connections 0.30 and 0.31; alkylnaphthalenes 0.53 and 0.53; carboxylic (fatty) acids 1.12 and 1.14; unidentified polycyclic aromatic hydrocarbons 0.19 and 0.19; connections required subsquent identification 0.48 and 0.50 respectively. The authors note that approximately 2/3 of the mass of organic substances is leached from the aquifer, and 1/3 are the products of their biotransformation by autochthonous microbes, mainly hydrocarbon oxidizing (60÷500 cells/mL). Sulfate-reducing microbes in the process of vital activity transform sulfate into hydrogen sulfide, which, in turn, is assimilated by thion microbes, transforming again into sulfate. It is interesting that an additional source of polyphenols is fallen leaves, from where they seep through the soil to the aquifer.[11] Therefore, Naftussya water is a unique ecosystem and is quite rightly nominated as «Living Water».[12]

On the other hand, the gastrointestinal tract also is a complex ecosystem that associates a resident microbiota and cells of various phenotypes lining the epithelial wall expressing complex metabolic activities. The resident microbiota in the digestive tract is a heterogeneous microbial ecosystem containing up to 10^{14} colony-forming units (CFUs) of bacteria. The intestinal microbiota plays an important role in normal gut function and maintaining host health.[13-24]

Aim

Based on the above, we assumed that one of the mechanisms of the therapeutic effect of Naftussya water in chronic diseases accompanied by dysbacteriosis, in particular pyelonephritis, is the interaction of external and internal ecosystems. This article is the first in a series in support of the hypothesis.

Material and methods

Ethics approval

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

Participants

The object of clinical-physiological observation were residents of the city of Truskavets' (21 men aged 24-67 years and 8 women 33-76 years) with chronic pyelonephritis in remission.

Study design and procedure

The day before, samples of morning urine and feces was collected, in which was determined the leukocyturia and bacteriuria levels and components of microbiota respectively. Unified methods are applied. Then daily urine was collected, in which was determined the concentration of electrolytes: calcium (by reaction with arsenase III), magnesium (by reaction with colgamite), phosphates (phosphate-molybdate method), chloride (mercury-rhodanidine method), sodium and potassium (flamming photometry); as well as nitric metabolites: creatinine (by Jaffe's color reaction by Popper's method), urea (urease method by reaction with phenolhypochlorite), uric acid (uricase method). The same

metabolic parameters were determined in plasma. The analysis carried out according to instructions Goryachkovskiy, 1998 with the use of analyzers "Reflotron" (BRD) and "Pointe-180" (USA) and corresponding sets of reagents.[25]

Urinary syndrome was assessed by quantitative and quantitative-qualitative levels of bacteriuria and leukocyturia. To qualitatively assess the manifestations of pyelonephritis, a single-point Popovych's[26,27] scale, built on the basis Harrington's desirability function,[28] was used. In particular, bacteriuria over 10^6 CFU/mL is quantified at 0.9 points (strongly expressed), within $(0.3\div1.0)\bullet10^6$ CFU/mL – 0.715 p (more than average, but not strong), 10^5 CFU/mL – 0.5 p (moderately expressed), $(0.2\div0.5)\bullet10^5$ CFU/mL – 0.285 p (weakly expressed), $(0.01\div0.1)\bullet10^5$ CFU/mL – 0.1 p (very weak), less than $0.01\bullet10^5$ CFU/mL - 0 p (absent). Leukocyturia over $60\bullet10^3$/mL – 0.715 p, within $(20\div60)\bullet10^3$/mL – 0.5 p, $(4\div20)\bullet10^3$/mL – 0.285 p, $(2\div4)\bullet10^3$/mL – 0.1 p, less than $2\bullet10^3$/mL – 0 p.

The inclusion criteria were the presence of pronounced urinary syndrome (bacteriuria: 0.285÷0.715 points; leukocyturia: 0.1÷0.5 points) with preservation of functional renal reserve (≥10%), previously assessed by the Gozhenko's method.[29]

In portion of capillary blood counted up leukocytogram (lcg) (eosinophils, stub and segmentonucleary neutrophils, lymphocytes and monocytes) and calculated its adaptation index (Table 1) as well as strain index by Popovych.[30] The second version of the indices, built on the basis of the specified parameters of the authors of the concept of anti-stressor general adaptive reactions of the body, was applied.[31,32]

Table 1. Quantification of general adaptive reaction of the body, second version[30]

Leukocytogram Lymphocytes level, %	General adaptive reaction of body	Eosinophils: 1–4.5 %; stub neutrophils: 3–5.5 %; monocytes: 5–7 %; leukocytes: 4–6 G/l	Eosinophils: <1;>4.5% stub neutrophils: <3; >5.5; monocytes: <5; >7; leukocytes: <4; >6 G/l
<21	Stress	1.22	0.02
21–27	Training	1.46	0.74
28–33	Quiet Activation	1.95	0.98
34–43.5	Heightened Activation	1.70	0.50
≥44	Overactivation	0.26	

Strain index-2 = $[(Eo/2.75\text{-}1)^2 + (SN/4.25\text{-}1)^2 + (Mon/6\text{-}1)^2 + (Leu/5\text{-}1)^2]/4$.

Parameters of phagocytic function of neutrophils estimated as described by Kovbasnyuk.[33] The objects of phagocytosis served daily cultures of Staphylococcus aureus (ATCC N 25423 F49) as typical specimen for Gram-positive Bacteria and Escherichia coli (O55 K59) as typical representative of Gram-negative Bacteria. Take into account the following parameters of Phagocytosis: activity (percentage of neutrophils, in which found microbes – Hamburger's Phagocytic Index, PhI), intensity (number of microbes absorbed one phagocytes – Microbial Count MC or Right's Index) and completeness (percentage of dead microbes – Killing Index, KI). On the basis of the registered partial parameters of phagocytosis, taking into account the content of neutrophils (N) in 1 L of blood, the integral parameter – the BacteriCidal Capacity of Neutrophils – was calculated by the formula Popovych et al, 2003:[26]

$$BCCN\ (10^9\ Bact/L) = N\ (10^9/L)\bullet PhI\ (\%)\bullet MC\ (Bact/Phag)\bullet KI\ (\%)\bullet10^{-4}.$$

After the initial testing, the patients received a one-week course of balneotherapy by Naftussya water (3 ml/kg 1 hour before meals three times a day) from the Opaka deposit, which is located next to the Truskavets' and Skhidnytsya deposits. On the second day after the end of the course, re-testing was conducted.

Statistical analysis

Statistical processing was performed using a software package "Microsoft Excell" and "Statistica 6.4 StatSoft Inc" (Tulsa, OK, USA).

Results

Adhering to the Truskavetsian Scientific School's analytical algorithm, the actual/raw parameters were normalized by recalculation by the equations:

$$Z = 4\bullet(V - N)/(Max - Min) = (V - N)/SD = (V/N - 1)/Cv,$$ where

V is the actual value; N is the normal (reference) value; SD and Cv are the standard deviation and coefficient of variation respectively.

Further, profiles (Fig. 1) of normalized parameters were created, the levels of which differ significantly before and after treatment, as well as several parameters which according to the following discriminant analysis were still recognizable, despite the insignificant value of Student's t criterion.

Fig. 1. Profiles of variables whose normalized levels (Z±SE) are changing under the influence of the Naftussya water

Another approach to quantifying effects is to calculate the direct differences between the final and initial parameters levels of each patient (Fig. 2).

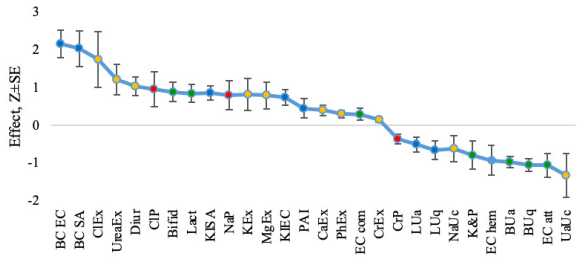

Fig. 2. The effects of the Naftussya water as direct differences of normalized variables (Z±SE)

As you can see, the weekly use of bioactive Naftussya water from the Opaka deposit causes a favorable normalizing effect on the microbiota: it increases the reduced content of Bifidobacteria and Lactobacilli, instead it reduces the increased content of Klebsiela and Proteus as well as pathogenic strains of E. coli (hemolytic and with attenuated enzymatic capacity), while the content of E. coli common increases.

Reduction of dysbacteriosis is accompanied by an increase in the reduced bactericidal capacity of blood neutrophils against E. coli and Staphylococcus aureus, and reduction of bacteriuria and leukocyturia.

At the same time, the elevated level of creatinine in the plasma decreases, instead, the decreased levels of sodium and chloride increase.

As expected, the daily diuresis and excretion of urea, creatinine, phosphates, calcium, magnesium and chloride increases, but not sodium and uric acid, the concentrations of which in the urine decrease.

Thus, the reduced levels of the parameters increase, and the increased ones decrease, as a rule, to normal. That is, there is a normalizing (ambivalence-equilibratory) effect of the Naftussya water as one of the attributes of adaptogensaccording to the good old "law of initial level".[34-36] At the same time, normal levels of some parameters also increase or decrease, albeit slightly. The described physiologically beneficial effects are interpreted as adaptogenic, which is confirmed by an increase in the reduced Popovych's adaptation index.[20] The dynamics of the adaptation index reflects an increase in the share of harmonious (normal) adaptation reactions from 6.9% to 24.2%, instead of a decrease in the share of disharmonious (premorbid) adaptation reactions from 82.8% to 65.5%, however, in 3 patients (10.3%) the pathological reaction of overactivation still remained.

The previously selected variables were further subjected to discriminant analysis[37] with the aim not so much to discover which of them are formally characteristic, but to visualize the integral state of each patient. The forward stepwise program included only 10 variables in the discriminant model (Tables 2 and 3), including those subject to non-significant (t<2,02) effects according to the Student's criterion, while other variables were outside the model, despite significant (*)

Table 2. Discriminant function analysis summary[a]

Variables currently in the model	State (n) and Mean±SE			Parameters of Wilks' Statistics					
	Before (29)	After (29)	Effect (29)	Wilks' Λ	Partial Λ	F-remove (1,47)	p	Tolerancy	Refer Cv SD
Bacteriuria actual, lg CFU/mL	2.11 0.08	1.16 0.17	-0.95 0.15*	0.345	0.999	0.03	0.860	0.159	0 0.98
Bacteriuria qualitative, points	0.5 0.03	0.25 0.04	-0.25 0.04*	0.369	0.934	3.30	0.076	0.148	0 0.24
Leukocyturia qualitative, point	0.2 0.03	0.1 0.02	-0.1 0.04*	0.371	0.928	3.64	0.062	0.341	0 0.15
Klebsiela and *Proteus faeces*, %	18.9 3.5	10.3 2.2	-8.6 4.1*	0.358	0.962	1.85	0.180	0.145	0 11
E. coli common, lg CFU/g	8.17 0.05	8.29 0.05	+0.12 0.06*	0.389	0.886	6.06	0.018	0.169	8.66 0.045
Killing index vs *S aureus*, %	45.2 1.1	52.4 1.6	+7.2 1.6*	0.388	0.887	5.97	0.018	0.717	58.9 0.142
Popovych's adaptation index-2	0.74 0.06	0.93 0.09	+0.19 0.11	0.355	0.971	1.43	0.238	0.820	1.705 0.245
Chloride plasma, mM/L	100.9 1.2	104 1.1	+3.2 1.5*	0.368	0.937	3.18	0.081	0.870	101.5 0.032
Chloride excretion, mM/24 h	172 12	223 20	+50 21*	0.379	0.909	4.73	0.035	0.768	167.5 0.172
Sodium Urine concentration, mM/L	118 4	104 6	-14 8	0.398	0.865	7.32	0.009	0.792	110 0.211

[a] Step 10, N of vars in model: 10; Grouping: 2 grps; Wilks' Lambda: 0,3446; approx. $F_{(11)}$=8,9; $p<10^{-6}$; in each column, the first line is the average, the second – SE; in norm column – the average and Cv or SD. The "Effect" and "Norm" columns are not the result of discriminant analysis

Table 3. Summary of stepwise analysis of discriminant variables ranked by criterion Λ

Variables currently in the model	F to enter	p-level	Λ	F-value	p
Bacteriuria actual, lg CFU/mL	25	10^{-5}	0.692	25	10^{-5}
Killing index vs *S. aureus*, %	12.8	0.001	0.561	21.6	10^{-6}
Sodium urine concentration, mM/L	4.52	0.038	0.517	16.8	10^{-6}
Leukocyturia qualitative, point	3.45	0.069	0.486	14	10^{-6}
Popovych's adaptation index-2	3.36	0.073	0.456	12.4	10^{-6}
Chloride excretion, mM/24 h	4.06	0.049	0.423	11.6	10^{-6}
Bacteriuria qualitative, points	2.12	0.151	0.405	10.5	10^{-6}
E. coli common, lg CFU/g	3.14	0.083	0.381	9.96	10^{-6}
Chloride plasma, mM/L	3.04	0.087	0.358	9.56	10^{-6}
Klebsiela&Proteus faeces, %	1.85	0.18	0.345	8.94	10^{-6}

changes (Tables 4 and 5). On the face of it, the Wilks' and Student's statistics do not match completely.

On the basis of the raw coefficients and constant (Table 6), the individual values of the canonical discriminant roots were calculated with the following visualization in Fig. 3.

The level of the root after the course of using the Naftussya water in almost everyone patients, with two exceptions, is lower than the initial level to one degree or another. The variability of the body's responses to adaptogens and stressors is due to individual reactivity.[38-40] This reflects both increasing levels of variables represented in the root inversely and decreasing levels of variables that are positively correlated with the root (Table 7).

Table 4. Microbiota and Phagocytosis variables currently not in the discriminant model

Variables currently not in the model	State (n) and Mean±SE			Parameters of Wilks' Statistics					
	Before (29)	After (29)	Effect (29)	Wilks' Λ	Partial Λ	F to enter	p-level	Tolerancy	Refer Cv SD
Leukocyturia actual, lg L/mL	3.56 0.11	3.24 0.1	-0.32 0.13*	0.344	0.998	0.11	0.748	0.135	3 0.21
Bifidobacteria faeces, lg CFU/g	4.83 0.21	5.84 0.2	+1.01 0.29*	0.344	0.997	0.16	0.690	0.406	6.94 0.164
Lactobacilli faeces, lg CFU/g	5.38 0.25	6.62 0.25	+1.24 0.35*	0.344	0.997	0.14	0.707	0.417	8.1 0.179
E. coli attenuated faeces, %	75.6 3.4	57.2 4.8	-18.4 5.6*	0.339	0.984	0.76	0.388	0.384	17.4 1
E. coli hemolytic faeces, %	33.8 8.1	10.8 4.7	-23.0 9.9*	0.344	0.997	0.15	0.704	0.618	0 **25**
Killing index vs *E. coli*, %	40.1 1.2	47.3 1.7	+7.2 2.0*	0.341	0.989	0.51	0.477	0.378	62 0.156
Bactericidity vs *S. aureus*, 10^9 bacteria/L	81 3	103 5	+22 5	0.345	1	0.00	0.983	0.404	106 0.1
Bactericidity vs *E. coli*, 10^9 Bacteria/L	74 2	95 4	+21 4	0.343	0.995	0.23	0.633	0.080	99 0.1

Table 5. Metabolic variables currently not in the discriminant model

Variables currently not in the model	State (n) and Mean±SE			Parameters of Wilks' Statistics					
	Before (29)	After (29)	Effect (29)	Wilks' Λ	Partial Λ	F to enter	p-level	Tolerancy	Refer Cv
Diuresis, L/24h	1.61 0.13	2.01 0.13	+0.40 0.10*	0.344	0.998	0.11	0.740	0.398	1.4 0.274
Uric acid urine concentration, mM/L	2.52 0.34	1.82 0.1	-0.70 0.31*	0.339	0.984	0.72	0.399	0.669	2.14 0.25
Urea excretion, mM/24 h	462 38	566 41	+104 35*	0.344	0.999	0.07	0.798	0.411	458 0.186
Creatinine excretion, mM/24 h	7.06 0.6	8.87 0.8	+1.81 0.82*	0.344	0.997	0.13	0.717	0.776	11 0.3
Calcium excretion, mM/24 h	3.74 0.42	5.54 0.57	+1.80 0.62*	0.343	0.996	0.2	0.658	0.441	4.38 0.214
Phosphate excretion, mM/24 h	18.3 1.7	26.2 2.7	+7.9 2.4*	0.343	0.996	0.18	0.672	0.612	25.2 0.294
Magnesium excretion, mM/24 h	4.00 0.31	4.84 0.39	+0.84 0.36*	0.343	0.996	0.19	0.664	0.553	4.1 0.256
Potassium excretion, mM/24 h	58.3 5.7	72.5 6.0	+14.2 7.4	0.343	0.996	0.19	0.669	0.766	65 0.269
Creatinine plasma, μM/L	87.6 2.9	82.6 1.8	-5.0 1.9*	0.342	0.993	0.34	0.564	0.77	77 0.17
Sodium plasma, mM/L	141.8 1.5	145.8 1.4	+4.0 1.9*	0.344	0.999	0.05	0.821	0.426	145 0.034

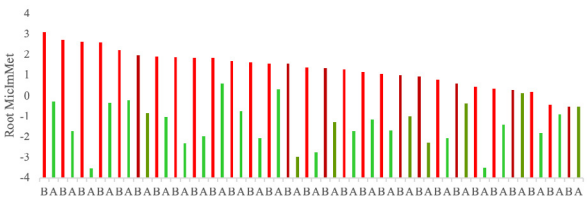

Fig. 3. Individual values (M±SE) of discriminant Root before (B) and after (A) course of intake of the Naftussya water. Women are highlighted in shades of colors

Table 6. Standardized and raw coefficients and constant for discriminant variables

	Coefficients	
Variables	Standardized	Raw
Bacteriuria actual, lg CFU/mL	0.08	0.11
Killing index vs *S. aureus*, %	-0.49	-0.069
Sodium urine, mM/L	0.51	0.018
Leukocyturia qualitative, points	0.567	3.859
Popovych's adaptation index-2	-0.234	-0.561
Chloride excretion, mM/24 h	-0.426	-0.005
Bacteriuria qualitative, points	0.823	4.272
E. coli com., lg CFU/g	1.015	3.839
Chloride plasma, mM/L	-0.333	-0.053
Klebsiela&Proteus faeces, %	0.633	0.04
	Constant	-26.29
	Eigenvalue	1.90
Squared Mahalanobis Distance=7.35; $F_{(11)}$=8.9; $p<10^{-6}$		
Canonical R=0.810; Wilks' Λ=0.3446; $\chi^2_{(10)}$=54; $p<10^{-6}$		

Table 7. Effects of Naftussya water as differences between levels (Z±SE) after and before treatment

Clusters and Variables	R	Before (29)	After (29)	Effect (29)
Bacteriuria actual	0.484	+2.16±0.09	+1.18±0.17	-0.97±0.15*
Bacteriuria qualitative	0.476	+2.08±0.13	+1.05±0.17	-1.04±0.16*
Leukocyturia qualitative	0.245	+1.33±0.21	+0.69±0.15	-0.65±0.24*
Leukocyturia actual		+0.88±0.18	+0.38±0.16	-0.50±0.20*
Klebsiela&Proteus faeces	0.201	+1.72±0.32	+0.93±0.20	-0.78±0.37*
E. coli hemolytic faeces		+1.35±0.32	+0.43±0.19	-0.92±0.39*
E. coli attenuated faeces		+3.34±0.19	+2.29±0.27	-1.05±0.32*
Sodium urine concentration	0.185	+0.36±0.18	-0.25±0.26	-0.61±0.35
Uric acid urine concentration		+0.71±0.64	-0.61±0.19	-1.32±0.58*
Creatinine Plasma		+1.34±0.20	+0.99±0.13	-0.35±0.13*
Killing index vs *S. aureus*	-0.373	-1.64±0.13	-0.78±0.19	+0.86±0.19*
Killing index vs *E. coli*		-2.27±0.13	-1.52±0.18	+0.75±0.21*
Bactericidity vs *S. aureus*		-2.32±0.30	-0.28±0.45	+2.04±0.47
Bactericidity vs *E. coli*		-2.55±0.20	-0.39±0.40	+2.16±0.36
Bifidobacteria faeces		-1.86±0.19	-0.97±0.18	+0.89±0.26*
Lactobacilli faeces		-1.87±0.17	-1.02±0.17	+0.85±0.24*
E. coli common	-0.163	-1.26±0.13	-0.96±0.13	+0.30±0.16
Chloride excretion	-0.212	+0.16±0.41	+1.91±0.69	+1.75±0.73*
Chloride plasma	-0.187	-0.19±0.37	+0.78±0.34	+0.97±0.46*
Sodium plasma		-0.65±0.31	+0.15±0.28	+0.80±0.38*
Urea excretion		+0.05±0.45	+1.27±0.48	+1.22±0.41*
Diuresis		+0.56±0.33	+1.60±0.33	+1.04±0.25*
Potassium excretion		-0.39±0.33	+0.43±0.35	+0.82±0.42
Magnesium excretion		-0.09±0.30	+0.71±0.37	+0.80±0.35*
Calcium excretion		+0.85±0.10	+1.27±0.13	+0.41±0.14*
Phosphate excretion		+0.73±0.07	+1.04±0.11	+0.31±0.1*
Creatinine excretion		+0.64±0.05	+0.81±0.07	+0.16±0.07*
Popovych's adaptation index-2	-0.169	-2.31±0.15	-1.85±0.22	+0.46±0.26

The accuracy of the retrospective classification of effects by calculating individual classification functions based on its coefficients and constants (Table 8) is 91,4% (two and three mistakes before and after treatment responsibility) (Table 9).

Discussion

It seems to us that the primary effect of Naftussya bioactive water is a significant increase (though without normalization) of a significantly reduced content in the microbiota of *Bifidobacteria* and *Lactobacilli*. These beneficial bacteria and the cells lining the gastrointestinal epithelium are two partners that properly and/or synergistically function

to promote an efficient host system of defence. One of the basic physiological functions of the resident microbiota is that it functions as a microbial barrier against microbial pathogens. The mechanisms by which the species of the microbiota exert this barrier effect are as follows: bacterial interference, acid and pH effects, antimicrobial substances, immunomodulation.[13,14,16]

Table 8. Coefficients and constants of classification functions

	Clusters	Before	After
Variables		**0.500**	**0.500**
Bacteriuria actual, lg CFU/mL		-8.630	-8.928
Killing index vs *S. aureus*, %		-1.761	-1.575
Sodium urine concentration, mM/L		0.870	0.821
Leukocyturia qualitative, point		-14.88	-25.34
Popovych's adaptation index-2		51.64	53.16
Chloride excretion, mM/24 h		-0.116	-0.103
Bacteriuria qualitative, points		229.9	218.3
Escherichia coli com., lg CFU/g		676.6	666.2
Chloride plasma, mM/L		-0.018	0.127
***Klebsiela&Proteus faeces*, %**		9.923	9.815
	Constants	-2925	-2853

Table 9. Classification matrix

Group	Rows: Observed classifications Columns: Predicted classifications		
	Percent correct	B p=0.50000	A p=0.50000
Before	93.1	27	2
After	89.7	3	26
Total	91.4	30	28

With regard to the cohort of patients with chronic pyelonephritis observed by us, the data that pyelonephritogenic Escherichia coli was highly suppressed by Lactobacillus rhamnosus and both *Bifidobacteria* strains are of particular interest.[15] This gives us reason to assume that inhibition of the growth of pyelonephritogenic *E. coli* in the intestine by probiotics reduces its translocation to the kidneys via lymph and/or blood. In addition, circulating bacteria are destroyed by neutrophils, whose bactericidal capacity increases significantly. The result is a decrease in bacteriuria, as well as leukocyturia as a marker of pyelonephritis.

The mechanism of the increase in the bactericidal capacity of neutrophils that we discovered requires a separate discussion. We know that feeding of mice with *Bifidobacteria* or *Lactobacilli* strains resulted in significant increase in the phagocytic activity of peripheral blood leukocytes and peritoneal macrophages compared to that in control mice. Administration of *Lactobacillus rhamnosus* to healthy volunteers is followed by a relative increased proportion of polymorphonuclear cells showing phagocytic activity. The phagocytic capacity of polymorphonuclear and mononuclear phagocytes in elderly subjects was also elevated after *Bifidobacter lactis* consumption.[14]

Thus, Naftussya bioactive water increases the bactericidal ability of neutrophils through the mediation of gut probiotics, that is, it manifests itself as a prebiotic.

At the same time, direct effects on phagocytes (as well as others immunocytes) through their aryl hydrocarbon receptors (AhR) are quite real.[41,42]

This assumption is based on the fact that cells within the immune system, such as macrophages, dendritic cells, granulocytes, natural killer cells and lymphocytes (T cells and B cells), express AhR. The expression of the AhR in a majority of immune cell types and the expression of multiple xenobiotic- or dioxin-responsive elements (XREs/DREs) in the promoter region of many genes that regulate the immune response demonstrates the importance of this receptor in immunological processes.[43-47] Researchers have discovered a wide range of AhR ligands, both natural and synthetic, including environmental contaminants, dietary compounds, microbial byproducts, and endogenous mediators. Typically components of environmental pollutants: polycyclic aromatic hydrocarbons such as benzo(a)pyrene, anthracene, and 3-methylcholanthrene as well as halogenated aromatic hydrocarbons such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls.[48]

On the other hand, among the organic substances of Naftussya water probable AhR ligands are present (alkylbenzene, alkenylbenzene, esters of aromatic acids, alkyl phenols, alkylnaphthalenes, polycyclic aromatic hydrocarbons). By the way, probiotics themselves are capable of producing an endogenous ligand of AhR 6-Formylindolo[3,2-b] Carbazole (FICZ).[49]

Ubiquitous AhRs are also expressed by neurons, so a neuro-immune mechanism of phagocytosis activation is also possible, the analysis of which will be the topic of the next article.[50,51] So let us limit ourselves to the announcement: Naftussya bioactive water has a modulating effect on the autonomic and central nervous systems, which, in turn, activate phagocytosis, as well as modulate cellular and humoral immunity.[33,52-59]

Conclusion

The healing effect of Naftussya bioactive water is the result of the interaction of external and internal ecosystems. Naftussya bioactive water increases the bactericidal ability of neutrophils through the mediation of gut probiotics, that is, it manifests itself as a prebiotic. At the same time, direct effects on phagocytes (as well as others immunocytes) through their aryl hydrocarbon receptors are quite real. The next our article will consider the role of the nervous and endocrine systems in this interaction.

In conclusion, we want to put forward the concept of Naftussya bioactive water as synbiotic.

It is known that prebiotics are a group of biological nutrients that are capable of being degraded by microflora in the gastrointestinal tract, primarily *Lactobacilli* and *Bifidobacteria*. When prebiotics are ingested, either as a food additive or as a supplement, the colonic microflora degrade them, producing short-chain fatty acids (SCFA),

which are simultaneously released in the colon and absorbed into the blood circulatory system. The two major groups of prebiotics that have been extensively studied in relation to human health are fructo-oligosaccharides (FOS) and galactooligosaccharides (GOS). The candidature of a compound to be regarded as a prebiotic is a function of how much of dietary fiber it contains.[60] Prebiotics are either natural or synthetic non-digestible (non-) carbohydrate substances that boost the proliferation of gut microbes. Undigested FOS in the large intestine are utilised by the beneficial microorganisms for the synthesis of short-chain fatty acids for their own growth.[61] Generally, non-digestible carbohydrates are considered prebiotic. However, our data indicate the ability of another group of substances to boost the proliferation of gut microbes.

In May 2019, the International Scientific Association for Probiotics and Prebiotics (ISAPP) convened a panel of nutritionists, physiologists and microbiologists to review the definition and scope of synbiotics. The panel updated the definition of a synbiotic to "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host". The panel concluded that defining synbiotics as simply a mixture of probiotics and prebiotics could suppress the innovation of synbiotics that are designed to function cooperatively. Requiring that each component must meet the evidence and dose requirements for probiotics and prebiotics individually could also present an obstacle. Rather, the panel clarified that a complementary synbiotic, which has not been designed so that its component parts function cooperatively, must be composed of a probiotic plus a prebiotic, whereas a synergistic synbiotic does not need to be so. A synergistic synbiotic is a synbiotic for which the substrate is designed to be selectively utilized by the co-administered microorganisms. This Consensus Statement further explores the levels of evidence (existing and required), safety, effects upon targets and implications for stakeholders of the synbiotic concept.[62]

As follows from the above, Naftussya bioactive water fully meets the requirements for synbiotics.

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Author contributions

Conceptualization, I.P.; Methodology, I.P.; Software, I.P. and W.Z.; Validation, I.P. and W.Z.; Formal Analysis, I.P. and W.Z.; Investigation, V.F., H.K., I.B., O.V., I.K., O.L. and T.S.; Resources, W.Z.; Data Curation, I.P. and W.Z.; Writing – Original Draft Preparation, I.P. and W.Z.; Writing – Review & Editing, I.P. and W.Z.; Visualization, I.P. and W.Z.; Supervision, I.P. and W.Z.; Project Administration, I.P. and W.Z.; Funding Acquisition, V.F., H.K., I.B., O.V., I.K., O.L. and T.S.

Conflicts of interest

The authors declare no competing interests.

Data availability

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

Ethics approval

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

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ORIGINAL PAPER

The needs of children questionnaire – Turkish cross-cultural adaptation

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ABSTRACT

Introduction and aim. Determining the needs of children hospitalized for treatment is important in terms of identifying children who are more at risk and developing support systems for the child and the family. We aimed to test the validity and reliability of the Turkish needs of children questionnaire (NCQ) and cross-culturally adapt it to the Turkish language.

Material and methods. This cross-sectional study was conducted using a total of 160 children aged 5-16 years who were hospitalized between May 2021 and May 2022. The linguistic, content validity, construct validity, and internal consistency of NCQ were assessed.

Results. NCQ had a four-factor structure consisting of two categories and explained 76% of the total variance. The Cronbach's alpha coefficients were 0.748, 0.799, 0.821, and 0.802 for the subscales of Caring, Information, Activities, and Relationships, respectively; and 0.893 for the total score. Inter-item correlations ranged from 0.149 to 0.702 ($p<0.05$).

Conclusion. NCQ has a high level of validity and reliability for Turkish society. Turkish children aged 5 to 16 years were able to comprehend this instrument and express their needs and feelings about their hospitalization period.

Keywords. children, hospital, reliability, Turkey, validity

Introduction

Physical, behavioral and psychological differences of children, their continuing growth and development, and their need for adults to meet their basic needs even when they are healthy increase the importance of healthcare provided by pediatric health professionals in determining and meeting children's needs when they are hospitalized.[1,2] Disease-and-treatment-related variables, child's own characteristics and familial factors are significant determinants on their compliance with hospital conditions and their level of psychological exposure to these conditions.[3,4] According to the developmental biopsychosocial model, biological factors, developmental characteristics, psychological factors, risk factors associated with the disease, and social factors have significant roles in the child's reactions to illness and treatment.[5]

Hospital setting is a foreign setting for a hospitalized child. The child has no information about the hospital, health professionals, the equipment used at the hospital, and procedures to be applied. In addition, in hospitalization process, school-age children, just as other children, have various needs such as not falling behind in their academic life, doing activities, playing games, getting information about the procedures applied, having their parents by their side, establishing effective communication with them, and meeting their emotional needs. Meeting these needs is important in terms of supporting the child's development and improvement of his/her individuality.[6–8] By determining the needs of children receiving inpatient treatment, children who are at higher risk can be recognized more easily and relevant support systems can be developed for them and their family.

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Thus, traumatic effects of illness and hospitalization on children can be minimized.[9] Due to changing social and cultural values over time, advancing treatment modalities, and increasing technological opportunities, this subject is up-to-date and open to change in every period and should be understood well enough to meet the needs of hospitalized children.[10] By determining needs of children, an optimal efficiency can be obtained in pediatric treatment and care, improving their well-being.[11] To maximize children's positive healthcare experiences, a questionnaire is required to assess whether the quality of care in hospitals is consistent with what children perceive as important and necessary.[12]

Self-report is the best assessment method in children and is considered the gold standard.[13,14] In today's modern world, children still have high levels of anxiety/fear/psychosocial problems due to hospitalization, leading researchers to discover new assessment methods for children.[11] There is a need for self-report scales for children, considering their developmental characteristics.[12]

The needs of children questionnaire (NCQ) was developed by Foster et al. in English language.[15] The scale was developed in order to determine the psychosocial, physical, and emotional needs of school-age children (aged 5-16 years) hospitalized in pediatric services based on self-report. Psychometric properties of the questionnaire were evaluated after school-age children completed their needs in four pediatric categories in Australia and New Zealand. These categories are Caring, Information, Activities, and Relationships. The NCQ was developed between 2013-2017 in three stages. Content adequacy evaluation, questionnaire management, factor analysis, internal consistency evaluation, and construct validity were performed. The NCQ was firstly tested by Foster et al. in Australia and New Zealand.[15] The scale was finalized as a 16-item 4-category scale. The Cronbach's alpha coefficient for combined samples was 0.93.

It reports as easy to use and useful. In this context, it is the first questionnaire in which the needs of hospitalized children are determined and their fulfillment is evaluated.[15] To the best of our knowledge, there is no easy-to-use scale in Turkish based on self-report of children hospitalized in pediatric services by which their psychosocial, physical, and emotional needs are evaluated. A tool that is culturally and developmentally appropriate, valid, and reliable can contribute to the determination of the needs of Turkish children hospitalized in pediatric services.

Aim

We aimed to cross-culturally adapt the NCQ that is used to determine the psychosocial, physical and emotional needs of school-age children based on their own self-reports into the Turkish and test the validity and reliability of its Turkish version. The research questions:

- Is the Turkish version of the Needs of Children Questionnaire (NCQ) instrument a valid instrument?
- Is the Turkish version of the Needs of Children Questionnaire (NCQ) instrument a reliable instrument?

Material and methods

Ethical approval

An ethical approval was obtained from an ethics committee of a university (IRB number: 2021-SBB-0249, Decision no: 9, Date: 31.05.2021). Permission was obtained from Mandie Foster, who developed the scale, via e-mail to use the scale in the study. We obtained a written consent from parents of the children included in the study.

Participants

The cross-sectional study study was conducted with the participation of children aged between 5-16 years who were hospitalized in Bartin Obstetrics and Pediatrics Hospital located in the West Black Sea region of Turkey. In scale improvement studies, the sample size should be 5-10 times of the total number of scale items used in the study.[16] We used normative sample in this current study. The normative sample is the sample from which norms are obtained and consists only of a part of individuals from a reference population. The reference population refers to a larger group of people, to whom the analytic sample is being compared.[17] Therefore, as the NCQ consists of 16 items, a total of 160 children (other than those used in the pre-application) who met the study inclusion criteria were included in the sample. Study inclusion criteria were as follows: (1) being a child aged between 5-16 years old who can communicate in Turkish well and (2) being hospitalized for more than 24 hours (3) agreeing to participate in the study (4) having a parental approval to participate in the study.

Data collection

We collected data after the children and the parents were informed about the purpose of the study and the confidentiality of the data. The children were asked to complete the questionnaire on their last day in hospital. The Descriptive Information Form and The Needs of Children Questionnaire were the data collection tools.

Descriptive information form

This form includes questions about children's age, gender, length of hospital stay, chronic disease, hospitalized clinic, age and education level of caregiver.

The needs of children questionnaire (NCQ)

It consists of 16 items and two subscales: importance and fulfilment. This is a three-point Likert type scale, scoring as 3=very important/always, 2=important/sometimes, and 1=not important/never. Scores ob-

tained for each item are summed up to find total scale score. A higher scale score indicates greater perceived importance and fulfilment.

Cross-cultural adaptation

We used the guide for the cross-cultural adaptation of self-report scales. Our steps were translation, semantic (semantic) annotations, expert panel, pilot study and cognitive review, having prefinal form and adaptation process.[16,18]

Translation

The scale was translated by two bilingual (fluent in Turkish and English) translators independently from the study. Each of the translators performed this process separately. Later, the translators gathered their own translations and exchanged ideas until they came up with a common product. Two translators then translated the questionnaire back into its English, completely blind to the original version.

Back translation

The back translations were produced by two persons who able to speak both languages (Turkish and English) and are non-experts. The reason for applying this method is to find problematic words and to prevent inferences that professional translators can make. When consensus was reached, the draft scale was produced for the next step.[16]

Synthesis

The two translators came together to synthesize the results of the translations. Starting from the original form, in addition to the translations of the first translator and the second translator, first a synthesis of these translations was made (a co-translation was produced). In the process, consensus was achieved on each of the issues addressed and how they were resolved, with a written report carefully documenting the synthesis process.[16]

Expert committee review

The scale was presented to expert opinion for scope and content validity. Nine expert opinions, including four faculty members from the department of Pediatric Nursing, three faculty members from the department of Psychiatry Nursing, one pediatrics specialist, and one child development specialist, were taken. The experts were shown the original and draft forms of the scale and they were asked to score the items between 1 (not relevant) and 4 (highly relevant). The consistency between expert opinions was evaluated. The Lawshe content validity index (CVI) was used for the item-level and the scale-level CVI of NCQ.[19] The experts found the Turkish and English forms appropriate. The language experts evaluated the final form of the scale.

Pretesting

The last step of the adaptation was pilot study.[16] In such studies, it is enough to collect data from 10-15 people for the pre-application.[16,20] Upon expert opinion, a pilot study was conducted on 20 children aged between 5-16 years in order to check the children's comprehension of scale items. Each child filled the questionnaire and was asked to express what the children understood was meant by each item. The children in the pilot study stated that the scale was easy and understandable. Thus, no changes have been made in the Turkish version of the scale, and the researchers decided to apply the questionnaire to the study sample.

Data evaluation

Frequencies and percentages, arithmetic means, and medians were used for the descriptive statistics. We used the IBM SPSS Version 22.0 (Armonk, NY, USA) package program and AMOS Graphics to test internal consistency and content and construct validity. Content validity was evaluated by CVR and CVI. Validity analyzes were performed with the exploratory factor analysis (EFA). The suitability of the sample size to start the analysis was decided by Bartlett's Test of Sphericity and Keiser-Mayer-Olkin (KMO). Varimax rotation was used in EFA. The Cronbach's alpha coefficient was used for internal consistency. The statistical significance of the results was determined in a 95% confidence interval, and $p<0.05$ was accepted as statistically significant.

Results

Demographic variables

Most of the children were between the ages of 8-10 (44.5%) years and girls (57.5%). In addition, most of them hospitalized for 1-2 days (61.2%) and in medical clinics (72.5%). The mean age of the children was 10.03±1.98 (median=10) (Table 1).

Table 1. Demographic characteristics of children

Demographic variables	Mean±SD	Median
Age	10.03±1.98	10
	n	**%**
Age		
5–7 years	35	21.8
8–10 years	71	44.5
11–16 years	54	33.7
Gender		
Girl	92	57.5
Boy	68	42.5
Length of stay		
1–2 days	98	61.2
3–4 days	38	23.7
5–7 days	24	15.1
>7 days	0	0
Hospitalized clinical unit		
Medical	116	72.5
Surgical	44	27.5

Content validity

Nine experts were consulted for the content validity of the NCQ. Considering the number of experts as nine, the minimum CVR should be 0.78.[19] By taking the average of total CVRs for all items, the CVI was calculated as 0.78. Considering CVI=∑CVR/Number of Items and as provided CVI=CVR, the content validity of the scale was statistically significant (Table 2).

Table 2. Content validity results

Items	Content validity ratios	Content validity index
To know I am safe and well looked after	100%	78%
To not see other children sad or upset	100%	
To feel the staff care about me	100%	
To have mum, dad or my family help care for me	100%	
That staff talk to me about the medicines I am having	100%	
That staff tell me my test results	100%	
To talk about how my illness may affect me	78%	
To have staff show me how the machines work	100%	
To get back to school	100%	
To have books to read	100%	
To have special treats after a test (presents)	100%	
To be able to do arts and crafts	100%	
To be able to go to the playroom	78%	
That I choose when I have visitors (family and/or friends)	100%	
To have the same nurse or doctor care for me	78%	
That staff listen to me	100%	

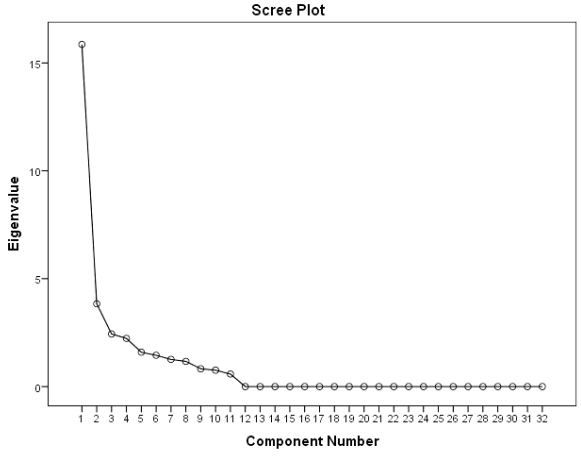

Fig. 1. Slope of scree plot

Construct validity

The Kaiser Meyer-Olkin (KMO) coefficient was found as 0.774 (Table 3). High values of KMO (more than 0.7) generally indicate that a factor analysis may be useful with the data.[21] A factor analysis was performed as the KMO value was higher than 0.70 (acceptable value).[21]

According to the explanatory factor analysis (EFA), the NCQ was found to have four factors with an eigenvalue above 1 (Table 3, Figure 1). The eigenvalue of the first factor was 15.85 and the variance it explained was 49.55; the eigenvalue of the second factor was 3.83, the variance it explained was 11.98; the eigenvalue of the third factor was 2.43 and the variance it explained was 7.62; and the eigenvalue of the fourth factor was 2.23 and the variance it explained was 6.97. The total variance explained was 76.13. It is sufficient for total vari-

Tablo 3. Exploratory factor analysis: pattern matrix

Items	Factors				Kaiser-Meyer-Olkin Measure
	Caring	Information	Activities	Relationships	
To know I am safe and well looked after	0.76				0.77
To not see other children sad or upset	0.77				
To feel the staff care about me	0.73				
To have mum, dad or my family help care for me	0.66				
That staff talk to me about the medicines I am having		0.78			
That staff tell me my test results		0.72			
To talk about how my illness may affect me		0.77			
To have staff show me how the machines work		0.77		0.56	
To get back to school		0.61			
To have books to read			0.4		
To have special treats after a test (presents)			0.67		
To be able to do arts and crafts			0.65		
To be able to go to the playroom			0.66		
That I choose when I have visitors (family and/or friends)				0.57	
To have the same nurse or doctor care for me				0.62	
That staff listen to me				0.55	
Eigenvalue	15.85	3.83	2.43	2.23	
Explained variance	49.55	11.98	7.62	6.97	
Total variance explained	49.55	61.53	69.15	76.13	

Table 4. Item-total score correlations and internal consistency results

Items	Factors			
	Caring	Information	Activities	Relationships
To know I am safe and well looked after	0.60			
To not see other children sad or upset	0.54			
To feel the staff care about me	0.50			
To have mum, dad or my family help care for me	0.51			
That staff talk to me about the medicines I am having		0.62		
That staff tell me my test results		0.58		
To talk about how my illness may affect me		0.59		
To have staff show me how the machines work		0.65		
To get back to school		0.53		
To have books to read			0.57	
To have special treats after a test (presents)			0.62	
To be able to do arts and crafts			0.64	
To be able to go to the playroom			0.72	
That I choose when I have visitors (family and/or friends)				0.6
To have the same nurse or doctor care for me				0.74
That staff listen to me				0.62
Cronbach's alpha (factors)	0.74	0.79	0.82	0.8
Cronbach's alpha (total)			0.89	

ance explained in multifactorial structures to vary between 40% and 60%. Table 2 shows the factor loadings of scale items according to EFA.

Reliability

The NCQ had high internal consistency (the Cronbach's alpha coefficients were 0.74, 0.79, 0.82, and 0.80 for the subscales of Caring, Information, Activities, and Relationships, respectively; and 0.893 for the total scale). Item-total score correlations of the NCQ varied between 0.53 and 0.82 (Table 4). Inter-item correlations of the NCQ were ranged from 0.15-0.70 ($p<0.05$) (Table 5).

Discussion

This study aimed to cross-culturally adapt NCQ and assess the Turkish validity and reliability of NCQ, which was developed to determine the needs of hospitalized children. NCQ had four-factor structure consisting of two categories and explained 76% of the total variance. NCQ showed high internal consistency (the Cronbach's alpha coefficients were 0.74, 0.79, 0.82, and 0.80 for the subscales of Caring, Information, Activities, and Relationships, respectively; and 0.89 for the total scale). Item-total score correlations of the NCQ varied between 0.53 and 0.82. Inter-item correlations of the NCQ were ranged from 0.15-0.70. Inter-item correlations values to be acceptable, must be greater than 0.30 and less than 0.80. Inter-item correlation values between 0.15 to 0.50 depicts a good result. lower than 0.15 means items are not correlated well.[22]

The construct validity of the NCQ was assessed in this study, performing EFA. Performing EFA is essential for testing construct validity in scale adaptation and development studies.[23] As a result of EFA, the NCQ was found to have a four-factor structure, explaining 76% of the total variance.

In this study, the item total score correlations of the NCQ ranged from 0.53 to 0.82. Item-total score correlation gives information about whether the item measures the quality measured by the remaining items of the scale. The lower the total score correlation value of the item, the lower its share in the scale.[24] Item-total score correlation coefficient should have a positive value and be greater than +0.20. Items that do not fulfill this condition should be removed from the scale and the remaining items and the reliability of the scale should be checked again.[25] Foster et al. found the item-total score correlations of the NCQ between 0.50 and 0.77.[15] In this study, the item-total score correlations of the NCQ were found to be higher than those determined by Foster et al.[15]

Cronbach's alpha coefficient was used to determine the internal consistency of the NCQ. In this study, the Cronbach's alpha coefficients were found to be 0.74, 0.79, 0.82, and 0.80 for the subscales of Caring, Information, Activities, and Relationships, respectively; and 0.89 for the total scale. These values suggest that the NCQ has high reliability.[26] The higher the Cronbach's alpha coefficient, the more compatible the items in the scale and the more they collaborate to measure the same feature.[27] Foster et al. reported the Cronbach's alpha coefficients as 0.41, 0.47, 0.74, and 0.47 for the subscales of caring, information, activities, and relationships, respectively; and 0.69 for the total scale.[15]

Parallel forms reliability, one of the methods used for scale reliability, can be used when an alternative or equivalent form of the tested scale is available or created.[24] In this study, no scale was used as a parallel form to the NCQ. In Turkey, there is no scale to determine the psychosocial, physical and emotional needs of children based on their self-reports. Foster et al. also used no parallel form in the original study of the scale.[15]

In this study, most of the children had a short hospital stay (1-2 days). Most of the self-report measures in children were performed using children with chronic diseases.[28,29] Therefore, a time interval is needed for

Table 5. Inter-item correlation matrix[a]

	ACT1	ACT2	ACT3	ACT4	INF1	INF2	INF3	INF4	INF5	REL1	REL2	REL3	CAR1	CAR2	CAR3	CAR4
ACT1	1															
ACT2	0.53**	1														
ACT3	0.41**	0.51**	1													
ACT4	0.48**	0.55**	0.51**	1												
INF1	0.46**	0.56**	0.5**	0.7**	1											
INF2	0.26**	0.23**	0.24**	0.36**	0.49**	1										
INF3	0.24**	0.3**	0.15*	0.36**	0.35**	0.59**	1									
INF4	0.29**	0.34**	0.22**	0.32**	0.46**	0.27**	0.40**	1								
INF5	0.68**	0.62**	0.4**	0.49**	0.53**	0.32**	0.32**	0.31**	1							
REL1	0.4**	0.52**	0.37**	0.55**	0.59**	0.43**	0.46**	0.45**	0.53**	1						
REL2	0.56**	0.63**	0.31**	0.5**	0.53**	0.36**	0.34**	0.42**	0.65**	0.53**	1					
REL3	0.53**	0.53**	0.41**	0.48**	0.46**	0.26**	0.24**	0.29**	0.68**	0.4**	0.56**	1				
CAR1	0.53**	0.54**	0.51**	0.55**	0.56**	0.23**	0.3**	0.34**	0.62**	0.52**	0.63**	0.53**	1			
CAR2	0.41**	0.51**	0.50**	0.51**	0.5**	0.24**	0.15*	0.22**	0.4**	0.37**	0.31**	0.41**	0.51**	1		
CAR3	0.48**	0.55**	0.51**	0.5**	0.7**	0.36**	0.36**	0.32**	0.49**	0.55**	0.5**	0.48**	0.55**	0.51**	1	
CAR4	0.46**	0.56**	0.50**	0.70**	0.51**	0.49**	0.35**	0.46**	0.53**	0.59**	0.53**	0.46**	0.56**	0.50**	0.70**	1

[a] ACT – activities; CAR – caring; INF – information; REL – relationships; RES – resources; * $p<0.05$, ** $p<0.01$

test-retest applications. In the retests performed in a brief time, participants can remember their previous answers, thus affecting the reliability of the scale negatively. A reliability study needs a time interval ranging from 1 to 24 weeks.[30,31] In this study, children had short-term hospitalizations due to acute illnesses. It would therefore be unethical to assess the test-retest reliability of the scale. For this reason, the test-retest reliability was not tested in the original study of the scale.[15]

Physical, physiological, behavioral and psychological differences of children, their continuing growth and development, and their need for adults to meet their basic needs even when they are healthy increase the importance of healthcare provided by pediatric health professionals in determining and meeting children's needs when they are hospitalized.[1,2] Pediatric health professionals can learn the needs of their patients in the most accurate way from their own statements. Health professionals who know the needs of their patients can fully apply their care. Therefore, it is recommended that the scale be used by pediatric health professionals to evaluate the psychosocial, physical and emotional needs of hospitalized children in Turkey.

Conclusion

The NCQ, which was developed to determine the psychosocial, physical and emotional needs of school-age children based on their self-reports, has a high level of validity and reliability in Turkey. Therefore, it is recommended that the scale be used to evaluate the psychosocial, physical and emotional needs of hospitalized children in Turkish society. Its validity and reliability are recommended to be assessed in children with chronic diseases by using a larger sample.

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Declarations

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Author contributions

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

Conflicts of interest

The authors declare that there is no conflict of interest.

Data availability

Data available on request from the authors.

Ethics approval

An ethical approval was obtained from an ethics committee of a university (IRB number: 2021-SBB-0249, Decision no: 9, Date: 31.05.2021).

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ORIGINAL PAPER

Home care experiences of mothers of children with tracheostomies – a qualitative study

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ABSTRACT

Introduction and aim. Parents of children who are addicted to technology have many problems in home care. This study aimed to describe the home care experiences of mothers of children with tracheostomies.

Material and methods. The study adopted Husserl's phenomenological method, a qualitative research design. The sample consisted of 23 mothers of children with tracheostomies followed up in the pediatric pulmonology outpatient clinic of a university hospital. All participants cared for their children at home. Data were collected using a sociodemographic questionnaire and a semi-structured interview questionnaire. All interviews were recorded and transcribed.

Results. Children (12 girls and 11 boys) had a mean age of 3.43±3.326 years. The mean age of tracheostomy insertion was 2.8±2.508 years. Seventeen children were on ventilator support. All participants were mothers with a mean age of 32.34±6.00 years. Half the mothers had primary school degrees (52.2%). The interviews revealed one main theme (burnout), three subthemes (social isolation, perception of competence, and regrets), and five categories (burden of care, fear, awareness, decisions, and role confusion).

Conclusion. Mothers of children with tracheostomies experience numerous problems when they provide home care. They mostly have difficulty improving themselves and enduring role confusion. We must address the issues mothers of children with tracheostomies face during home care to reduce the prevalence of potential complications and improve the quality of care for both them and their children.

Keywords. care, child, experience, mother, nurse, phenomenology, tracheostomy

Introduction

The latest developments in the healthcare system and the integration of technology into care allow parents to care for their children with complex health needs at home. A tracheostomy is a surgical procedure to create an opening through the neck into the trachea to help a person breathe. In the United States, 5000 pediatric tracheostomies are performed annually.[1,2] Parents providing home care in the post-tracheostomy period are likely to experience many life-threatening problems, leading to an increased burden of care.[3,4]

Parents of children with tracheostomies experience physiological, mental, and social problems such as anxiety, depression, and social isolation.[5,6] Caring for tracheostomized and ventilator-dependent children at home affects parents' work and social lives.[4] In Türkiye, it is mostly mothers who take care of children with complex care needs.[7] Mothers do not consider themselves competent despite receiving training in caregiving and medical devices.[8] Parents are reluctant to accept their children with tracheostomies and have difficulty communicating and integrating socially.[9] Parents spend too

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much time caring for their children and endure a heavy burden related to changes in parental roles, helplessness, and lack of professional support.[10] The main challenges faced by family members of children with tracheostomies are coping with the new situation, new care demands, difficulty acquiring material, and limitations on social life.[11] Both children with tracheostomies and their caregivers (mostly parents) experience psychosocial problems. Therefore, we need to find solutions to those problems to support parents psychologically and help them experience less burden of care.[12] There is a large body of research on the care-related experiences of parents of children with tracheostomies. However, there is limited data on how providing care at home affects mothers of children with tracheostomies.[3,11,13,14]

Aim

In Turkey, mothers are regarded as the primary caregivers, meaning they experience the homecare process much more differently than fathers. Therefore, this study focused on the home care experiences of mothers of children with tracheostomies.

Research question

What are the experiences of mothers caring for their children with tracheostomies at home?

Material and methods

Ethical approval

Ethics approval for the conduction of study was obtained from the Research Ethics Committee
of the local state university (Ref No: 2021/8/11) before data collection.

Study design

This study adopted a phenomenological approach, which is a qualitative research method. Phenomenology is an appropriate method for determining lived experiences and problems.[15] The study was reported using the 21-item Standards for Reporting Qualitative Research (SRQR) developed by O'Brien et al.[16]

Setting and participants

The data were collected between March and April 2021. The study population consisted of all parents of children with tracheostomies followed up at the pediatric pulmonary polyclinic of a university hospital. The sample consisted of 23 parents who agreed to participate in the study. The inclusion criteria were (1) having a child with tracheostomy under 18 years of age, (2) having provided home care for at least six months, (3) being able to use computers or smartphones to approve the consent form and to be interviewed, and (4) having Internet access. The exclusion criteria were (1) having lost a child with tracheostomy before and (2) having difficulty accessing/using technology.

Data collection

The data were collected using a sociodemographic questionnaire and a semi-structured interview questionnaire developed by the researchers. Interviews were conducted using the semi-structured interview questionnaire developed based on SPIDER.[17] The semi-structured interview questionnaire consisted of six items on parents' earliest experiences after tracheostomy, the home care process, challenges, and the need for training (Table 1). Two clinical nurses and two nursing academics were consulted for the intelligibility and relevance of the interview questions, which were revised based on their feedback.

Table 1. Interview questions

Topics	Questions
Changes in parents' lives after tracheostomy	1. How has your life changed since your child underwent a tracheostomy? 2. In what way do you help your children with tracheostomy care? 3. When do you think your child needs you the most throughout the day?
The challenges of home care after tracheostomy	4. Have you had any problems with the home care you are providing for your child with a tracheostomy? Can you tell us about those problems?
Social life after tracheostomy	5. Has caring for your child with a tracheostomy limited your social life? In what way? Can you explain, please?
The need for training in tracheostomy care	6. In what areas do you need support regarding your child's tracheostomy care?

Interview

The parents were interviewed online at their convenience. The researchers contacted the parents on WhatsApp and sent a Google Meet link. The parents were alone during the interviews. During the interviews, their children were cared for by other caregivers (father, grandmother, grandfather, etc.). The researchers received verbal consent to record the interviews and took notes during the interviews. There were at least two researchers during each interview. One of the researchers led the interview, while the other asked follow-up questions and took notes about the interviewee's facial expressions and gestures. One of the researchers was a nursing academic, while the other was a pediatric nurse. Each interview lasted 30-45 minutes.

Methodological rigor

The scientific rigor criteria of credibility and transferability (validity) and consistency and confirmability (reliability) were checked. Audit trail and expert feedback were used for confirmability. Two academics and a clinical nurse checked the semi-structured interview questionnaire for consistency. The moderator summarized the participants' responses and read them to them. In this way, repetitive questioning and participant control were achieved for credibility. A purposive sampling method was used for transferability. The sample consisted of 23 mothers. Data saturation was reached after fifteen interviews. However, eight more interviews were conducted. Those participants gave similar answers.

Therefore, the interviews were terminated afterward. Findings cannot be generalized to all patients with tracheostomies due to the medical reasons for tracheostomy and the fact that the age groups are different.[18]

Data analysis

The study adhered to the seven steps of Colaizzi's phenomenological data analysis.[19] The researchers listened to the interviews repeatedly and then transcribed them. They followed Colaizzi's steps to evaluate phenomenological data and group common themes. Afterward, each researcher consolidated the results and developed subthemes. The data were analyzed using the interactive analysis technique developed by Miles and Huberman.[20] The interrater agreement was calculated as 0.890. The researchers agreed on one main theme, three subthemes, and five categories.

Table 2. The steps of Colaizzi's phenomenological data analysis[a]

1.	Description of all participants' answers by careful reading*
2.	Defining the expressions directly related to the researched phenomenon**
3.	Formulation of key expressions identified**
4.	Creating theme sets from formulated expressions**
5.	Making a broad definition of the researched phenomenon**
6.	Determining the basic structure of the researched phenomenon
7.	The researcher giving feedback to the participants regarding the phenomenon determined***

[a] * the texts recorded by the reporter were read and combined by both researchers within the first 72 hours of the interviews to avoid data loss; ** Miles and Huberman's formula was used to provide a consensus on statements and to understand and group them; *** the researchers had the participants confirm the statements they were unsure about without adding their comments

Results

Children (12 girls and 11 boys) had a mean age of 3.43±3.326 years. The mean age of tracheostomy insertion was 2.8±2.508 years. Seventeen children were on ventilator support. All participant parents were mothers with a mean age of 32.34±6 years. Half the mothers had primary school degrees (Table 3).

The data were coded, yielding one main theme (burnout), three subthemes (social isolation, challenging self-improvement, and regrets), and five categories (burden of care, fear, awareness, decisions, and role confusion). The greatest challenge for parents was burnout. However, they also suffered from social isolation, had difficulty improving themselves, and had regrets. Table 4 explains the themes.

Theme

Burnout

The main theme was "burnout." Participants reported social isolation while caring for their tech-dependent children at home due to the limitations of tech-dependent life. They stated that social isolation made it difficult for them to share the burden of care and caused them to see their own self-efficacy as inadequate. They reported being caught between their roles as parents and caregivers. They noted that they regretted some of their decisions, which resulted in burnout. They remarked that they continued to take care of their children at home but drifted into a deep burnout over time. The main theme of "burnout" consisted of the sub-themes of social isolation, perception of competence, and regrets.

Table 3. Demographic characteristics

Participant	Child Age (years)	Child Gender	Age of tracheostomy	Mechanical Ventilation Support	Parent Age (year)	Parent Education (degree)
P1	1.5	Boy	4 years	Yes	42	Primary school
P2	2	Girl	1.5 years	Yes	26	High school
P3	1	Boy	1 year	Yes	38	Primary school
P4	2.5	Girl	2 years	Yes	29	Middle school
P5	2	Boy	1 year	Yes	28	High school
P6	1	Girl	8 months	No	33	High school
P7	1	Girl	6 years	Yes	44	Primary school
P8	6	Boy	5 years	No	26	Middle school
P9	6	Girl	5 years	Yes	30	Bachelor's
P10	4	Girl	4 years	Yes	25	Primary school
P11	3	Girl	3 years	No	33	Bachelor's
P12	1.5	Boy	1.5 years	Yes	23	High school
P13	1	Boy	1 year	Yes	35	Primary school
P14	18	Boy	12 years	Yes	43	Primary school
P15	11	Girl	1 year	Yes	32	Primary school
P16	1	Boy	1 year	No	25	High school
P17	3	Boy	3 years	Yes	30	Primary school
P18	1.5	Girl	1.5 years	No	30	Primary school
P19	1.5	Boy	7 months	Yes	38	Literate
P20	1.5	Girl	1.5 years	Yes	28	Middle school
P21	3	Boy	2.5 years	Yes	39	Primary school
P22	2	Boy	2 years	Yes	33	Primary school
P23	4	Girl	3 years	No	34	Primary school

Table 4. Theme and subthemes

Main Theme/Subtheme	Category	Subcategory
*Burnout		
Social isolation	(1) Burden of care	(1) Limited social acceptance
		(2) Tech-dependent life
Perception of competence	(1) Fear	(1) Losing the child
		(2) Cannula blockage and dislodgement
	(2) Awareness	(1) The need for structured training
		(2) Learning by living
Regrets	(1) Decisions	(1) Decision For Tracheostomy Formation
		(2) Switching to ventilator support
		(3) Home care
	(2) Role confusion	(1) Acceptance by siblings
		(2) Feeling incompetent as a mother

Subthemes

Social isolation

Participants were socially isolated mainly due to the burden of care, resulting in burnout. This was because being socially isolated prevented participants from using the social support systems that could help them feel less burden of care.

Burden of care

The first category was "burden of care," consisting of the subcategories "limited social acceptance" and "tech-dependent life". The first subcategory is limited social acceptance. Participants were uncomfortable about how other parents treated them and could not participate in social life due to their children's health conditions. The second subcategory is Tech-dependent life. Participants stated that they had almost no social life because they could not leave home as their children had to lead tech-dependent lives (aspirator, ventilator, monitor, etc.). The following are some quotations:

"I don't want them looking at my kid like he is any different from them. That's why I don't have much social life." P16

"I can't leave home. I keep thinking about my kid even when I go to the supermarket for shopping." P1

Perception of competence

The second theme was "perception of competence." Participants stated that they had a tough time getting used to caring for their children in the hospital and at home. The theme consisted of two subcategories: "fear" and "awareness."

Fear

Participants had concerns about taking responsibility for the care of their children as they were afraid of making mistakes or losing their children. The category "fear" consisted of two subcategories: "losing the child" and "cannula blockage and dislodgement." The first subcategory is *"Losing the child"*. Participants noted that they were terrified of losing their children because of a mistake they might end up making during the care. The second subcategory is *"Cannula blockage and dislodgement"*. Participants remarked that one of the greatest challenges of homecare was the blockage and dislodgement of the cannula. The following are some quotations:

"I'm afraid of losing my child, like, what if an earthquake hits? I can't leave her behind; I can't have that on my conscience." P21

"I'm terrified that the cannula might come off. If it's gonna happen, I hope it's gonna happen when we're in the hospital. The training was very nice, but I don't dare to fix it; I'm afraid of hurting my kid." P9

"I'm scared that the cannula might get clogged. Once his saturation was almost below 40, I didn't know what to do. I was afraid of losing him." P5

Awareness

The category "awareness" was about the fact that participants realized they needed the training to care for their tech-dependent children at home. They stated that they even had difficulty getting their tech-dependent children to care for themselves. They said that they did not know the principles of care. The category "awareness" consisted of two subcategories: "the need for structured training" and "learning by living.". The first subcategory was *"The need for structured training"*. Participants received training before discharge. However, they were too stressed out to understand the training because their children were in the intensive care unit. They noted that the home setting was completely different from the hospital and that they did not dare to put what they learned into practice.

The second subcategory was "*Learning by living*". Participants stated that they experienced burnout mostly because they had to learn some things by trial and error. They felt incompetent, although they received training. However, they added that their home care experiences raised their awareness. The following are some quotes: For example,

"I got trained but I just couldn't understand anything because I was stressed and freaked out back then. So I think they should give us training again." P19

"I learned everything from the problems I had about tracheostomy care. But there must be another way. We should get more support. It was pretty hard for me to take over care at home. One shouldn't have to learn tracheostomy care from mistakes and problems." P18

Regrets

"Regrets" was the third theme. Participants believed they were responsible for the decisions they made about caring for their tech-dependent children. They noted that they had difficulty taking care of their non-tech-dependent children because they had to devote most of their time to caring for their tech-dependent children. The theme "regrets" consisted of two categories: "decisions" and "role confusion."

Decisions

The category "decisions" was about the decisions made by participants about caring for their tech-dependent children. The category consisted of three subcategories: "*decision for tracheostomy formation*" "switching to ventilator support," and "home care". The first subcategories is *"Decision For Tracheostomy Formation"*. Participants stated that they regretted their decision about the tracheostomy procedure. They believed it was a premature decision as they did not consider the possibility that their children might get better without a tracheostomy. The second subcategories is *"Switching to ventilator support"*. Ventilator support was another challenge for participants because being tech-dependent made things much more difficult. Third subcategory is "*Home care*". For participants, home care itself was a challenge. Participants who needed training and had difficulty maintaining self-care had doubts about their decisions about providing home care as they believed that it was a mistake. The following are some quotes:

"My kid had osteogenesis imperfecta, so it was pretty hard for us to decide the right time for the tracheostomy procedure. On the one hand, he couldn't breathe, and on the other hand, his neck might have been broken." P8

"I freaked out the first time I saw my kid. I just lost my head. I had training, but home care was pretty hard. I sometimes have doubts about the tracheostomy procedure. I wish my kid didn't need to get ventilator support." P17

"I said 'No' when they wanted to discharge my kid because I was afraid that I wasn't gonna be able to care for her at home. But they discharged us anyway." P13

Role confusion

The category "role confusion" referred to the changes in family processes that participants experienced after their children underwent tracheostomies. The category consisted of two subcategories: "acceptance by siblings" and "feeling incompetent as a mother." The first subcategory was *"Acceptance by siblings"*. Participants stated that caring for their children with tracheostomies at home also affected their siblings. Participants noted that tech-dependent life and long hospital stays impacted every aspect of life at home. They added that their non-tech-dependent children were unsure about what they were supposed to feel for their siblings with tracheostomies. The second subcategory was *"Feeling incompetent as a mother"*. Participants stated that they felt incompetent when it came to taking care of their tech-dependent children. They believed they encountered problems with caring for their tech-dependent children because of their own incompetence. The following are some quotes:

"*My non-tech-dependent children want to go out and play in the park, but I can't take them there because I'm afraid that my tech-dependent children might get an infection*." P3

"*My non-tech-dependent children keep asking me when we'll have friends over or when we'll go and visit them*." P4

"I still can't do some things, like aspiration or dressing. My husband does those things. I feel bad, and I want to do them, but I just can't look at the hole" P10

"*I have an 11-year-old daughter, too. Our life has changed completely. I couldn't take care of her. We were completely isolated at home. I found myself in a situation where I couldn't even notice that she needed me*." P6

Discussion

This study focused on the home care experiences of mothers of children with tracheostomies. The findings yielded one main theme (burnout) and three subthemes (social isolation, challenging self-improvement, and regrets).

Participants stated that society should recognize the increasing burden of care associated with chronic diseases and help them cope with their problems. Caring for children with complex health needs at home places a heavy burden on parents. Because of this burden, they are socially isolated and suffer from sleep disturbances and temporal limitations. Parents of children with tracheostomies are physically and psychologically overwhelmed at home after discharge.[21] They have difficulty managing their time because they have other responsibilities. In other words, they have little time and energy left to care for their non-tech-dependent children because they spend most of their time caring for their tech-dependent children. This causes social isolation, anxiety, and communication problems. They also have a low quality of time because they have difficulty understanding their children's health conditions.[3]

Swain and Acharya reported that the tracheostomy tube created social stigma and caused anxiety in social interactions.[9] More and more children with complex health needs are dependent on technological devices.[22] Therefore, we should support home care to improve parents' and their children's physical, emotional, and mental health.[23] Our participants reported that they struggled with the increased burden of care after their children were discharged. They added that they were socially isolated because they could not share their burden with other family members and feared that they would not be accepted by society.

The number of children with tracheostomies is increasing day by day. However, home care technologies are advancing, allowing parents of children with tracheostomies to execute numerous complex skills at home. In other words, parents of children with tracheostomies assume the responsibilities of healthcare professionals over time.[24] Therefore, nurses should help children with tracheostomies develop those skills. They should also provide them with training programs and assess how much they can adapt to the new situation. Thus, they can resolve many complex and complicated variables during home care. In other words, they can minimize the problems parents of children with tracheostomies experience during home care." On the other hand, those parents should design their homes to provide care more easily and encounter fewer complications.[25] After discharge, they need to learn several challenging skills related to home care at the same time because they face numerous complicated problems during the process.[26] They have difficulty caring for their children with tracheostomies at home, although they receive training from healthcare professionals.[27] When faced with challenges, they often turn to unreliable websites to meet their information needs.[26,28] Inaccurate sources of information and poor information management reduce the quality of home care.[27] The more training the parents receive, the fewer complications their children develop and the less often they are hospitalized.[29] However, parents do not feel ready for home care.[30] Changing the tube, bleed-

ing, infections, and tube blockage are the most common problems experienced by parents during home care.[31,32] Breathing problems children with tracheostomies experience are a cause for concern for parents.[33] Therefore, parents should be provided with post-discharge training to help them manage life-threatening situations during home care.[13] Our participants also stated that the training they received in the hospital was not long-lasting. They noted that they went through a tough self-developmental process after they began caring for their tech-dependent child at home. In other words, they learned by trial and error, which was painful for them. They also remarked that the fear of losing their children was the greatest force that pushed them to improve themselves. Research also shows that parents fear life-threatening complications (i.e., tube blockage and dislodgement). Our participants recommended that healthcare professionals regularly provide structured training during the home care process instead of one-time training in the hospital.

Medical technologies have extended the life expectancy of children with complex health needs. Parents have to make numerous medical decisions, including tracheostomy.[34] According to Nageswaran et al., parents turn to different support systems (clergy, medical staff, etc.) before making medical decisions for their children. They receive adequate support but have gaps in their knowledge.[14] Parents prioritize the interests of their children when making medical decisions. However, they have difficulty making medical decisions when they do not fully understand the objectives of complex treatments (tracheostomy or respiratory support).[35] The more health professionals respect parents' self-efficacy and decisions about pain and symptom control, the more accurate their decisions will be.[36] Our participants noted that they regretted their past decisions, such as giving the O.K. to the tracheostomy procedure, ventilator support, or home care. They also remarked that life-threatening complications their children experienced made them regret their decisions.

Our participants also noted that the whole process of home care affected their families. Siblings of tech-dependent children grow up experiencing many emotions (pain, grief, anxiety, loss, etc.).[37] Effective communication helps strengthen family bonds and protect mental health.[38] Siblings of children with life-threatening diseases have more emotional and behavioral problems and lower quality of life.[2] Children with complex health needs have a greater impact on family members. This impact is associated with socioeconomic status, support systems, year of diagnosis, and home care support.[39] Family members communicate less often, and siblings have more unmet needs when tech-dependent children are at home.[40] Parents experience pain as they care for their tech-dependent children because they have gaps in their knowledge and have difficulty meeting their non-tech-dependent children's needs.[41] Our participants also had difficulty meeting their non-tech-dependent children's needs, which was a great problem. They found it difficult to manage family processes because their Our participants also had difficulty meeting their non-tech-dependent children's needs, which was a great problem had difficulty communicating with their tech-dependent siblings and accepting their health conditions. They noted that this caused them to feel more and more incompetent.

Mothers who actively care for children with tracheostomies at home experience numerous spiritual and tracheostomy care issues. Training programs for healthcare professionals reduce unplanned hospitalizations and emergencies during home care regarding children with tracheostomies.[42] Home care and repeated hospitalizations cause job losses and financial problems for parents. Financial problems result in an increased burden of care and mortality and morbidity rates and poor quality of life.[43] Mothers believe that their children with tracheostomies have difficulty socializing and meeting their educational needs.[44] We should take into account the cultural structure and education level in Turkey and develop standardized training programs and determine their impact. Nurses should support mothers for post-discharge home care and follow up on them regularly to help them overcome their problems.

Conclusion

This study focused on the experiences of mothers of children with tracheostomies. Mothers caring for their children with tracheostomies at home are socially isolated. Moreover, they have difficulty improving their care because they have gaps in their knowledge. They also regret some of their decisions and find it difficult to navigate family relationships. Therefore, we need healthcare policies to improve home care services to help them overcome these challenges. We need to set criteria and timelines for the discharge of children with complex care needs and their parents. Healthcare professionals should provide family-centered care to support discharge processes and protect family processes. We should ensure that they receive community-based specialized healthcare services. In this respect, pediatric nurses should take an active role in home care services. We need to set up short-term care clinics for parents and improve home health care. If we set up social networking sites led by pediatric nurses, we can help mothers experience less social isolation. Our results will guide pediatric nurses in the way they train mothers of children with tracheostomies. In other words, they will raise pediatric nurses' awareness of family-centered care and adaptation to social life rather than focusing only on life-threatening complications related to home care.

Declarations

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Author contributions

Conceptualization, H.D. and E.G.; Methodology, H.D. and E.G.; Software, H.D. and E.G.; Validation, H.D. and E.G.; Formal Analysis, H.D. and E.G.; Investigation, H.D. and E.G.; Resources, H.D. and E.G.; Data Curation, H.D. and E.G.; Writing – Original Draft Preparation, H.D. and E.G.; Writing – Review & Editing, H.D.; Visualization, H.D. and E.G.; Supervision, H.D.; Project Administration, H.D.

Conflicts of interest

No potential conflict of interest was reported by the authors.

Data availability

The statistical analysis plan will be made available for research purposes upon request to the corresponding author.

Ethics approval

Ethics approval for the conduction of study was obtained from the Research Ethics Committee
of the local state university (Ref No: 2021/8/11) before data collection.

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ORIGINAL PAPER

Analgesic effects of ethyl chloride spray in venepuncture – a prospective, randomized, controlled, single-blind study

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ABSTRACT

Introduction and aim. This study evaluated whether ethyl chloride spray had an analgesic effect on pain intensity caused by venepuncture compared to a placebo.

Material and methods. A total of 339 patients were randomly divided into two groups: The group in which ethyl chloride spray was applied (n=212) and the placebo group (n=127). The analgesic efficacy of ethyl chloride spray was compared with the placebo group using the Visual Analog Scale (VAS).

Results. When the analgesic efficacy of ethyl chloride spray was compared with the placebo group, the VAS score was 4 [interquartile range (IQR): 1.0] for the ethyl chloride spray group and 5 (IQR: 2.0) for the placebo group. The efficacy of ethyl chloride spray in reducing pain was statistically significant compared to the placebo ($p<0.001$).

Conclusion. Ethyl chloride spray has analgesic activity in venepuncture. Therefore, this spray can be used at the emergency departments to reduce pain intensity in patients undergoing such interventions.

Keywords. analgesia, anesthesia, ethyl chloride, pain, venepuncture

Introduction

Venepuncture is an intervention that is frequently used in emergency departments. However, due to its invasive nature, patients experience anxiety and fear during this intervention. For some patients, this may be the first negative experience in emergency departments; therefore, it is important for patient comfort to relieve their anxiety before the intervention.[1] To this end, non-pharmacological or pharmacological methods containing pharmacological agents have been used in the literature for pain control in venepuncture.[2] Among these methods are the Valsalva maneuver, eutectic mixture of local anesthetics (EMLA), cytotherapeutic local anesthetic agents, and watching television for pediatric patients.[2-5] These methods are advantageous because they are not invasive, and nurses can use them independently.

Ethyl chloride spray is a non-invasive local anesthetic agent. When sprayed on a body surface, it shows its effects within seconds by numbing nerve endings through cooling tissues up to -20°C. This effect lasts only a short time, 2-3 minutes, and does not disturb the patient as the anesthetic effect wears off.[6] We consider that the use of ethyl chloride spray will contribute to clinical practice as a practical method since it can increase patient comfort through its anesthetic effect without an invasive intervention and can be independently used by nurses.

Aim

This study aimed to evaluate whether ethyl chloride spray had an analgesic effect on pain intensity caused by venepuncture compared to a placebo.

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Material and methods

Study design and setting

This study was conducted with a randomized controlled design from January 1, 2022, through January 30, 2022, at the emergency department of a tertiary hospital. For venous puncture sampling, ethyl chloride spray (Clorethyl Cooling Spray, EBT healthcare services, Bursa, Turkey, Bursa, Turkey) was compared with a placebo (distilled cold water). Written informed consent was obtained from all the patients in the study. The study started after obtaining the Ethical Approval from the Clinical Research Ethics Committee of Erzurum Training and Research Hospital (Number: 37732058-6027, dated: 22/12/2021). This study complied with the principles of Good Clinical Practice of the Declaration of Helsinki and carried out according to the CONSORT directive.

Sample size and patients

G-Power 3.1 software was used to determine the sample size for study. To calculate the sample size, a medium effect size of 0.5, type 1 error of 0.05, and power of 0.80 were used. The sample size for the study was calculated as a minimum of 127 patients in each group (254 patients with a 1:1 allocation ratio) at 10% loss. However, we obtained a larger sample size by including 212 patients in the ethyl chloride spray group and 127 patients in the placebo group.

The study included patients aged older than 18 years and younger than 65 years, who required venepuncture at the emergency department according to the medical criteria. Patients who were allergic to ethyl chloride, pregnant and breastfeeding women, patients who had taken analgesia within 24 hours, those with problems in verbal communication, unconscious patients, patients with peripheral neuropathies, those with a diagnosis of Raynaud's phenomenon, those with skin abrasions, and those with an infection in the region of intervention were excluded from the study. Only the cases in which the first or second access attempt was successful were included in the study. Patients that underwent three or more interventions were excluded.

Randomization and primary outcome

The patients were divided into two groups as ethyl chloride spray and placebo. Ethyl chloride spray and sterile water kept at 4°C were stored separately in the same closed cans numbered 1 and 2, respectively. Only the practitioner knew which drug was in which can. Patient selection was randomly performed according to the preference of the practitioner. The nurse decided on the method to be used in each patient. The patients, on the other hand, did not know the agent applied to them. Ethyl chloride spray kept at 4°C was applied to one group, while sterile water kept at 4°C was applied as a placebo to the other group. It was undertaken by two different nurses with at least six and 10 years of experience in the field. The primary outcome was pain scores evaluated using the 10-point Visual Analog Scale (VAS) in the patients that underwent venous puncture sampling.

Study variables and intervention

The sequential number of patients for the study group was documented in a file. For the placebo group, 127 patients were documented sequentially as a list. The nurse decided on the method to be used in each patient. The patient number of each patient who underwent the procedure was determined, and detailed data about the patients were recorded on the previously prepared forms. The sample size was completed by drawing a line on the sequence number of the patients who underwent the procedure. During the venepuncture procedure, after the patient's vein was palpated; the median vein in the antecubital region was preferred since it provides easier access. This region was first cleaned using cotton wool and 70% alcohol. Then, it was sprayed three times from a distance of 15 cm with the first or second spray can according to the nurse's preference. After waiting for 30 seconds, venous puncture was performed preferably with a pink 20-gauge cannula. All patients were cannulated with a pink 20-gauge. All the interventions at the emergency department were performed by two different nurses with at least six and 10 years of experience in the field. During the procedure, the arm movements of the patients were observed and recorded. After the procedure, the patients were followed up in terms of bleeding, swelling, and redness. Then, the volunteers were asked to mark the degree of pain they felt during the venepuncture intervention from 0 (no pain) to 10 (most severe pain) on a 10-cm horizontal VAS scale. They were also asked to rate their pain character as 0 ('no pain'), 1 ('oppressive pain'), 2 ('dull pain'), and 3 ('sharp pain').[7]

Statistical analysis

Statistical analysis was performed using SPSS software version 25.0 (IBM Corp., Armonk, NY, USA). The distribution of variables was evaluated for normality using the Kolmogorov-Smirnov test. Descriptive statistics were given as frequency (n) and percentage (%) values for categorical variables. The comparison of groups for variables with a normal distribution was made with Student's t-test, and group comparisons for variables that did not have a normal distribution were undertaken with the Mann-Whitney U test. For 2×2 comparisons between categorical variables, the Pearson chi-square test was used if the expected value was >5, the chi-square Yates test if 3-5, and the Fisher's exact test if <3. A p value of <0.05 was considered statistically significant.

Results

Patient populations and characteristics

The study initially included 385 patients; however, 46 patients that did not meet the inclusion criteria were excluded. As a result, a total of 339 patients were included in the sample (Figure 1). Data obtained from the 339 patients were analyzed using SPSS, with no other exclusion.

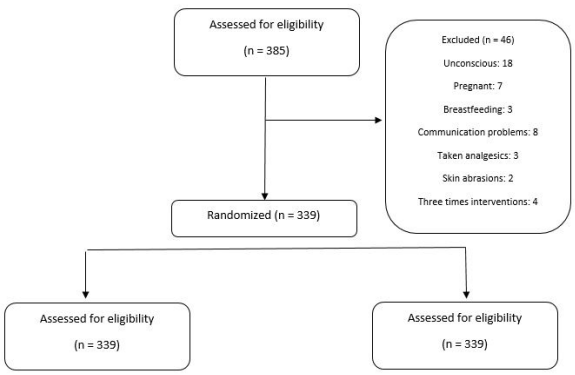

Fig. 1. CONSORT flow diagram of the study

The demographic and characteristic features of the patients are given in Table 1.

Table 1. Patients' demographic and characteristic features of the study groups [a]

Variables		Placebo (n=127)	Ethyl chloride (n=212)	p
Age (years), median (IQR)		61 (34)	42 (35)	<0.001*
Gender				
	Male, n (%)	62 (48.8%)	104 (49.1%)	0.966**
	Female, n (%)	65 (51.2%)	108 (50.9%)	
Attempts number				
	First, n (%)	115 (90.6%)	207 (97.6%)	0.004**
	Second, n (%)	12 (9.4%)	5 (2.4%)	
Wrist movement				
	No, n (%)	115 (90.6%)	194 (91.5%)	0.581**
	Little, n (%)	12 (9.4%)	18 (8.5%)	
Region of intervention				
	Antecubital, n (%)	120 (94.5%)	203 (95.8%)	0.595**
	Hand, n (%)	7 (5.5%)	9 (4.2%)	

[a] * – Mann-Whitney U Test; ** – Chi-square test

The mean age of the placebo group was 61 [interquartile range (IQR: 34)], and the mean age of the ethyl chloride group was 42 (IQR: 35), revealing a significant difference between the groups (p<0.001). The rate of male patients was 48.8% in the placebo group and 49.1% in the ethyl chloride spray group, with no significant difference between the groups (p=0.966). Intravenous access was achieved at the first attempt in 90.6% of the patients in the placebo group and 97.6% of those in the ethyl chloride spray group, and the difference between the groups was statistically significant (p=0.004). The success rate in the first attempt was significantly higher in ethyl chloride spray group. In the placebo group, wrist movement was absent in 90.6% and little in 9.4% of the patients, while in the ethyl chloride group, these rates were 91.5% and 8.5%, respectively, with no statistically significant difference between the groups (p=0.581). The area of intervention was the antecubital region in 94.5% of the patients in the placebo group and 95.8% of those in the ethyl chloride spray group, and the difference was not statistically significant (p=0.595).

Comparison of groups

The median VAS score was 3 (IQR: 2.0) in the ethyl chloride spray group and 5 (IQR: 2.0) in the placebo group. The efficacy of ethyl chloride spray in reducing pain was statistically significant compared to the placebo (p=0.000) (Figure-2). In the evaluation of pain character, 2.4% of the patients in the placebo group and 22.2% of those in the ethyl chloride spray group reported that they did not feel any pain. Thus, the rate of patients feeling no pain was statistically significantly higher in the ethyl chloride group compared to the placebo group (p = 0.000) (Table-2).

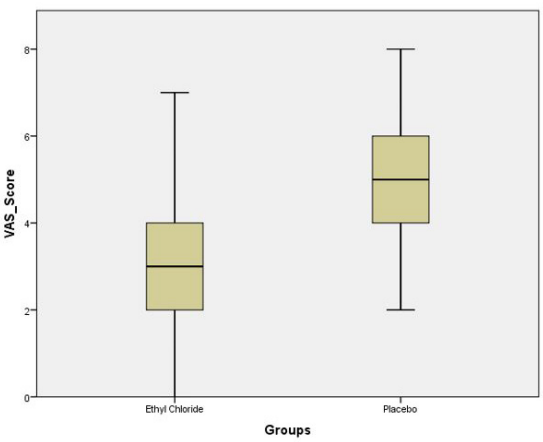

Fig. 2. Primary outcome: pain intensity in ethyl chloride spray and placebo groups

Table 2. Assessment of pain intensity in the study groups

Variables		Placebo (n=127)	Ethyl chloride (n=212)	p
VAS score (median, IQR)		5 (2)	3 (2)	<0.001*
Pain character, n (%)				
	No pain	3 (2.4%)	47 (22.2%)	
	Oppressive	6 (4.7%)	63 (29.7%)	<0.0001**
	Dull	3 (2.4%)	79 (37.3%)	
	Sharp	115 (90.6%)	23 (10.8%)	

[a] * – Mann-Whitney U test; ** – Chi-square test; VAS – Visual Analog Scale; IQR – Interquartile range

Discussion

Pain commonly occurs in intravenous access interventions, but it is actually preventable.[8] For patient comfort, it would be ideal to use a low-cost, easy-to-apply, non-invasive, and short-acting analgesic technique to reduce pain. In this study, we examined the role of ethyl chloride spray in pain reduction during venepuncture by comparing it

with a placebo. Patients treated with ethyl chloride spray reported significantly less pain during this intervention compared to the placebo group. The patients in the placebo group felt significantly more pain.

Pain relief is a human right for all patients undergoing painful interventions. Intravenous venepuncture is also a painful procedure that is applied through the skin. In order to reduce pain during this intervention, in addition to local anesthetic drugs, there are local vapor cooling sprays, such as ethyl chloride and fluorohydrocarbons that provide anesthetic effects on the skin. With the sudden evaporation of the volatile liquid in these cold sprays, there is a rapid drop in skin temperature, the skin becomes temporarily desensitized, and as a result all sensations including pain are interrupted.[9-10] In our study, topically applied ethyl chloride spray was used for analgesia. With the sudden evaporation of the cold spray, there was a significant reduction in pain in the patients that underwent venepuncture.

In the literature, studies evaluating the efficacy of ethyl chloride spray report controversial results.[11] Selby et al. compared EMLA cream, lignocaine, and ethyl chloride spray in relieving pain in venous cannulation; however, they found no significant difference between these agents in terms of pain reduction.[4] In a similar study, ethyl chloride was sprayed continuously for 10 seconds to reduce pain during venepuncture, and it was found to be significantly effective in reducing pain.[12] In another study, Rao et al. compared the efficacy of ethyl chloride sprayed from a distance of 5 cm in reducing pain during venepuncture between once- and twice-sprayed groups. The authors concluded that twice-sprayed ethyl chloride was more effective than a single spray application.[13] In our study, ethyl chloride was sprayed three times from a distance of 15 cm. This group was compared with distilled water as a placebo. Similar to the studies in the literature, we observed a significant decrease in pain experienced by the patients in the ethyl chloride spray group during venepuncture. In light of these findings, we can state that despite the differences in the distance or number of applications used in the literature, the common conclusion is that ethyl chloride spray has an analgesic effect.

Local anesthetic agents can be used to reduce pain, but since most of these agents are administered invasively, they also cause pain. It is also necessary to wait for a while for the analgesia effect to appear, which increases the duration of the whole intervention. In a previous study, the analgesic effect of non-invasive lidocaine spray compared with a placebo in radial artery cannulation, but no significant difference was found.[14] In another study, it was reported that lignocaine spray was more effective than ethyl chloride spray. However, in that study, ethyl chloride spray was sprayed twice from a distance of 10 cm. The reason for different results may be different techniques used in the application of ethyl chloride[11]. Non-invasive analgesic creams applied to the skin have also been used to reduce pain and shown to be effective. However, when these applications are examined, it is noted that creams were applied several times for their analgesic effect, and this took a long time.[15-17] These methods are not practical for use in the emergency department because they have disadvantages related to their application and they are time consuming. In the current study, ethyl chloride spray, which is easy to apply and can be used independently by nurses, was preferred for analgesia. As a result, it significantly reduced pain compared to the placebo.

Study limitations

Since the pain threshold is a relative concept for each person, the VAS score of patients may vary individually. The evaluation of pain with a scale that is dependent on the patient constitutes a limitation of our study. Another limitation is that cannulation in different regions can have different pain intensities.

Conclusion

This study demonstrated the topical analgesic effect of ethyl chloride spray compared to a placebo in patients undergoing venepuncture. Therefore, we consider that the use of ethyl chloride spray during venepuncture in the emergency department will both increase patient comfort and reduce pain.

Declarations

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Author contributions

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

Conflicts of interest

No conflict of interest was declared by the authors.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was approved by the local ethics committee (Ethics Committee of Erzurum Training and Research Hospital date: 22.12.2021 decision number: 37732058-6027)

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ORIGINAL PAPER

Contribution of semiquantitative analysis with dynamic contrast enhanced magnetic resonance imaging to the differential diagnosis of focal liver lesions

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ABSTRACT

Introduction and aim. We aimed to evaluate the usefulness of dynamic contrast-enhanced (DCE) MRI semiquantitative analysis values in focal liver lesions (FLL) to provide additional qualities that can be used in daily practice in the differential diagnosis of lesions.

Material and methods. This retrospective study included 91 patients with liver masses on DCE-MRI. The sensitivity and specificity of time intensity curves (TIC) and semiquantitative analysis values were evaluated to differentiate benign and malignant lesions.

Results. The study included 91 patients (376 lesions), aged between 28-81 years. Of the lesions, 303 were malignant and 73 were benign. In TIC semiquantitative analysis, it was found that "Tpeak" and "wash-out" rate values showed differences, especially in the differentiation of HCC, metastasis, and hemangioma. Area under curve, maximum relative enhancement, and "wash-in" and "wash-out" values of metastases and hemangiomas were different. Brevity of enhancement values of HSK, hemangiomas, and metastases were found to be different. The risk of malignancy was found to be high when the "wash-out" ratio was above 0.08 (sensitivity: 64.3%, specificity: 70.4%).

Conclusion. We think that the 0.08 threshold value we found for the washout ratio with DCE-MRI semiquantitative analysis data will be useful in daily practice in the differentiation of malignant and benign FLL.

Keywords. adult liver cancer, benign hepatoma, perfusion imaging

Introduction

Focal lesions of the liver (FLL) include epithelial, mesenchymal, mixed group primary benign or malignant tumors and secondary lesions. Common lesions with clinical significance include hemangioma, hepatic adenoma (HA), focal nodular hyperplasia (FNH), hepatocellular carcinoma (HCC), intrahepatic cholangiocellular carcinoma (IHCCC), and metastases.[1-4]

Magnetic resonance (MR) perfusion imaging is a quantitative technique that provides information about tissue microcirculation at levels below the spatial resolution of conventional imaging techniques.[5]

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Düzkalır HG, Kış N, Urgun DA, Ağaçlı MO, Kılıçoğlu ZG. *Contribution of semiquantitative analysis with dynamic contrast enhanced magnetic resonance imaging to the differential diagnosis of focal liver lesions*. Eur J Clin Exp Med. 2023;21(2):344–356. doi: 10.15584/ejcem.2023.2.6.

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), which requires intravenous administration of gadolinium contrast, is often used to study FLL because it gives information about things like the growth of blood vessels in the tumor, its stage, its ability to spread to other parts of the body, and how it reacts to anti-tumor therapy.[1,6] Peak arterial contrast enhancement analysis is required to determine the intrinsic tissue properties of lesions and to detect neovascularization in its early stages.[7] The methods that can be used in the analysis of this contrast enhancement are grouped as: visual assessment, which may be subjective; semi-quantitative analysis obtained from time-intensity curves (TIC), which are automatically measured with the post-processing technique in the available software and measure the changes in contrast concentrations over time; and quantitative analysis, which requires highly complex formulas and a lot of time.[8] Therefore, we used a semiquantitative analysis technique in our study.

Aim

The aim of our study was to evaluate the usefulness of semi-quantitative analysis of abdominal DCE-MRI images in patients with diffuse malignant or benign FLL in providing additional features that can be easily used in daily practice to differentiate between lesions.

Material and methods

Ethical approval

This study does not contain any studies with human participants or animals performed by any of the authors. All data was processed anonymously, according to the privacy legislation.

Study design and study population

This study was carried out as a retrospective observational cross-sectional study at a single center. Patients aged between 18 and 85 years who underwent abdominal DCE-MRI in our clinic and who were found to have local liver lesions were retrospectively evaluated, and those who fulfilled the inclusion criteria were included in the study. Inclusion criteria:

- to have a primary or metastatic, benign or cancerous, focal lesion confirmed by imaging and/or clinical diagnostic criteria and histopathology;
- there are no MR contraindications (renal failure, respiratory failure, allergy, claustrophobia, and so on);
- to have optimum image quality for measurements.

Patients with an impaired general condition, an inability to establish respiratory cooperation, or an inappropriate condition for MRI (MR-incompatible prostheses, a cardiac pacemaker, etc.), those with artefactual images and masses smaller than 1 cm, were excluded from the study because optimal measurement could not be performed. A total of 376 (n) liver masses in 91 patients (F=41, M=50) (aged between 28 and 81) were included in the study. In patients with multiple lesions, lesions over 1 cm were included in the study. The hemangiomas included in the study (n=60) were diagnosed with MR appearance features and typical contrast enhancement patterns. Thirty-three of these lesions were already followed up with radiological imaging methods (ultrasound, computerized tomography, or MR).

Of the 203 metastatic masses in the study, 102 of these lesions were diagnosed as metastases in patients with known pathological diagnoses of primary malignancies (breast cancer, gastrointestinal tract, pancreas, etc.) during routine follow-up. The remaining 101 metastatic liver masses were biopsy-diagnosed lesions. Of the four adenoma patients included in the study as primary liver tumors, one had typical radiological imaging features; two were further confirmed by dynamic contrast-enhanced MRI with a liver-specific contrast agent; and one was definitively diagnosed by biopsy.

Among the patients included in the study, there was one case of focal nodular hyperplasia (FNH) and one case of malignant hemangioendothelioma diagnosed by imaging methods and biopsy. One patient was being followed up for an angiomyolipoma (AML). FNH and AML were excluded from the evaluation since they were numerically insufficient.

In 13 of our 15 hepatocellular carcinoma (HCC) cases, the radiological (American Liver Association; AASLD) and laboratory diagnostic criteria available in the literature were taken as references.[8] Histopathological diagnosis was available in two HCC cases. Ten of the HCC cases were followed up because of cirrhosis, seven because of chronic HBV, and four because of chronic HCV. Masses evaluated as dysplastic or regenerative nodules according to MR signal characteristics were not included in the study. One of the patients with IHCCC (n=2) was diagnosed histopathologically, and the other was diagnosed with typical radiological imaging features and clinical findings.

There was no diagnostic change in the clinical and radiological follow-up of all patients.

MRI techniques

Patients were asked not to take food for at least two hours before the examination, technical information was given, and informed consent was obtained. Subsequently, an IV cannula was inserted into one of the antecubital veins. All patients underwent routine upper abdominal dynamic contrast-enhanced MR examinations on a 1.5 Tesla MR machine (Philips Achieva) with a phased-array coil. In all cases, FOV was placed and a slice plan was applied to visualize the whole liver in the axial plane, and the liver was centralized to

reduce artifacts in the dome. Imaging included these sequences:

- T2-weighted axial plane turbo spin eko (TSE) with fat suppression (TR/TE: 386/80; tilt angle: 90°; slice thickness: 7.5 mm; FOV: 375),
- TSE T2 weighted (TR/TE: 524/80), TSE long TE T2 weighted (TR/TE: 520/200),
- T1 weighted gradient echo (TR/TE: 181/4.6 (in phase), 181/2.3 (out of phase), flip angle: 80°).
- Diffusion-weighted imaging (TR/TE: 1666/76; flip angle: 90°; slice thickness: 7.5 mm; slice spacing: 1.5 mm; FOV (field of view): average 375; matrix: 152x124) (single-shot echo-planar sequence, b=0 and b=1000 mm^2/s),
- T1-weighted fat-suppressed dynamic contrast gradient echo (TR/TE: 4.1/1.9, flip angle: 10°, FOV: average 400, matrix: 196x224, slice thickness: 4 mm, slice spacing: 2 mm). The dynamic series were taken in five phases to avoid missing the arterial phase due to possible technical problems.

After the contrast-enhanced exam, all of the patients were kept under observation for about 45 minutes. If there were no problems, the patients were sent home.

MRI analysis techniques

Using the quantitative analysis measurement in the software on the workstation, TIC curves were automatically made from DCE cross-sectional images, and semiquantitative analysis values for all lesions were taken from this screen (Fig. 1). All images were measured by a single radiologist with at least 3 years of abdominal radiology experience.

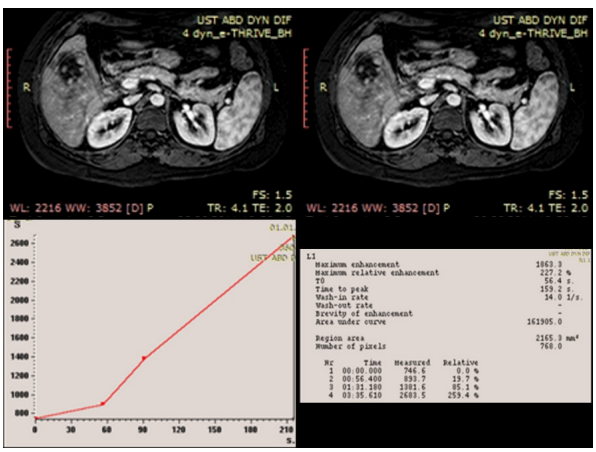


Fig. 1. Hemangioma (in a 39-year-old woman): Early arterial and late phase images of the lesion in dynamic series and semiquantitative analysis values with TIC

In the measurements, if the lesion had a homogeneous internal structure and was round in shape, the region of interest (ROI) was placed in a way to include the entire lesion without extending beyond the mass (Fig. 2). In non-round homogenous lesions, the borders of the mass were drawn with a "free-hand" ROI. For lesions below 2.5 cm, the ROI was measured to cover the entire lesion. For lesions of 2.5 cm or more and lesions with heterogeneous internal structure, measurements were made both by placing an ROI covering the entire lesion and by placing an ROI on the solid peripheral part of the lesion that retains the most contrast (Fig. 3). The average of three measurements from the same section was taken in large lesions.

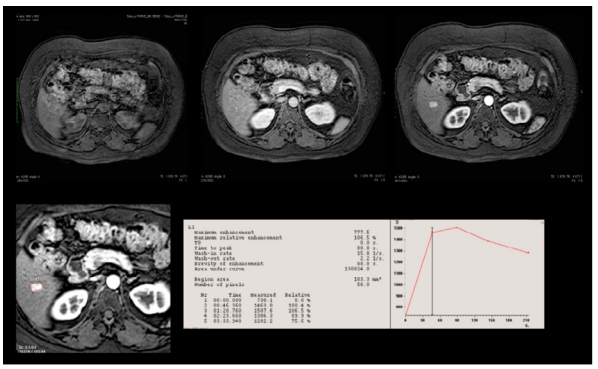


Fig. 2. Adenoma: (46 years old, female) A-TSE T2A, B- DWI b 1000, C, D, E- Time-intensity curve and semiquantitative analysis values of the lesion in the arterial phase and the whole lesion in the dynamic series

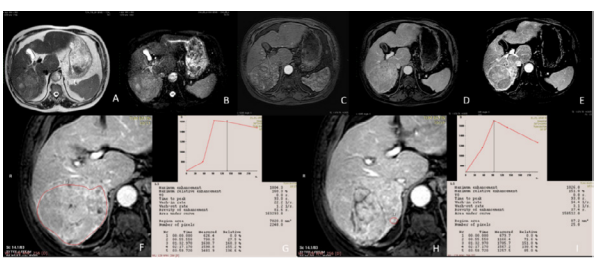


Fig. 3. Hepatocellular carcinoma: (62 years-male) A-TSE T2A, B- DWI b 1000, C, D, E- Lesion in dynamic series, F, G- Time-intensity curve and semiquantitative analysis values of the whole lesion in arterial phase in dynamic series, H, I- Time-intensity curve and semiquantitative analysis values of the partial lesion in dynamic series

In order to compare the lesion values, measurements were made from the right lobe posterior segment of normal liver tissue in each patient, with 1-cm-diameter ROIs placed in 3 different localizations in each section and averaged. In patients who could not be measured due to massive lesions in the right lobe posterior, measurements were made from other segments of the right lobe or the left lobe, which were at least 2 cm away from the lesions and did not contain vascular structures. Automatically obtained TIC curves were grouped as type 1, type 2, and type 3 patterns (Figure 4).

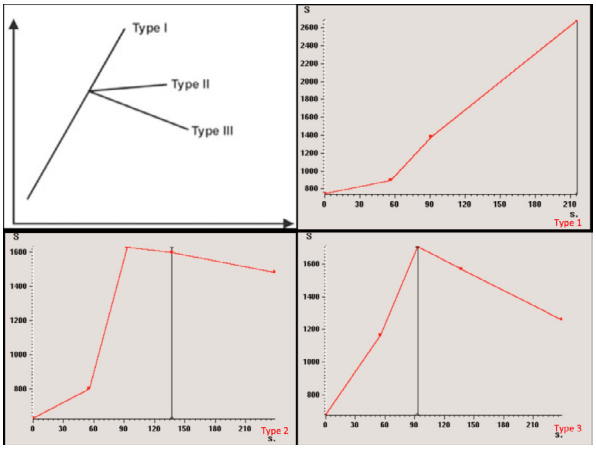


Fig. 4. Types of TIC curves

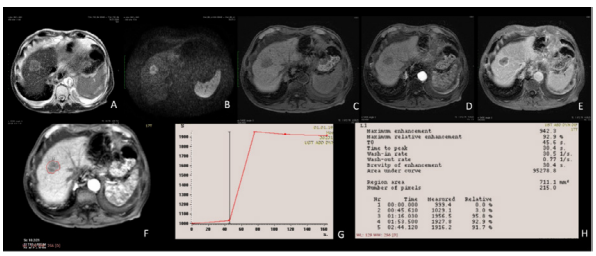


Fig. 5. TIC curve: maximum intensity (1), maximum contrast enhancement (2), time to peak (3), wash-in (4) and wash-out (5) rates, and brevity of enhancement (6)

The following parameters were obtained from the TIC curve (Fig. 5):

- T0: the moment of contrast arrival in the tissue.
- S0: intensity before contrast arrival
- Maximum Intensity: The peak value of the curve
- Maximum contrast enhancement: the difference between the peak value and S0
- Time to peak (uptake rate) (TTP): the time difference between T0 and the time of peak intensity
- "Wash-in rate (staining rate): The tangent of maximum tangency between T0 and peak intensity time. It indicates the maximum rate of contrast medium uptake. This allows the early, strong contrast uptake of tumor tissue to be adequately estimated.
- "Wash-out" rate: the tangent of maximum steepness between the time of peak intensity and the last measurement point It indicates the maximum clearance rate of the contrast medium.
- "Brevity of enhancement" (BOE): The time between "wash-in" and "wash-out."
- Relative contrast enhancement (RCE): the percentage of signal intensity that increases between post-contrast and pre-contrast signal intensities, respectively.
- Maximum relative contrast enhancement (MRE): percentage of the signal intensity increase between the maximum post-contrast and pre-contrast signal intensities.
- The area under the curve (AUC): the integral of the curve divided by the area under the TIC.

Statistical analysis

Statistical analyses were performed using MedCalc for Windows (MedCalc Software, Ostend, Belgium). Statistical differences in TIC data were evaluated by an ANOVA test, and differences between groups were evaluated by the Student-Newman-Keuls test. Frequency and the chi-square test were used to compare the morphological characteristics of the lesions in the T_1 and T_2 weighted series. The receiver operating characteristic (ROC) curve method was used to determine the "cut-off" (threshold value) for the benign-malignant differentiation of the lesion groups. The statistical significance level was set at $p<0.05$.

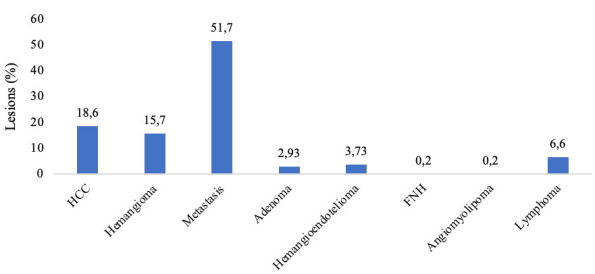
Results

Patient data

The number of patients in our study was 91 (M: 50 (54.9%); F: 41 (45.1%)). The age distribution was 28–81 years; the mean age was 55±15.7 years.

The total number of liver lesions evaluated was 376. Lesion sizes were 1–12.5 cm (mean 2.7 cm). 81% (n=303) of the lesions were malignant, and 19% (n=73) were benign.

The lesions included hemangiomas, HCC, metastases, adenomas, hemangioendothelioma, FNH, angiomyolipoma, and lymphoma. The distribution of the lesions is shown in Graph 1.



Graph 1. Distribution of lesions according to their types

Primary benign lesions (20%) were hemangiomas (n=60), adenomas (n=11), FNH (n=1), and AML (n=1). Primary malignant lesions included HCC (n=70), IHCCC (n=11), and malignant epitheloid hemangioendothelioma (n=14). Metastases (n=208) were predominantly gastrointestinal, including colorectal, gastric, esophageal, neuroendocrine carcinoma, gastrointestinal stomal tumor, gynecologic, prostate, pancreatic, and breast cancer (Fig. 6 and 7). The primary and metastatic distributions of the lesions are shown in Graph 2.

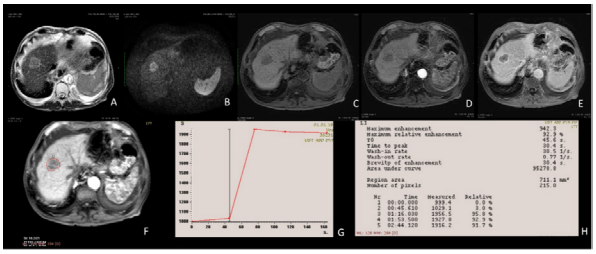
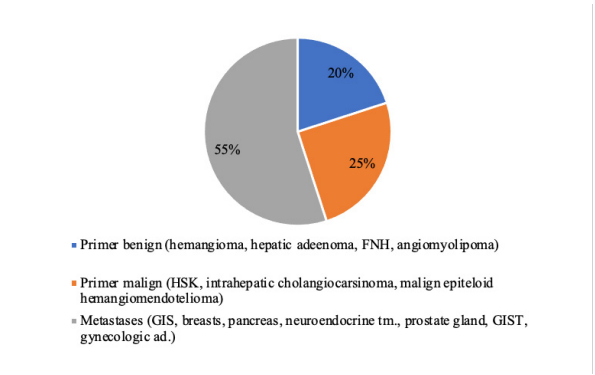


Fig. 6. Metastasis (stomach metastasis): (68 years old- male) A-TSE T2A, B- DWI b 1000, C, D, E- lesion in dynamic series, F, G, H- lesion in arterial phase in dynamic series, time-intensity curve and semiquantitative analysis values of the whole lesion



Graph 2. Distribution of primary and metastatic lesions

TIC semiquantitative analysis

The data for the TIC curves of the lesions are given in Table 1.

A paired comparison of AUC, MRE (F ratio: 3.602, p=0.001), and "wash-in" and "wash-out" (F ratio: 6.85, p<0.001) values of metastases and hemangiomas showed differences. Maximum enhancement and MRE values are different between hemangioendotheliomas and other groups (Graph 3). The wash-in rate of HCC was faster than metastases and hemangiomas (F ratio: 6.26, p<0.001). Metastases washed in more slowly than hemangiomas (Graph 4). There was a difference in TTP values in HCC, metastasis, and hemangiomas (p<0.001, F ratio: 11.63) (Graph 5).

The brevity of enhancement values were significantly different in HCC, hemangiomas, and metastases (F ratio: 3.51, p=0.001) (Table 2). The "wash in rate/wash out rate" value was higher in hemangiomas compared to metastases and HCC (F ratio: 2.43, P<0.02) (Graph 6). HCC washes out faster than metastases and hemangiomas (Graph 7).

In the ROC curve analysis used to determine the effectiveness of the "wash-out rate" value in the benign-malignant discrimination of lesion groups, the threshold value was found to be 0.08 (p<0.0001, 95% confidence interval: 0.61%–0.71%). At this cut-off value,

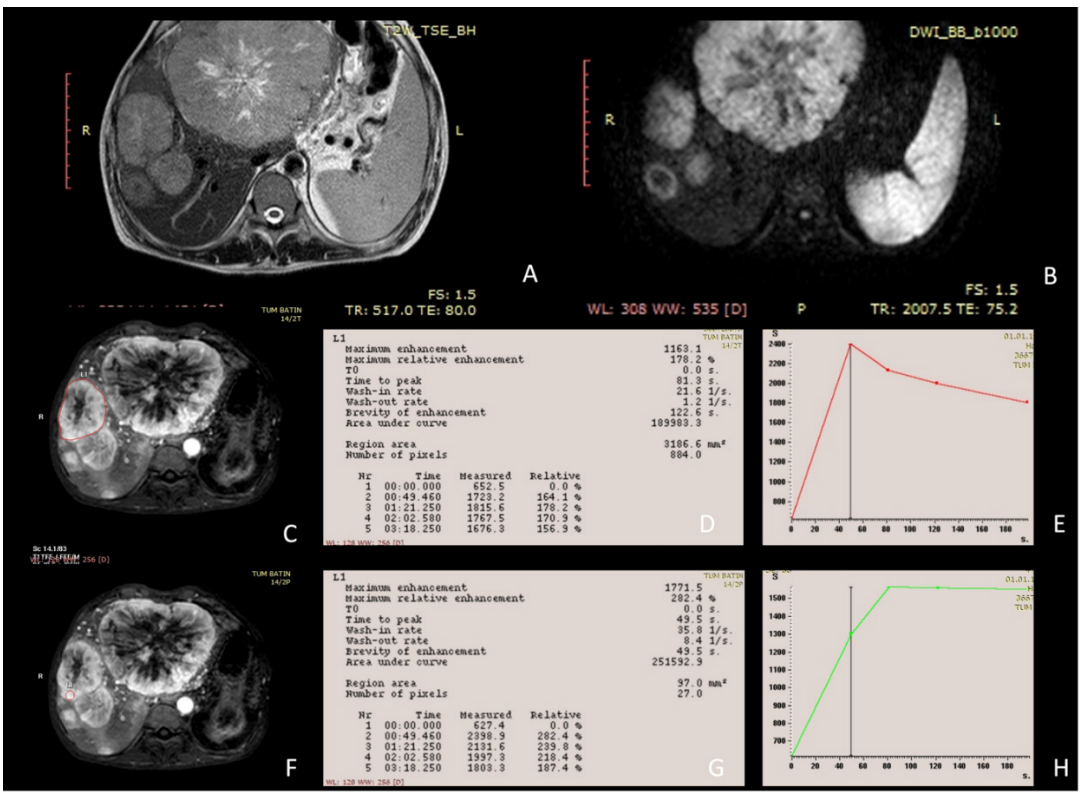
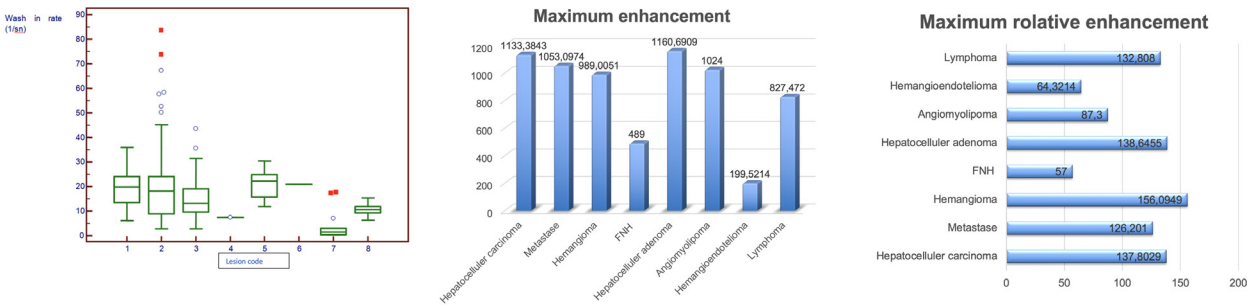


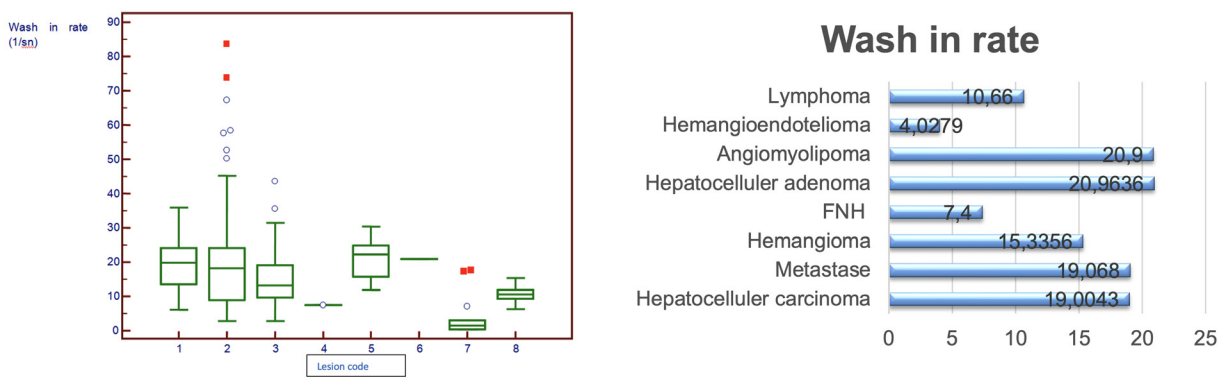
Fig. 7. Metastasis (neuroendocrine tumor): (41 years-male) A- TSE T2A, B- DWI b 1000, C, D, E- lesion in arterial phase in dynamic series, time-intensity curve and semiquantitative analysis values of the whole lesion, F, G, H- lesion in arterial phase in dynamic series, time-intensity curve and semiquantitative analysis values of the partial lesion

Table 1. Distribution of TIC data of the lesions

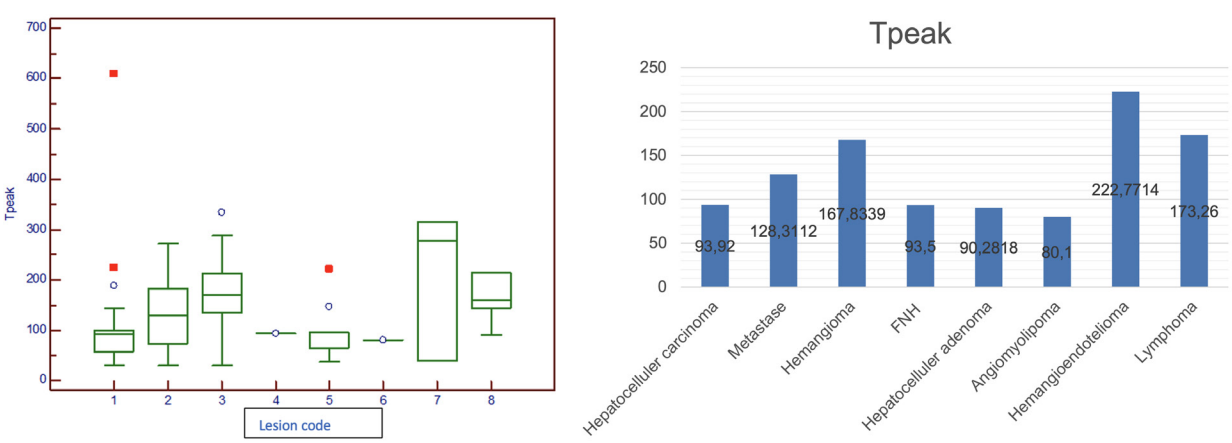
TIC MEAN/LESİONS	Hepatocelluler carcinoma	Metastase	Hemangioma	FNH	Hepatocelluler adenoma	Angiomyolipoma	Hemangioendotelioma	Lymphoma
AUC	176586.29	172292.02	130598.98	100745.	250616.27	191545	45594.14	101438.96
Brevity of enhancement	74.25	39.36	15.21	41.5	50.86	80.1	10.91	25.51
Maximum enhancement	1133.38	1053.09	989.01	489	1160.69	1024	199.52	827.47
Maximum rolative enhancement	137.8	126.2	156.09	57	138.64	87.3	64.32	132.81
T0	8.53	17.74	25.81	0	16.69	46.9	13.64	21.48
Tpeak	93.92	128.31	167.83	93.5	90.28	80.1	222.77	173.26
Wash in rate	19	19.07	15.33	7.4	20.96	20.9	4.03	10.66
Wash out rate	3.59	1.38	0.68	1	3.53	1.9	0.11	1.89
Wash in /wash out rate	10.01	23.47	69.99	7.4	8.34	11	28.56	20.24



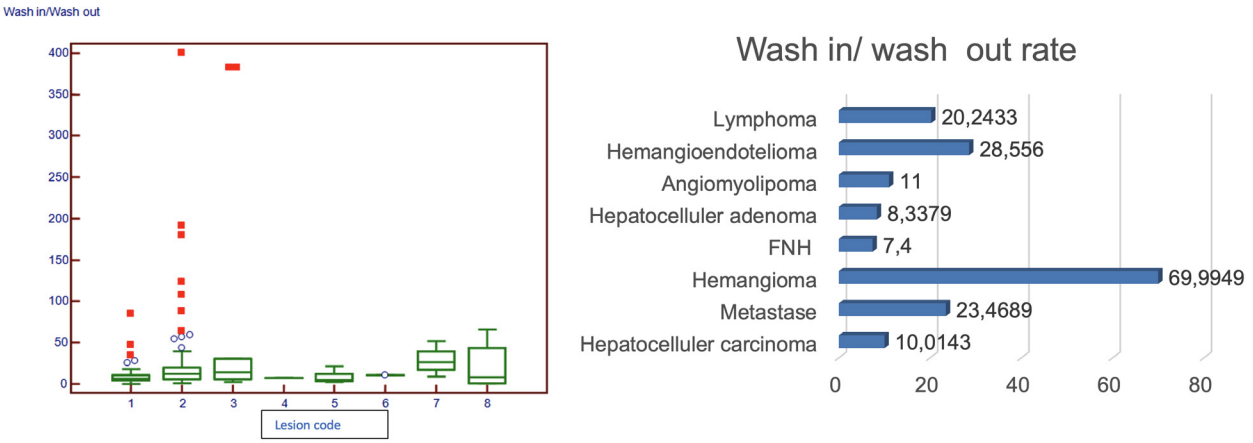
Graph 3. Distribution of the ME and MRE values of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)



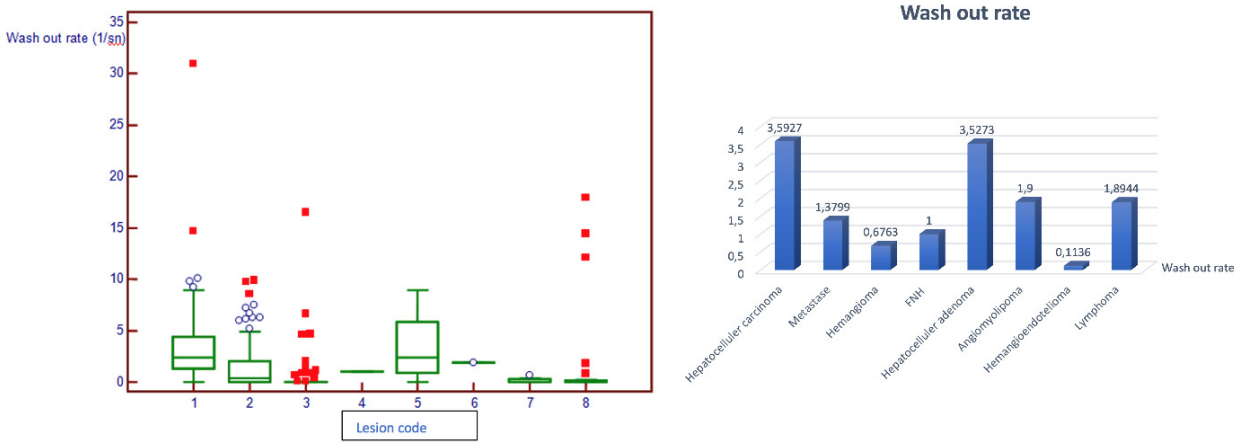
Graph 4. The distribution of the "wash-in" rates of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)



Graph 5. Distribution of the TTP values of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)



Graph 6. Distribution of "Wash in rate"/"Wash out rate" values of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)

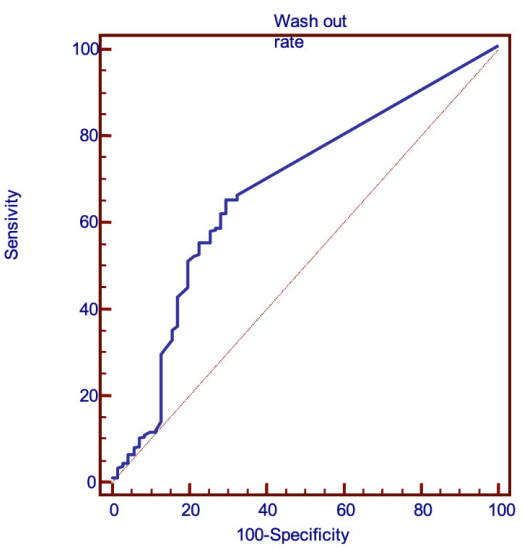


Graph 7. The distribution of the "Wash Out Rate" values of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)

sensitivity was 64.3% (95% confidence interval: 58.3%–70.2%) and specificity was 70.4% (95% confidence interval: 58.4%–80.7%). The positive LR (likelihood ratio) was 2.18, and the negative LR was 0.51 (Graph 8).

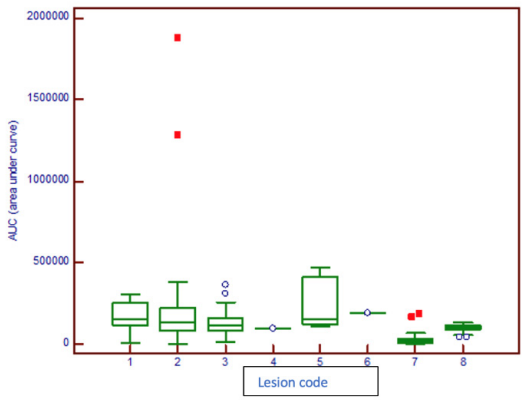
Table 2. A comparison of the BOE values of the lesions (1 = hepatocellular carcinoma, 2 = metastasis, 3 = hemangioma, 4 = FNH, 5 = hepatocellular adenoma, 6 = angiomyolipoma, 7 = hemangioendothelioma, 8 = lymphoma)

Lesions	n	Mean	Different (p<0.05) from factor
(1)1	70	74.25	(2)(3)(7)(8)
(2)2	194	39.36	(1)(3)(7)
(3)3	59	15.21	(1)(2)
(4)4	1	41.5	
(5)5	11	50.86	
(6)6	1	80.1	
(7)7	14	10.91	(1)(2)
(8)8	25	25.51	(1)

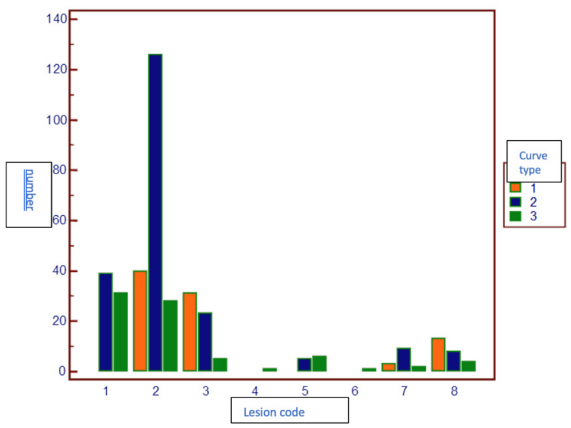


Graph 8. "Wash-out rate" ROC curve analysis

The "area under curve" and "wash-in" values of hemangioendothelioma and lymphomas are different from those of HCC, metastases, and hepatic adenoma (Graph 9).



Graph 9. The distribution of the "AUC" values of the lesions (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)



Graph 10. Distribution of lesions based on the type of curve (1 = HCC, 2 = Metastasis, 3 = Hemangioma, 4 = FNH, 5 = Adenoma, 6 = Angiomyolipoma, 7 = Hemangioendothelioma, 8 = Lymphoma)

TIC curve analysis

The distribution of lesions according to curve types is shown in Graph 10. In our hemangioma cases, there were type I and type II curve patterns, predominantly type I. In hemangiomas over 2.5 cm, ROIs measured from the periphery of the lesion showed a type I curve almost completely. In hemangiomas and metastases, type I contrast enhancement curves were observed.

Contrast enhancement was present in the arterial phase in our HCC lesions. The type III contrast enhancement curve was observed in the majority (61.4%). All 27 lesions with a type II curve developed in a cirrhotic background, with 13 measuring less than 2 cm and 7 measuring more than 5 cm. In 12 lesions (>3 cm) with a type II curve in ROI measurements including the whole lesion, a type III curve was observed in partial measurements.

Discussion

Accurate recognition and differentiation of FLL with noninvasive imaging techniques is important. Dynamic MRI can be used in clinical practice for noninvasive quantification of hepatic perfusion, which is essential in differential diagnosis.[9-11] The difference in changes in arterial and portal venous blood flow in benign and malignant lesions makes perfusion imaging complementary to conventional imaging in lesion detection and especially in characterization.[12] Perfusion MRI was first described for imaging regional and global blood flow in the heart, lung, and brain.[13] MR perfusion of the liver was reported in 1994 using gadolinium in rats.[14] Subsequently, several studies involving animal and human subjects were reported.[15] In the Materne study, tissue tracer concentration in rabbits was first estimated by empirical determination of the relationship between the pulse sequences used and the signal intensity and T1 values; then perfusion MR imaging was used to evaluate perfusion parameters in rabbits with and without cirrhosis and also in humans.[16,17] It has been suggested that perfusion imaging can be used as an in vivo marker of angiogenesis and even give more accurate results than histological examination, which is considered the gold standard for demonstrating angiogenesis.[18,19] Thanks to its high temporal and spatial resolution, DCE MRI increases the detectability of lesions even in less experienced observers. However, visual assessment of the wash-in and wash-out of lesions is sometimes difficult.[18] Therefore, it is advantageous to evaluate DCE MRI with functional maps and quantitative or semi-quantitative parameters.[17,18] The goal of quantification techniques is to reduce the variability caused by the selection of imaging systems, magnetic field strengths, sequences, and parameters so that patients and centers can be compared. Kinetic parameters of quantification techniques may also contribute to the understanding of tumor biology.[12] Therefore, for applicability in daily practice, obtaining quantitative data should be simplified, reproducible, and less time-consuming. Our study demonstrated the applicability of semi-quantitative parameters derived from DCE-MRI functional maps in daily practice.

Mathematical modeling of imaging data is used to get quantitative measurement parameters like vascular density, permeability, perfusion, extravascular space, and plasma volume. These parameters are related to the pathophysiology of the lesion.[20] In a study comparing perfusion parameters between benign and malignant liver lesions, Ippolito et al. discovered that benign lesions had higher values than malignant ones (RAE 33.8

vs. 49.13%; RVE 66.03 vs. 40.54%; RLE 80.63 vs. 47.52%; ME 776 vs. 448.78%; MRE 86.27 vs. 49.85%; TTP 146.95 vs. 183.79%).[13] The usefulness of the perfusion and permeability parameters obtained in the detection of HCC and the differentiation of liver metastases and HCC has been reported.[21-23] The authors also reported that distribution volume and perfusion can distinguish liver metastases from neuroendocrine tumors according to their enhancement patterns (i.e., hypo- or hyper-rich).[24] Both arterial fraction and arterial hepatic blood flow have been shown to be significantly higher in HCC. Portal venous blood flow and volume of distribution have been found to be significantly lower in HCC compared to the surrounding cirrhotic parenchyma, possibly due to significant changes in tumor microvascular architecture and angiogenesis.[25] In the literature, it has been reported that quantitative parameters may also be useful in monitoring treatment response.[25, 26] Applications of quantitative MR perfusion have been investigated in the monitoring of treatment response after locoregional and systemic therapies in HCC.[27] Consistent with Taouli et al.[25] Ippolito et al. found differences in semi-quantitative perfusion analysis in tumors with and without completed TACE treatment.[26] The authors looked at how well perfusion MR imaging could show early changes after treatment. They found that those who were not targeted with TACE had significantly lower portal venous hepatic blood flow and a higher arterial fraction. In a study using rodents as a preclinical model, Braren et al. found that measuring the extravascular extracellular volume fraction one day after trans-arterial embolization was linked to more tumor necrosis.[28] In fact, Michielsen et al. reported the usefulness of perfusion parameters evaluated before TACE in predicting progression-free survival.[29] Hsu et al. showed that the Ktrans value was well correlated with tumor response, progression-free survival, and overall survival in patients receiving systemic therapy for advanced HCC and suggested that this may be related to changes in tumor vascularization caused by anti-angiogenic therapy.[30] Some studies have reported that early perfusion changes in advanced HCC are valuable in predicting overall survival after systemic therapy.[31,32] Similar results have been reported for K trans and various perfusion parameters in patients with colorectal metastases treated with chemotherapy in combination with targeted therapies. Coenegrachts et al. showed that the constant ratio between extravascular extracellular space and blood plasma (i.e., kep = Ktrans/ve) was significantly higher in treatment responders than in non-responders at baseline, with a significant decrease in this group after treatment.[33] De Bruyne et al. also associated a >40% reduction in Ktrans after treatment with longer progression-free survival.[34] Like Hirashima et al., Cannella et al. stated that changes can be seen in the early post-treatment period and observed changes in both kep and Ktrans within one week after treatment, which may be helpful in predicting response to treatment.[6,35] But these quantitative data aren't very useful because they depend on a lot of different factors and take a lot of time to use.

Galbraith et al. reported that the use of complex pharmacokinetic modeling to generate fully quantitative parameters did not significantly alter the reproducibility of the technique and that simpler semiquantitative techniques were sufficiently reproducible in measuring relative changes in patients.[36] Some studies suggest that the semiquantitative DCE MRI perfusion parameters are different for hemangiomas and malignant tumors like HCC, cholangiocarcinoma, and metastases.[18,37]

In our study, the 0.08 threshold value we found for "wash-out" in DCE MR semiquantitative analysis is instructive in distinguishing between malignant and benign liver tumors. Ippolito et al. also reported that benign lesions showed higher values in semiquantitative analysis compared to malignant lesions, which is consistent with our findings.[38] It has also been reported that contrast enhancement and perfusion values in lesion groups may provide complementary quantitative information that may improve the final diagnostic accuracy if those with similar patterns can be clustered into subtypes. Therefore, we believe that the combination of functional information with morphological findings and research in larger case series with an increased number of subgroups for reproducible semiquantitative analysis may provide a standardized method that can be easily incorporated into the clinical workflow. Still, there will always be some variation because of changes in tissue blood flow, magnetic field changes, patient position, and body temperature.[18,39,40] In our study, the higher maximum relative enhancement of hemangiomas compared to metastases and the longer "wash-in" time of metastases compared to hemangiomas; the significantly shorter time between the arrival of contrast to the tissue and peak intensity time (TTP) in our HCC cases compared to metastases and hemangiomas; and the difference in BOE values in HCC, hemangiomas, and metastases (F ratio: 3.51, p=0.001) may be clues for the differential diagnosis. The difference may be explained by the fact that hemangiomas consist of blood-filled cavities lined with endothelium over a thin fibrous stroma and a large extracellular space, whereas malignant liver lesions show tumoral angiogenesis.[12,41] It has also been documented in the literature that HCCs are mainly supplied by the hepatic artery, whereas hypovascular metastases have a diffuse portal blood supply.[42] Abdullah et al. reported the usefulness of perfusion MRI in differentiating HCC and colorectal liver metastases.[43] DCE MRI arterial phase evaluation showed a positive predictive value of 82–90% and specificity of 80–99% for the diagnosis of hemangioma, HCC, and metastases.[18] In the literature,

the values of perfusion parameters (such as RAE, ME, and MRE) have been reported to be significantly higher in HCC lesions than in hypovascular metastases, consistent with the typical hypervascularity of HCC and the hypovascularity of metastases.[38] Although it is well documented that HCCs are mainly supplied by the hepatic artery and that hypovascular metastases have a diffuse portal blood supply[42], Alicioğlu et al. reported that no metric parameter could be identified to distinguish between HCCs and metastases.[18]

In metastases, differences may be observed depending on the degree of underlying hepatic arterial supply. However, there is some overlap between benign and malignant tumors. In this case, size may be effective; the lack of wash-in/wash-out phenomena in smaller tumors may be explained by the fact that angiogenesis has not yet developed. Another factor influencing behavior may be the degree of tumor cellular differentiation.[44] In our study, although the "wash in rate/wash out rate" value was higher in our haemangioma cases compared to metastasis and hepatocellular carcinoma (F ratio: 2.43, $p<0.02$), we could not obtain a reliable threshold value for discrimination in ROC curve analysis.

Although the number of lesions in our hemangioendothelioma and lymphoma cases was acceptable, the statistical data were homogenized because of the multiple lesions belonging to a small number of patients. However, a healthy interpretation can be made with the results of comprehensive analyses in subgroups.

Ippolito et al. and Donati et al. evaluated the diffusion and perfusion MRI features of FNH. They concluded that in semi-quantitative analysis, all lesions showed a rapid and marked increase followed by a rapid decay and then a slow decay, depending on the dominant arterial support, whereas the normal surrounding parenchyma showed a rapid increase followed by a plateau of slow decay; perfusion MRI may be an additional tool in accurately diagnosing FNH with the information it provides about the vascularity of the lesions.[38,45] It has been reported that functional-metric evaluation helps lesion characterization in the differentiation of hypervascular pseudolesions consisting of arterioportal shunts, which are frequently seen in cirrhosis or chronic hepatitis, and perilesional enhancement in metastatic lesions from true metastases.[18,46]

The degree of histopathological differentiation of the primary mass, as well as hypo- or hypervascularity, were found to be effective in observing a Type I contrast enhancement curve in metastases near hemangiomas in our study. However, since metastases were not classified in our study, no evaluation could be made in this direction. In future studies, the categorization of metastases considering the degree of vascularization and differentiation may help obtain meaningful clues for the differential diagnosis of the primary disease.

In clinical practice, quantification of hepatic blood flow has been reported for the assessment of liver metastases and chronic liver disease and for the study of the systemic availability of drugs.[9-11] Furthermore, hepatic perfusion parameters have been used to assess changes in sinusoidal permeability in cirrhosis.[15] The fact that all of the HCC lesions with a type II curve in our study developed on the background of chronic liver cirrhosis may be due to the limited specificity of arterial hypervascularity in cirrhotic livers, as well as inhomogeneity due to lesion size and poor sensitivity of portal and venous wash-out in lesions below 2 cm.[37] The observation of a type III curve in partial measurements in lesions >3 cm, which showed a type II curve in measurements including the whole lesion, suggested that partial measurements may be more sensitive due to the heterogeneity in the internal structure of HCC lesions with capsular staining. If the number of patients with HCC increases, the heterogeneity of the patient group and the variety of factors affecting the contrast kinetics of the lesion will increase, and the effects of the pattern difference in the curves due to mass, background liver, and measurement technique can be revealed in more detail.

Although diffusion-weighted (DW) MRI is widely used in clinical practice to differentiate focal liver lesions, it has been reported that quantitative ADC threshold values have variable accuracy depending on many factors, such as lesion type, b-values used for acquisition, and necrosis or fibrotic changes in malignant lesions, and that differentiation should not be made by ADC measurement alone due to the overlap of malignant and benign lesions and the differentiation of tumors.[47-49] Inclusion of parameters in algorithms will probably reduce the number of suspicious cases; however, in our study, we evaluated semiquantitative analysis data, not diffusion parameters.

Study limitations

Our study had some limitations. Firstly, although a large patient-lesion population was included in the study, statistical evaluation became impossible for some lesion types due to our limited number of cases. Secondly, the placement of ROIs by a single reader may be considered as a limitation. Thirdly, considering our cirrhotic patients, the surrounding liver used as reference tissue was not the same between the two groups. Furthermore, semiquantitative analysis may be affected by acquisition parameters, injection protocols, including contrast volume and injection rate, and physiological conditions such as respiratory movement.[50] Therefore, overlapping quantitative values of a single perfusion parameter may represent a bias in functional analysis, but multiparametric evaluations including conventional sequences and DWI may be the solution.

The current status of quantitative MRI in FLLs is somewhat paradoxical. Although numerous studies and clinics report promising results, their application in clinical practice is scarce. However, as seen in our study, quantitative data combined with qualitative imaging may provide solutions in various clinical situations. However, the clinical applications of perfusion imaging for FLLs are limited. The reasons for this include complex pharmacokinetic models caused by the fact that the liver is a mobile, blood-filled, flexible organ with double vascular access and fenestrated sinusoids, differences in imaging systems, non-standardized acquisition protocols, respiratory movements, possible iron overload, and starvation. This situation also makes it difficult to compare research studies. Most of the published studies are single-center and retrospective, and standardized acquisition parameters, post-procedural methods, or predicted results cannot be reported. The complex procedure required for perfusion quantification limits its use.[5,6] To overcome the limitations, the medical imaging community and the Alliance for Quantitative Imaging Biomarkers, the Radiological Society of North America, or the Biomarker Inventory are making a collective effort. The fact that new sequences, such as golden angle radial sparse parallel (GRASP) imaging, allow safe assessment of hepatic perfusion parameters with quantitative results comparable to perfusion CT, gives hope that next-generation sequences will make perfusion data more readily available.[51-53]

Conclusion

DCE MRI semiquantitative analysis of the abdomen may be useful in daily practice as a potential aid in differential diagnosis by providing noninvasive in vivo information about the nutrition and microvascular properties of lesions without increasing the application time. Furthermore, it may facilitate diagnostic studies in the detection and staging of hepatic diseases, treatment follow-up, and the development of anti-tumor drugs.

In our study, we thought that the difference in "Tpeak" and "wash-out" rate values in the semiquantitative analysis of DCE MR TIC in the differentiation of HCC, metastasis, and hemangioma and the 0.08 threshold value we found for "wash-out" in the ROC curve analysis in the differentiation of malignant and benign lesions of focal liver lesions may be guiding. Therefore, the comparison of semiquantitative analysis in larger case series in which the number of subgroups is increased may provide new opportunities as a reproducible, standardized method that can be easily combined with clinical workflow.

Declarations

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Author contributions

Conceptualization, H.G.D. and Z.G.K.; Methodology, H.G.D and Z.G.K; Software, M.O.A.; Validation, H.G.D., D.A.U and N.K.; Formal Analysis, H.G.D.; Investigation, H.G.D. and D.A.U; Resources, H.G.D. and D.A.U.; Data Curation, H.G.D. and N.K; Writing – Original Draft Preparation, H.G.D. and D.A.U; Writing – Review & Editing, H.G.D., Z.G.K; Visualization, H.G.D. and M.O.A; Supervision, H.G.D. and Z.G.K.

Conflicts of interest

Authors state no conflict of interest.

Data availability

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors. All data was processed anonymously, according to the privacy legislation.

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REVIEW PAPER

Relationship between obesity, insulin resistance and cell membrane properties

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ABSTRACT

Introduction and aim. The obesity is one of the greatest public health problems in developing countries and it is a triggering factor for diabetes associated with insulin resistance. The importance of cell membrane lipids as essential regulators of insulin resistance, since changes in the dynamic properties of the cell membrane (e.g., membrane fluidity), could be one of the events by which obesity affects insulin sensitivity. Thus, the insulin resistance may not only be a cause but also a consequence of lipid disorders such as dyslipidemia and/or cell membrane phospholipid composition change. The modification of plasma membrane lipid composition can change membrane biophysical properties and thus influencing protein-lipid interactions, enzymatic activity and regulation of surface receptors. Alterations in the lipid composition modify the fluidity of plasma membranes and the expression of membrane functions, such as receptor binding and enzyme activities. This review summarizes the current knowledge on the effects of the modulation of plasma membrane lipid composition and membrane fluidity in the functionality of membrane proteins involved in insulin activity, including the insulin receptor, glucose transport and Na^+/K^+ ATPase and, in turn, the key features of the metabolic syndrome.

Material and methods. References for that article were found through PubMed and Google Scholar, using terms: "obesity", "insulin resistance" and "membrane properties". The research was limited to abstracts and available full-text articles.

Analysis of the literature. There is a strong relationship between dietary lipids, membrane lipid profiles and insulin resistance. The changes in the dynamic properties of the cell membrane (e.g., membrane fluidity), could be one of the events by which obesity affects insulin sensitivity. The modification of plasma membrane lipid composition can change membrane biophysical properties and thus influencing protein–lipid interactions, enzymatic activity, and regulation of surface receptors. Modifications of membrane phospholipid composition could have a role in the insulin action by altering membrane fluidity and, as a consequence, the insulin signaling pathway.

Conclusion. As conclusion the membrane-lipid therapy approach can be used to treat important pathologies such as obesity and many others diseases such as : cancer, cardiovascular pathologies, neurodegenerative processes, obesity, metabolic disorders, inflammation, and infectious and autoimmune diseases. This pharmacological strategy aims to regulate cell functions by influencing lipid organization and membrane fluidity, inducing a concomitant modulation of membrane protein localization and activity which might serve to reverse the pathological state. Through this review we suggest an in-depth analysis of the membrane lipid therapy field, especially its molecular bases and its relevance to the development of innovative therapeutic approaches.

Keywords. insulin resistance, membrane properties, obesity

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Introduction

The metabolic syndrome (MetS) is becoming a matter of great concern throughout the world. MetS is a group of risk factors than can lead to heart disease and type 2 diabetes (T2DM): insulin resistance, high blood pressure and obesity. The overall prevalence of the metabolic syndrome in the USA is 33–39%, with significantly higher prevalence in women compared with men.[1,2] In European countries, the MetS has an estimated prevalence below 18% in Denmark or in Spain the prevalence is above 30%.[2,3] Obesity is a major risk cause of several comorbidities such as cardiovascular diseases, type II diabetes, cancers and other health problems.[4]

Overweight and obesity are significant risk factors for developing the MetS and are the major nutrition-related disorders worldwide. The prevalence of obesity is one of the greatest public health problems also in developing countries that have undergone important changes in lifestyle, eating habits and physical activity in the last years.[5] More than 1 billion people worldwide are obese, 650 million adults, 340 million adolescents and 39 million children. This number is still increasing. WHO estimates that by 2025, approximately 167 million people adults and children will become less healthy because they are overweight or obese. WHO estimates that 59% of adults are living with overweight or obesity, with more than half of adults in 50 out of 53 Member States in the European Region living with overweight or obesity. Levels are higher among males (63%) than among females (54%) across the WHO European Region and in most countries. Obesity occurs when dietary energy intake exceeds energy expenditure. Extrapolations of the literature findings to alterations of membrane function, relevant to the pathogenesis of obesity, are speculative, although attractive. Altered lipoprotein and phospholipid metabolism could be responsible for the perturbation of plasma membrane composition and physical properties; these would, in turn, affect the structural

and functional properties of membrane enzymes and yield, as a consequence, impaired ion transport and abnormal thermogenesis, which may be involved in the pathogenesis of obesity.

However, it should be mentioned that diet intervention is also a powerful tool to prevent the development of the obesity, healthy diets and particularly dietary fatty acids have been shown to have a protective role against the metabolic syndrome. Particularly, dietary fatty acids, among other mechanisms, by modifications of the lipid composition of the membranes in insulin-sensitive tissues.

Aim

The architectural influence of the plasma membrane on insulin action, particularly the molecular events regulating insulin receptor binding and glucose transport, is the focus of this review.

Material and methods

References for that article were found through PubMed and Google Scholar, using terms: "obesity", "insulin resistance" and "membrane properties". The research was limited to abstracts and available full-text articles.

Analysis of the literature

Dietary lipid and insulin resistance

Healthy diets rich in fruits, vegetables, grains, fish and low-fat dairy products have a protective role.[6] The quality of dietary fat is also determinant in the effect of diet on insulin sensitivity and the metabolic syndrome. Diets high in saturated fatty acids (SFA) impair both insulin sensitivity and blood lipids, while substituting carbohydrates or monounsaturated fatty acids (MUFA) for SFA revert these abnormalities in both healthy and diabetic subjects.[7-10]

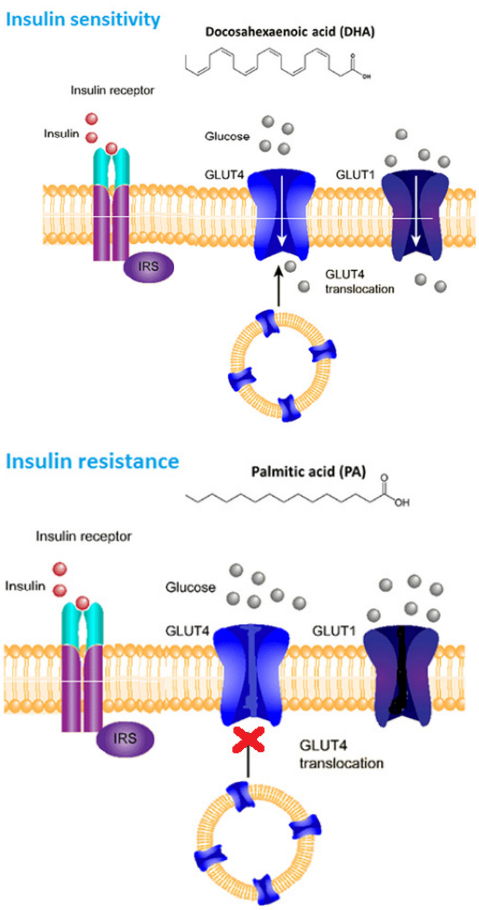

Fig. 1. Schematic diagram showing the role of dietary fatty acid on insulin signaling pathway

Cell membrane phospholipid composition is regulated by the fatty acid composition of dietary fat, being especially sensitive to n-6 and n-3 polyunsaturated fatty acids (PUFA), with a preference for the latter.[11,12] In contrast, membrane SFA and MUFA content is not as

dependent on the dietary fatty acid profile, as these fatty acids can be synthesized endogenously. Many studies have demonstrated a strong relationship between dietary lipids, membrane lipid profiles and insulin resistance, with high SFA diets leading to insulin resistance, whereas diets high in n-3, with a low n-6/ n-3 ratio, keeping insulin action at normal levels.[12-14] Many reports have shown that a high intake of dietary SFA significantly worsens insulin resistance, in particular through modifications in the composition of cell membrane phospholipids. Palmitic acid has been shown to have the capability to induce insulin resistance in the liver.[13] For instance, palmitic acid exposure resulted in the accumulation of ceramides and diacylglycerol, developing insulin resistance, reduction of hepatic GLUT-2 expression and decreased glucose uptake (Fig 1).

However studies of oleic acid have reported contradictory results. Some studies have shown an increase in insulin sensitivity with diets rich in oleic acid, whereas others have shown an inverse association between oleic acid and insulin sensitivity.[13,14]

The type and amount of dietary lipids influence the lipid composition of cell membranes and modulate the interactions with proteins involved in the regulation of insulin sensitivity but also processes associated with other components of the metabolic syndrome like dyslipidemia and hypertension. The effects, at least in part, are probably mediated by modification of the composition and structural properties of plasma membranes.[15]

Membrane properties and obesity

Membrane lipid composition and obesity

Changes in erythrocyte membrane phospholipid composition, especially in obese subjects, parallel those in membrane of other tissues providing a helpful model to study the effects of insulin resistance in plasma membrane.[16] The alterations in lipid composition and fluidity exist in erythrocytes from obese subjects.

Many studies have shown that erythrocyte membrane fatty acid composition in obese adolescents differs from that in age and sex matched lean controls, reflecting a decrease in n-3 PUFA and MUFA and an increase in SFA, especially in very-long chain SFA, like 24:0.[17] In addition, the erythrocyte membranes from obese subjects are characterized by a higher cholesterol/phospholipid ratio, which has been used as an index of membrane fluidity.[15] Membranes enriched in cholesterol and SFA and decreased in MUFA and PUFA showed enhancement of membrane rigidity, affecting the signaling pathways related to membrane proteins. It has also been proposed that insulin resistance may not only be a cause but also a consequence of lipid disorders such as dyslipidemia and/or cell membrane phospholipid composition abnormalities.

Min et al. described a decrease in the phospholipid phosphatidylethanolamine and arachidonic (20:4, AA) and docosahexaenoic (22:6, DHA) acids in red blood cells (RBC) from patients with gestational diabetes, often linked to obesity.[18] Other work of Cazzola et al. reported on an increase in the cholesterol/phospholipid ratio in RBC membranes from obese patients, together with a decrease in ω-3 fatty acids (e.g., DHA) and an increase in ω-6 (e.g., AA).[19] In the same way Pietiläinen et al. found increased proportions of palmitoleic acid (16:1) and AA, together with increased levels of ethanolamine plasmalogens in adipose tissue of the obese twins.[20] Recently, studies from Arranz group have found that, in children with obesity RBC membranes, saturated and trans-unsaturated fatty acyl chains were increased.[21] In a parallel study, they found that monounsaturated chains were decreased in obese children.[22]

The modification of plasma membrane lipid composition can change membrane biophysical properties and thus influencing protein-lipid interactions, enzymatic activity, and regulation of surface receptors.[23-25] Modifications of membrane phospholipid composition could have a role in the insulin action by altering membrane fluidity and, as a consequence, the insulin signaling pathway.

Membrane fluidity and obesity

Numerous studies have shown that changes in the dynamic properties of the cell membrane (e.g., membrane fluidity), could be one of the events by which obesity affects insulin sensitivity.[26] The particularity of this review is that we have focused on the nature of the phospholipids and sphingolipids presents in the membrane cell of the obese people and which may be the cause of this disease. Alterations in the lipid composition, particularly the relative proportions of cholesterol and phospholipids, modify the fluidity of plasma membranes and the expression of membrane functions, such as receptor binding and enzyme activities.[27] Many studies have demonstrated that some kind of lipids play a crucial role in the insulin-resistance. The insulin-resistant state was positively correlated with membrane sphingomyelin, phosphatidylethanolamine, and phosphatidylcholine contents, and negatively with phosphatidylinositol contents in the whole population. Multivariate regression analyses showed that two membrane parameters, phosphatidylethanolamine and sphingomyelin, were among the independent predictors of insulin resistance in the whole population, but also in the lean and the obese groups separately.[16] Intervention induced a significant reduction in body weight, fat mass, and insulin resistance. More important, the reduction in insulin resistance was directly associated with reduction in sphingomyelin and phosphatidylethanolamine contents. These results suggest that the abnormalities in the membrane phospholipid composition could be included

in the unfavorable lipid constellation of obesity, which correlated with impaired insulin sensitivity.

Measurement of membrane fluidity by steady-state fluorescence polarization is the most common technique to assess the physical state of the cell membrane.[28] The fluorescence anisotropy of a membrane probe is an inverse of the fluidity of the lipid region where it is situated. Various cellular functions that may be involved in insulin action, such as enzyme activity, ion and substrate transport, and receptor binding and capping, are modulated by the physical properties of the cell membrane. Beguinot and his collaborators demonstrated that erythrocyte membrane phospholipid composition is related to hyperinsulinemia in obese nondiabetic women.[29] This study shows that both the membrane cholesterol/phospholipids ratio and the fluorescence polarization of DPH in erythrocytes obtained from obese subjects were significantly higher than in erythrocytes from healthy subjects. The higher cholesterol/phospholipids ratio was generated by a net increase in cholesterol and a net decrease in total lipid-bound phosphorus; it was not associated with altered plasma concentrations of total cholesterol and triglycerides. The increased C/P ratio in erythrocytes is due to an average 2-fold increase in cholesterol with normal or elevated phospholipid levels compared to a 20% decrease in membrane phospholipids and a 17% increase in cholesterol in the erythrocytes from obese subjects.[30-31] More recently, surprising results was found by Sot and his collaborators, they show a clear tendency for obese patient RBC to exhibit a higher fluidity in their membranes, or, more specifically, at the polar–non-polar interface of the membrane bilayers than the control cohort. This group try to explain these results doing Lipidomics, they found significant changes in concentration of ω-3 and ω-6 fatty acids.[32] More precisely, obese patient RBCs undergo an increase is some ω-6 fatty acids such as arachidonic acid, while reducing ω-3 ones, such as DHA. In the other part, a significant reduction in SM is detected for obese patient RBC. Both events, SM reduction and, perhaps more decisively, the increase of ω-6 fatty acids seem to contribute to the afore mentioned fluidity of obese patient RBC membranes.

Membrane properties and insulin resistance

The mechanisms by which obesity predisposes to insulin resistance remain poorly understood. However, insulin resistance could be related to changes in cell membrane properties.

Many cellular functions involved in insulin action are modulated by the physical properties of the cell membrane, such as enzyme activity, ion and substrate transport, and receptor binding.[33] Consequences of structural alterations of plasma membrane properties include reduced Na^+-K^+ ATPase, decreased concentration of insulin receptors and decreased glucose transport.

Insulin secretion and insulin receptor binding

The β-cells respond to many nutrients in the blood circulation, including glucose, other monosaccharides, amino acids, and fatty acids, the amplitude of insulin secretion induced by glucose is much larger compared with that stimulated by protein or fat. The metabolism of glucose and other nutrients causes depolarization of the B-cells which subsequently causes an increase in intracellular Ca^{2+} and insulin secretion. Elevation of intracellular cAMP and activation of Ca^{2+}/phospholipid-dependent protein kinase C (PKC) have also been implicated in the regulation of insulin secretion. Glucose is also known to stimulate the generation of arachidonic acid (AA), and its metabolites, prostaglandins and hydroxyeicosatetranoic acids in pancreatic islets.[34,35] Arachidonic acid is known to release intracellular Ca^{2+} in several cell types, presumably from the endoplasmic reticulum and it is feasible that such a mechanism may underlie the effects of AA on insulin release since we have shown that elevations in cytosolic Ca^{2+} alone are sufficient to initiate insulin release.[36,37]

If AA is acting as a physiological fusogen by affecting membrane fluidity we might predict that structurally similar fatty acids would have similar effects, and the results of Band group demonstrate that eicosapentanoic acid and docosahexaenoic acid, but not eicosatrienoic acid, stimulate insulin release.[38] In contrast, oleic and linoleic acid are also produced in glucose stimulated islets and possess some fusogenic activity.[35,39] However, those fatty acids were relatively ineffective in stimulation of insulin secretion.[38]

The plasma membrane plays an important role in containing numerous proteins involved in receiving signals from hormones, growth factors, and other molecules. The first step of a metabolic cascade leading to glucose uptake is the binding of insulin to its receptor in the cell membrane. The activity of the insulin receptor, as well as its affinity to insulin depend on the fluidity of the cell membrane, which, in turn, are dependent on the membrane lipid composition. Increasing SFA content in phospholipids decrease membrane fluidity and leads to a decrease in the number of insulin receptors and the affinity of insulin to them. On contrary, increasing PUFA content in phospholipids increase membrane fluidity and improve insulin sensitivity.[40] Several studies have shown that insulin receptor signaling is impaired by low membrane fluidity, probably because lateral diffusion and localization to membrane microdomains is important for ligand binding and signaling. [41-43]

To demonstrate the relation between membrane fluidity and insulin sensitivity, the study of Tong and his collaborators was carried out with fifteen patients T2DM (ten male) and Twenty-one healthy white subjects (ten male) with normal glucose tolerance and no family history of diabetes mellitus.[44] The results shown

that the binding of insulin coincides with the conformational change and aggregation of insulin receptors.[45] The higher fluidity at the core of the diabetic leucocyte membranes may hinder conformational changes and aggregation of insulin receptors, resulting in impaired action of insulin. Interestingly, some data suggest that the antidiabetic drug metformin, by increasing membrane fluidity, may correct a protein configuration or configurations disturbed by the diabetic state.[26]

Na^+-K^+ ATPase

Sodium/potassium-ATPase is a membrane protein responsible for the active transport of Na^+ and K^+ ions across the plasma membranes of eukaryotes.[46,47] A reduction in Na^+/K^+-ATPase levels is associated with obesity and in several experimental systems, Na^+/K^+ ATPase is altered in response to changes in membrane lipid composition.[48-50] It could be speculated that membrane physical properties play a major role in maintaining ionic gradients across the bilayer, a process that involves a large amount of cellular energy.[51] In the same way, Iannello and his collaborators have shown that obesity may repress Na^+/K^+-ATPase enzyme activity, probably through the mediation of free fatty acids (FFAs), which are elevated in such cases.[52-54] FFAs, present in the membrane or as the products of phospholipase A2 (PLA2)-dependent regulatory pathway, tend to inhibit Na^+/K^+-ATPase.[55-46] Interestingly, Iannello et al. reported that Na^+/K^+-ATPase activity is reduced in the adipose tissue of obese hyperinsulinemic subjects.[52]

Glucose transport

GLUT are integral membrane proteins that contain 12 membrane spanning helices with both the amino and carboxyl termini exposed on the cytoplasmic side of the membrane. Specific transporter proteins (glucose transporters, GLUT) are required to facilitate glucose diffusion into cells according to a model of alternate conformation. GLUT4 is an insulin-regulated glucose transporter that is responsible for insulin-regulated glucose uptake into fat and muscle cells. In the basal state, GLUT4 cycles continuously between the plasma membrane and one or more intracellular compartments.[56] GLUT4 differs from other glucose transporters in that about 90% is sequestered in intracellular vesicles in the absence of insulin. Once the insulin receptor has been stimulated, the intracellular stores are translocated to muscle plasma membranes. A cascade of events culminates finally in membrane fusion with GLUT4 containing vesicles. These plasma membrane localized transporters subsequently facilitate the influx of plasma glucose into the cell.[56]

The plasma membrane is intricately involved in the initial (signal reception), intermediate (lipid and protein molecule compartmentalization), and final (GLUT4 intercalation) steps of this process. Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis and a smaller but important decrease in the rate of internalization by endocytosis.[57-60]

Glucose transport across the membranes could be influenced by membrane fluidity. Moderate increases in plasma membrane fluidity have been documented to increase glucose transport.[61,62] Furthermore, it has been shown that basal glucose transport is not fully active in fat cells and can be increased further by augmenting fluidity.[61] In direct support of that finding, insulin-stimulated glucose transport is decreased when fluidity diminishes.[62] The fatty acid composition of membrane phospholipids may influence glucose transport by GLUT. Garvey et al. observed that in patients with obesity, impaired glucose intolerance, T2DM and gestational diabetes impaired GLUT-4 function or translocation occurs.[63] Weijers et al. suggested that a shift from unsaturated towards SFA in phospholipid membranes counteracts the machinery responsible for GLUT4 insertion into plasma membrane, by creating a more tight packing of phospholipids and affecting glucose transport and insulin sensitivity.[57] In addition, cholesterol depletion from plasma membrane results in an increase in the basal-state plasma membrane level of GLUT4.[64] The finding that the endocytic rate of protein retrieval from the cell surface is cholesterol dependent provides an explanation for a buildup of this transporter at the plasma membrane.[64,65]

Activation of phosphatidylinositol-3 kinase (PI3K) is one of the important steps in insulin signaling downstream of IRS, as it is involved in the translocation of GLUT4 to the cell membrane in response to the insulin signal but its activity in response to insulin can be totally inhibited by fatty acids.[66] However, whether fatty acids act on PI3K directly or mediated by PKC is still unclear. On the other hand, it has been suggested that PUFA can act as ligands of peroxisome proliferator-activated receptor-gamma or modulate its expression, thus increasing GLUT4 transcription and synthesis, and improving insulin resistance.[67,68]

Conclusion

Membrane lipid composition, membrane lipid structure, and membrane lipid fluidity influence the localization of proteins in membrane microdomains via protein–lipid interactions, facilitating specific protein-protein interactions and their resulting signals. Therefore, regulating the membrane lipid composition through pharmaceutical or nutraceutical interventions can serve to normalize signals that have been altered under different pathological conditions.

Membrane lipid therapy has emerged as a novel and innovative therapeutic concept that facilitates the design/discovery of new molecules. Molecules developed using this strategy target the membrane lipid boundary of cells and/or internal organelles, where many cellular

functions occur. The development of such new drugs is aided by the identification of the factors regulating membrane lipid structures, and their roles in cell signaling and pathophysiological processes, and such information has allowed and will facilitate the design and discovery of novel molecules for the treatment of important diseases.

Declarations

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Author contributions

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

Conflicts of interest

The author have no conflicts of interest to declare.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

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REVIEW PAPER

Innate defenses to intestinal cell death in necrotizing enterocolitis – spotlight on macrophage efferocytosis and its efficacy in rescuing inflamed intestinal mucosa

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ABSTRACT

Introduction and aim. Necrotizing enterocolitis (NEC) is a grave gastrointestinal disease of preterm infants which is widely prevalent in the neonatal intensive care units. Current treatment options are very limited with high mortality and morbidity. With no disease specific interventions, understanding nascent cellular events that occur immediately after microbial insult can offer insights for devising novel treatment options for curtailing the disease progression in NEC. In this regard, intestinal cell death in NEC is a primordial cell-signaling event and is regarded as a harbinger of future pathological derangements such as increased intestinal permeability, intestinal dys-homeostasis, and systemic inflammation.

Material and methods. We performed PubMed search of relevant articles that describes the host response to intestinal cell death in NEC by cellular battalion including dendritic cells, lymphocytes, neutrophils and macrophages which are important in containing intestinal inflammation.

Analysis of the literature. We particularly focused this review on enumerating macrophage efferocytosis, and pertinent novel treatment modalities based on this physiological process that has inherent capability for down regulating inflammation and promoting tissue repair in NEC. We highlighted its mechanistic aspect including mediators, receptors and signaling mechanisms and its physiological significance.

Conclusion. Macrophage efferocytosis is an overlooked and undervalued physiological defense mechanism to clear the dying intestinal epithelial cells for facilitating tissue healing and restoring the intestinal homeostasis. Any impairment of this critical defense mechanism can result in rapid clinical progression and systemic complications. Understanding its importance in the pathogenesis of NEC is important for designing novel therapeutic interventions to attenuate disease progression.

Keywords. efferocytosis, inflammation and immune responses, intestinal cell death, macrophage, necrotizing enterocolitis

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Introduction

It has been estimated that 7 out of 100 very low birth weight infants (VLBW) develop necrotizing enterocolitis (NEC) in Neonatal Intensive Care Unit.[1] The pooled estimate of NEC by Quality Effect Models and Random Effect models is approximately 6% and 7% respectively.[1] In a prospective analysis study, examination of 473,895 VLBW infants born between 2006 and 2017 from 820 United States clinical centers revealed that, the incidence of medical and surgical NEC is approximately 58.3% and 41.7% respectively.[2] The risk factors that are responsible for NEC are classified into prenatal factors (maternal cocaine use, pregnancy-induced hypertension, maternal infections and decreased placental blood flow), intrapartum factors (maternal cardiac arrest, umbilical cord prolapse, chorioamnionitis and placental abruption) and clinical course factors (patent ductus arteriosus (PDA), endotracheal intubation, bag mask ventilation, hypothermia, hypoventilation, fortified breast milk, infections, antibiotics, feeding intolerance and H2 receptor antagonists.[3] Due to these risk factors, intestinal tissues of preterm infants are exposed to wide variety of pathological insults such as lipopolysaccaride (LPS), tumor necrosis factor-alpha (TNF-α), nitric oxide, interferon gamma (IFN-γ) and cytokines.[4,5] This makes them vulnerable to assorted types of intestinal cell death ranging from apoptosis, necrosis, pyroptosis, necroptosis to autophagy (Fig. 1).

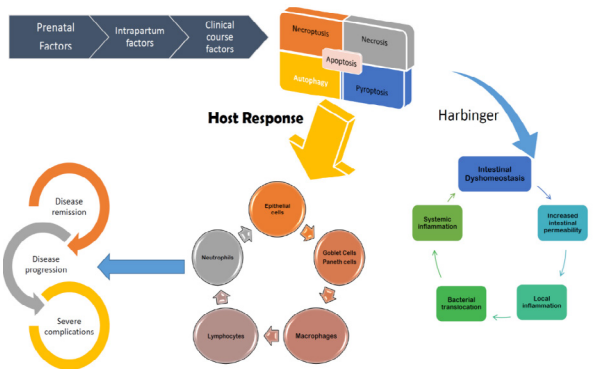

Fig. 1. Assorted cell death and host response in NEC. Preterm and very low birth weight infants are exposed to prenatal, intra-natal and clinical course risk factors. This results in assorted types of intestinal cell death ranging from apoptosis, necrosis, necroptosis autophagy, and pyroptosis. Intestinal cell death can be regarded as a harbinger of future pathological derangements such as changes in intestinal permeability, local inflammation, and translocation of bacterial load inside blood vessels. These changes lead to spread of systemic inflammatory response. Host response to intestinal cell death is mediated by cellular battalion ranging from epithelial cells, goblet cells, neutrophils, lymphocytes, dendritic cells and macrophages.
Depending on the robustness of host immune response, clinical outcomes of NEC can range from complete remission to clinical progression and systemic complications with associated mortality and morbidity

Apoptosis has been widely documented in the postmortem examination of intestinal tissues in the NEC patients.[6] Apoptosis can occur via caspase-8 mediated direct pathway or mitochondrial mediated indirect pathway, with both pathways converging on the secondary caspases (3 and 9) for final execution of the apoptotic cascade.[7] Previous work indicates that, apoptosis of the intestinal epithelial cells provides the first signal for subsequent intestinal necrosis and resulting pathological consequences.[6] Proliferation of pathogenic bacteria within the intestinal lumen releases toxic stimuli causing activation of inflammatory cascade and ultimately resulting in the intestinal necrosis.[4] Necroptosis is another form of cell death requiring the activation of receptor interacting protein kinases, as well as phosphorylation and membrane translocation of mixed lineage kinase domain-like protein due to the presence of damage associated molecular patterns (DAMPs) such as TNF-α.[8] Previous studies indicate that, activation of toll-like receptor 4 (TLR4) signaling is mainly responsible for the occurrence of necroptosis in the NEC animal models which is partially inhibited by the supplementation of breast milk.[9] Pyroptosis is another type of cell death primarily mediated by caspase-1 characterized by cellular swelling, osmotic lysis, release of pro-inflammatory cytokines, interleukin (IL) IL-1β and IL-18, nuclear condensation and DNA cleavage.[10] Pyroptosis is another kind of cell death implicated in the pathogenesis of NEC due to the documentation of increased mRNA levels of IL-1β, IL-18 and nucleotide-binding domain, leucine-rich repeat–containing proteins pyrin domain containing 3 (NLRP3) in the intestinal tissues of premature infants.[11] Although autophagy is generally regarded as a protective mechanism, it can mediate cell death, which can identified by the excess accumulation of large size autophagic vacuoles within the cytoplasm of dying cells.[12] According to a study performed by Yu et al., autophagy was the initial cellular event that heralded the subsequent onset of apoptosis in the intestinal epithelial cells in the rat model of NEC.[13] In the same study, administration of erythropoietin resulted in the protection against autophagy and apoptosis in the intestinal epithelial cells via protein kinase B/mammalian target of rapamycin and mitogen activated protein kinase (MAPK) pathways respectively.[13] The array of pathological findings that can be found on examination of the intestinal tissues on NEC infants can range from ischemia necrosis, inflammation, bacterial overgrowth, epithelial regeneration, fibrosis and granulation tissue formation.[14] Intestinal cell death in NEC is regarded as a nascent cellular event that heralds the onset of future

pathological derangements in the intestinal epithelium and surrounding intestinal milieu which can facilitate the rapid local disease progression and systemic complications. Specifically, it is a harbinger of leaky intestinal barrier, dissonance in intestinal homeostasis, local inflammation, and systemic inflammation (Fig.1). The key to clinical remission and cessation of disease progression from early stages of NEC rests on launching counteractive robust defense mechanisms for neutralizing the bacterial insult and invigorating anti-inflammatory pathways (Fig.1). This will eventually pave the way for tissue healing and restore of physiological intestinal homeostasis so that early clinical remission can occur without further delay (Fig.1). Disjointed, uncoordinated and unabated immune responses can be counterproductive and leads to rapid disease progression with associated local and systemic complications. This review will discuss physiological host defense mechanisms by epithelial cells, neutrophils, lymphocytes, and macrophages upon encountering intestinal cell death during primordial stages of NEC. We particularly discussed the physiological efferocytosis executed by macrophages in a detailed manner and highlighted its crucial role in facilitating tissue healing and restoring mucosal integrity by its inherent anti-inflammatory pathway activation. The step-by-step process of macrophage efferocytosis occurring in the intestinal milieu needs to be comprehended in systematic and methodical manner. We surmise that, this physiological host defense response can be manipulated and exploited for devising macrophage-based cell therapies, which can be potentially utilized for countering the clinical disease progression in the NEC disease models. This will ultimately be beneficial for optimizing the clinical outcomes and attenuating mortality as well as morbidity in NEC.

Aim

To understand and glean the innate immune cellular defense responses kickstarted in responsible to intestinal cell death in NEC

Material and methods

PubMed search of relevant articles describing host response particularly macrophage mediated responses in response to intestinal cell death in NEC were carefully reviewed and analyzed. Furthermore, pertinent treatment modalities based on the signaling mechanisms of macrophage efferocytosis were discussed. Comprehending this unrecognized physiological defense mechanism is crucial in devising future basic science research studies to understand its potential role in disease pathogenesis of NEC. These studies might become a physiological basis for crafting novel therapeutic interventions for derailing clinical progression in NEC.

Analysis of the literature

Host response to cell death in preterm and full infants

As of today, there are few studies documented that ascertain the differences between preterm and full-term infants in their ability to fight the microbial infections. In response to group B streptococcus infection, whole blood and cord blood monocytes from preterm infants responded with impaired cytokine profile as compared to the full-term infants.[15] Additionally, blood lymphocyte production of cytokines in response to group B streptococcus infection was poor in preterm and full-term infants as compared to the adults.[15] In concurrence with the above findings, incubation of cord blood from infants of different gestational ages with LPS revealed decreased secretion of TNF-α, IL-6 and granulocyte-colony stimulating factor (G-CSF) in the very low pre-term infants as compared to more advanced gestational age infants.[16,17] This emphasizes the notion that, pro-inflammatory cytokine secretion profile is directly proportional to the gestational age. Reduced secretion of pro-inflammatory cytokines (IL-1, IL-6, and IL-8) in the pre-term infants can be attributed to the reduced expression of TLR4 receptor signaling ligands (myeloid differentiation gene 88 and IRF5 interferon regulatory factor 5 [IRF5]) as well as impaired activation of the downstream signaling molecules such as mitogen activated protein kinase 14 and extracellular signal regulated kinase (ERK1/2) upon stimulation with bacterial LPS.[18,19] Analysis of the cord blood of preterm infants with gestational age (<28 weeks and 28-32 weeks) revealed decreased innate immune receptors (cluster of differentiation [CD14]), TLR2, TLR4 and myeloid differentiation protein 2 on the leukocytes.[20] As a result, preterm infants had reduced ability to defend against gram-positive and gram-negative infections and attenuated capacity for opsono-phagocytosis of the dead bacteria and these abnormalities were reversed partially by administration of IFN-γ.

Because of these impaired defense mechanisms, preterm infants are more likely to be prone to severe infections and clinical abnormalities as compared to the full-term infants. Accordingly, in a prospective observational cohort study, pre-term infants are more likely to develop late onset sepsis due to their impaired secretion of cytokines and immune hypo-responsiveness in response to staphylococcus epidermidis infection.[21,22] Pre-term infants with bronchopulmonary dysplasia are more likely to progress to chronic lung disease due to the increased presence of non-classical immature $CD14^{+}/CD16^{+}$ macrophages resulting in persistent inflammation in the lung tissues.[23] Increased presence of immature macrophages in the lungs of premature infants is due to failure of maturation of monocytes into mature macrophages, which makes them less prepared as well as inexperienced to handle any pertinent bacterial infections.

Preterm infants were shown to have lesser total white cell count, neutrophils, lymphocytes, monocytes and basophils at birth as compared to the full term infants.[24] The ability to fight bacterial infections in the preterm infants is usually subpar due to the presence of functional abnormalities such as decreased leukocyte recruitment, attenuated pattern recognition receptor function and diminished bacterial killing capacity.[19] Neutrophils which are the first responders to infections are powerless and incompetent in the preterm infants due to their functional defects such as decreased phagocytosis, slow mobility, and reduced antimicrobial protein secretion thereby predisposing them to severe bacterial and fungal infections.[25-28] Neutrophils in preterm infants are also demonstrated to possess diminished respiratory burst as well as defective neutrophil extraceullar traps (NETs) formation resulting in their reduced potency in executing intracellular and extracellular microbial killing.[29] In fact, the antimicrobial proteins and peptides that are secreted by neutrophils, macrophages and lymphocytes in response to microbial infections were shown to be proportional to the gestational age of the infant.[29]

In response to TLR agonists, preterm monocytes exhibited decreased phagocytosis, impaired activation of ERK1/2 and Extra-cellular signal-regulated kinase and nuclear factor – kappa B (NF-κB), reduced production of TNF-α as well as diminished acidification of bacterial phagosome underscoring paralyzed and futile early immune responses against bacterial infections in them.[30] Furthermore, monocytes and dendritic cells launch perturbed innate immune defenses in response to bacterial LPS in the very low birth weight pre-term infants, a factor associated with increased risk of severe bacterial inflammation with increased morbidity and mortality in them.[31] The ability of the monocytes and dendritic cells to ingest, process and present the foreign antigens to the T-lymphocytes in the neighboring lymph nodes (LNs) through major histocompatibility complex class II (MHC class II) for mounting an immune response is also impaired in the preterm infants.[32-34]

Evaluation of cord blood demonstrated reduced amounts of specific antibodies against Diphtheria, Tetanus, Pertussis, Neisseria meningitidis and Hemophilus influenza in the preterm infants less than 32 weeks of gestation as compared to the term infants due to decreased trans-placental transfer of protective maternal antibodies into their blood circulation.[35] Studies have shown that preterm infants usually have altered thymic 1 and 2 (Th1/Th2) lymphocyte ratio with predominance of anti-inflammatory Th2 phenotype and reduced production of IFN-γ making them susceptible for severe viral infections.[33] Analysis of B-lymphocytes in the preterm infants revealed multitude of abnormalities ranging from decreased expression of TNF-α receptor ligands (Traf interacting receptor for Tall1, B-cell maturation protein, and B-cell activating factor belonging to tumor necrosis factor family), diminished proliferation, lesser production of immunoglobulins such as IgA and IgG, disrupted isotype switching to altered B and T-lymphocyte interactions.[36] Adaptive immune responses are also suboptimal in the pre-term infants where administration of vaccines induced weaker protective B-cell antibody titers thereby offering lesser protection against infectious diseases.[37] In a prospective study studying the lymphocyte populations in the cord blood by flow cytometry, there is a predominance of naïve helper and cytotoxic T-lymphocytes generated by thymus and bone marrow in the preterm and term infants with lower counts of naïve T-lymphocyte populations in the infants with lower gestational age.[38] The classical and alternate complement pathways are also grossly impaired in the preterm infants thus making them vulnerable to microbial threats due to impaired leukocyte recruitment, opsonization and bacteria clearance.[39] Due to haphazard maturation and deficiency of anti-microbial defense mechanisms in the preterm infants, the chances of bacterial clearance in earlier stages of infection are very less thereby leading to rapid disease progression, severe disease pathology, and systemic spread of microbial infections.[40]

Cellular squad defenses to intestinal epithelial cell death in NEC

Gut mucosa cells including intestinal epithelial cells, Paneth cells and goblet cells.

In response to apoptosis and associated impairment of the intestinal barrier, intestinal mucosal cells including intestinal epithelial cells (IECs) try to compensate by mounting on specific defense mechanisms to mitigate the intestinal damage. IECs interact with microorganisms through pattern recognition receptors such as TLRs and nucleotide-binding domain, leucine-rich repeat-containing receptors to mediate secretion of pro-inflammatory mediators to curtail the bacterial induced intestinal damage (Fig. 2).[41]

Lamina propria of the intestinal mucosa along with gut associated lymphoid tissue has many IgA producing plasma cells.[42] As a part of mucosal immune response, IECs secrete polymeric immunoglobulin receptor (pIgR) which binds to the IgA on the basolateral side of IECs. Next IgA-pIgR complex gets internalized and migrates to the luminal or apical side of IECs.[43,44] Once the pIgR-IgA complex enters the apical side of IECs, secretory IgA enters the intestinal lumen and offers protection against bacteria or viruses in the lumen to curb further intestinal apoptosis (Fig. 2).[44] Additionally, anti-bacterial peptides such as stem cell factor, intestinal alkaline phosphatase and acyloxyacyl hydrolase, secreted by IECs offers protection against bacterial proliferation and invasion.[41,44] Furthermore, keratinocyte growth

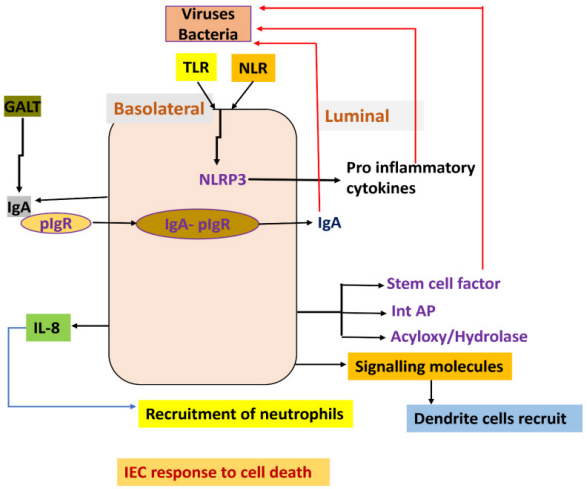

Fig. 2. Intestinal epithelial cell response to cell death. Viruses and bacteria activate Toll like and NOD like receptors on intestinal cells which results in NLRP3 inflammasomes with the resultant production of pro-inflammatory cytokines. This can also result in recruitment of poly IgA-polymeric immunoglobulin receptor complex from basolateral side to luminal side of intestinal epithelial cells. These pathological events produce additional pro-inflammatory cytokines and other tissue factors that results in the recruitment of neutrophils and dendritic cells towards the intestinal milieu that serves to amplify the immune response to neutralize the microbial threat and contain the spread of infection

factor which is secreted by intraepithelial lymphocytes helps to protect the IECs and restore the integrity of intestinal epithelial barrier.[44] During bacterial infections, epithelial cells secreted signaling molecules to promote the recruitment of dendritic cells from the blood stream to the intestinal mucosa. This results in upregulation of occludins locally leading to opening of tight junctions between the epithelial cells thereby permitting the dendritic cells to sample the pathogenic bacteria. Eventually these bacterial peptides are processed and presented to the lymphocytes in the neighboring lymph nodes, resulting in an immune response against the bacterial threats.[45] The basolateral surface of IECs usually secrete IL-8, which is responsible for recruitment of neutrophils to the intestinal lumen for setting in motion a robust antimicrobial response to contain their spread.[46] Moreover, monocyte chemoattractant protein-1 (MCP-1) secreted by Paneth cells and goblet cells has been shown to downregulate TNF-alpha mediated IEC apoptosis via reducing the migration of plasmacytoid dendritic cells.[47] Furthermore, Paneth cells secrete anti-microbial defensin peptides which are instrumental in strengthening the innate immune response by neutralizing the threat imposed by gram positive and gram negative bacterial infections.[48] Goblet cells which are interspersed between IECs also been shown to contribute to the anti-microbial immune response through secretion of mucin as well as trefoil like peptides and resistin-like molecule β molecules.[49] Previous studies demonstrated that, IECs undergoes apoptosis only 12-18 hours after bacterial entry with upregulation of TNF-alpha and nitric oxide.[50] Taking advantage of this delayed onset of apoptosis, IECs and intestinal mucosa through coordinated effort will be able to launch a sturdy mucosal immune response with a cellular battalion comprising of dendritic cells, lymphocytes, neutrophils and macrophages to partially neutralize the bacterial insult, subdue the microbial spread and restore the intestinal mucosal integrity in the initial stages of disease.[50]

Dendritic cells or antigen presenting cells (APC)

During bacterial infections, $CD103^{+}CD11b^{+}$ dendritic cells (DCs) are the primary dendritic cells that are responsible for delivering the ingested cargo to the mesenteric lymph nodes (MLNs) for eliciting a vigorous immune response.[51] During the physiological conditions, overcrowding at the villus tip can result in apoptosis of the IECs through spingosine-1 phosphate (S1P) and rho associated kinase dependent caspase-3 signaling. Once the apoptotic IECs undergo shedding, DCs are most important immune cells that sample the apoptotic IECs and transport them to the MLNs for initiating an immune tolerance or immune response.[52] LPS released during bacterial infections can cause disruption of tight junctions between the epithelial cells through secretion of zonula occluding-1 (ZO-1) and this will permit the DCs to sample the intestinal lumen for bacterial products for orchestrating the immune response.[45] In this regard, $CD4^{+}/OX41^{+}$ DCs can be regarded as strong antigen-presenting cells (APCs) whereas $CD4^{-}/OX41^{-}$ DCs are relatively weak APCs with regard to their ability to sample, process and respond to the foreign antigens (Fig. 3).[53]

The evidence for this finding comes from studies revealing APCs with cytoplasmic epithelial DNA and epithelial cytokeratin extensively localized to lamina propria, Peyer's patches and MLNs.[54] During homeostasis, dendritic cells are responsible for mounting a comprehensive immunosuppression program by downregulating genes associated with inflammasome (NLRP3, caspase-1 and IL-1-β) and mitogen activated protein (MAP) kinase resulting in a non-inflammatory apoptosis.[52] However, during inflammatory bowel conditions, increased TNF-alpha induced excessive and unregulated apoptosis and necroptosis through caspase-8 and RIPK1/RIPK3 respectively results in disruption of this protective immunosuppression program mediated by dendritic cells.[52] Subsequently, dendritic cells can directly ingest the apoptotic IECs in the lamina propria with the help of αvβ5 integrin and CD36 receptors and then migrate to the mesenteric lymph nodes

in chemokine receptor 7 dependent manner to mount an robust immune response.[55,56] Antigen uptake by the dendritic cells can also occur indirectly and be classified as M-cell dependent, neonatal Fc receptor dependent and goblet cell dependent.[57] Once apoptotic IECs or foreign antigens are ingested by dendritic cells, next steps including degradation, processing, transportation and loading of antigen proteins on MHC class II molecules will have to proceed in a systemic manner for presentation to T-lymphocytes in MLNs (Fig. 3).[58,59] After migration to the MLNs, dendritic cells also upregulate the expression of α4β7 integrin and chemokine receptor 9 (CCR9) on the naïve T-lymphocytes and this will promote their homing to the intestinal mucosa for inciting an immune response to future bacterial infections.[60,61] Moreover, dendritic cells can secrete IL-10 and IL-12 by themselves upon exposure with LPS or *Streptococcus faecium* and these cytokines will also be responsible for shaping the immune responses against bacterial infections (Fig. 3).[62] Furthermore, dendritic cells can stimulate intestinal IgA production through secretion of B-cell activating factor belonging to the TNF family, a proliferation-inducing ligand and retinoic acid for amplifying the immune response against bacterial insults (Fig. 3).[57]

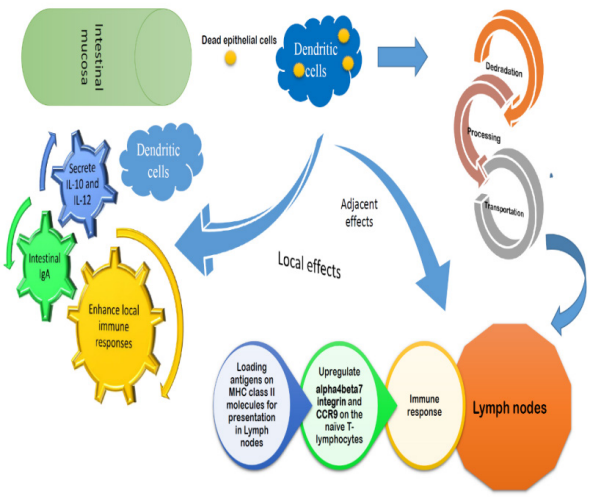

Fig. 3. Schematic representation of dentritic cell responses to dead intestinal epithelial cells. Occurrence of intestinal cell death can initiate dendritic cell recruitment. Ingestion of dead epithelial cells by dendritic cells results in degradation, processing, and presentation of antigens on MHC class II for presentation to T-lymphocytes for initiation of immune response. These events then trigger immune cells response in lymph nodes. Furthermore, involving up-regulation of α4β7 integrin and CCR9 on T cells surface can their activation and migration towards to the site of intestinal injury. The dead epithelial cells are entrapped by dendritic cells that produce local effects at the site by secreting IL-10, IL-12, cytokines, initiating IgA and local immune cell responses

Lymphocytes

During homeostatic apoptosis or contact with commensal bacteria, APCs aided antigen presentation in MLNs results in stimulation of intestinal lymphocytes. Fork head box-3 protein $CD4^+$T and $CD8^+$ regulatory (reg) lymphocytes which expresses higher concentration of IL-9, IL-10 and IL-13 are specifically recruited to the intestinal epithelial cells undergoing apoptosis.[57] CD4 T^+ helper cells and CD8 T^+ reg lymphocytes secreted cytokines such as IL-10, IL-17, IL-22, and transforming growth factor – beta (TGF-β) are responsible for restoring and healing the injured intestinal epithelium during physiological processes such as tissue development, regeneration and healing.[63] It is important to understand that, Th17 lymphocytes are implicated in the pathogenesis of multiple chronic inflammatory conditions whereas T reg lymphocytes are deemed to be protective through suppression of inflammation, immune tolerance and remodeling of tissues.[64] Naïve T-cells with their specific cytokine profile implicated in the pathogenesis of inflammatory bowel disease are usually classified into three groups namely Th1 (IFN-gamma, TNF-alpha), Th2 (IL-4, IL-5 and IL13) and Th17 (IL-17A, IL-17F, IL-21 and IL-22) lymphocytes (Fig. 4).[65,66]

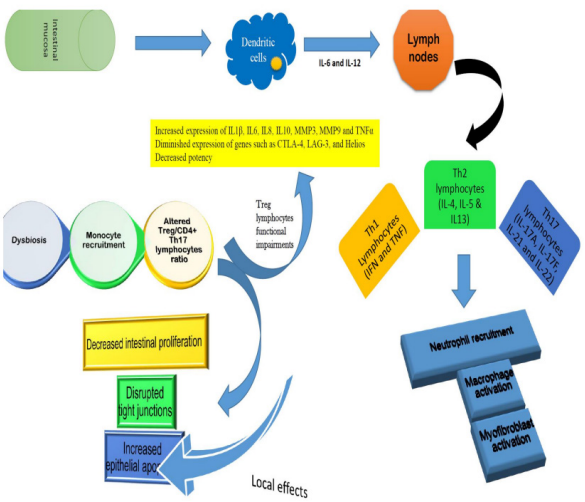

Fig. 4. Lymphocytes responses towards the intestinal cell death. Ingestion of dead intestinal cells by dendritic cells and their subsequent migration towards lymph nodes results in Th1, Th2 and TH17 dependent secretion of IF-γ, TNF-α, IL-4, IL-5, IL-13, IL-17A, IL-17F, IL-21, IL-22 cytokines. These cytokines cause neutrophil recruitment, macrophage and myofibroblast activation leading towards increased epithelial cell apoptosis. Moreover, the intestinal dysbiosis due to infection causes monocyte recruitment, altered Treg/$CD4^+$ lymphocyte ratio finally contributing towards the decreased intestinal proliferation, disruption of tight junctions and intestinal apoptosis

Activated Th1 lymphocytes secreted cytokines (IFN-γ and TNF-α) that execute intestinal epithelial

apoptosis through macrophage activation and via myofibroblast induced production of matrix metalloproteinases (MMPs) that provoke tissue matrix degradation.[65] Th2 lymphocytes induced production of IL-13 is also implicated in instigating increased intestinal epithelial apoptosis and disruption of intestinal epithelial barrier integrity.[65] Th17 lymphocytes induced release of IL-17 can stir up the recruitment of neutrophils from the blood stream into the intestinal mucosa for potentiating the microbial killing activity.[65] In the presence of bacterial or viral infection, CD 103$^+$ APC induced antigen presentation can activate Th17, Th1 and cytotoxic lymphocytes by TLR stimulation via IL-6 and IL-12 p40 subunit secretion.[57] The ratio of Treg/CD4$^+$lymphocytes to Treg/CD8$^+$lymphocytes in the NEC intestinal tissues is substantially lowered as compared to the surgical control intestinal tissues of age-matched gestational age.[67] Alteration of Treg/CD4$^+$ / CD4$^+$ T lymphocyte ratio secondary to dysbiosis of the intestinal bacteria one of important risk factors for developing NEC in premature infants (Fig. 4).[68] The authors in a recently concluded clinical research study observed that, about 60% depletion of Treg lymphocytes in the NEC intestinal tissues has been associated with increased gene expression of IL1β, IL6, IL8, IL10, MMP3, MMP9 and TNFα and accompanying intestinal inflammation as compared to age-matched gestational controls.[67,69] Treg lymphocytes in NEC intestinal tissues also exhibited several functional impairments including decreased potency in suppressing IL17 production and CD4$^+$CD25$^-$ T conventional cell proliferation in addition to diminished expression of genes cytotoxic lymphocyte associated protein 4, ligand lymphocyte activation gene 3, and IKZF2 zinc finger protein.[64] Downregulated expression and functional aberrations of Treg lymphocytes can fuel unabated and exacerbated inflammatory response thereby kindling severe intestinal lesions in NEC. Monocyte activation and recruitment to the intestinal mucosa during bacterial infections is shown to exacerbate altered Treg/CD4$^+$ Th17 lymphocytes ratio locally via upregulation of TNF- α and IL-6 as well as downregulation of IL-10 and TGF-β thereby contributing to the intestinal injury in NEC.[70] During bacterial infections, LPS-TLR4 signaling in the IECs leads to CCL25 dependent recruitment of CD4$^+$ Th17 lymphocytes and associated downregulation of Fork head box-3 protein (Treg lymphocytes) leading to exacerbated inflammatory response in NEC.[71] IL-17 secreted from the CD4+ Th17 lymphocytes was demonstrated to provoke decreased enterocyte proliferation, disruption of tight junctions and increased epithelial apoptosis leading to intestinal injury in NEC (Fig. 4).[71]

Neutrophils

Neutrophils are recruited to the inflamed intestinal epithelium as a first line defense mechanism in response to bacterial infections. Their primary responsibilities include killing of microorganisms, healing of injured mucosa, attenuation of inflammation and restoring the intestinal homeostasis. During chronic inflammation, IL-17 and IL-23 secreted by Th17 lymphocytes and dendritic cells/macrophages respectively are mainly responsible for the increased transcription of granulocyte monocyte colony stimulating factor (GM-CSF) in the bone marrow.[72,73] Increased GM-CSF in the bone marrow results in increased production of neutrophils and their subsequent release into the peripheral blood stream (Fig. 5).[73] To reach the intestinal lumen at the site of inflammation, neutrophils must penetrate through the endothelial, interstitial and epithelial barrier by trans-endothelial and trans-epithelial migration.[73-75] The numerous sequential steps in the trans-endothelial migration of neutrophils include tethering, rolling, arrest, adhesion, crawling and paracellular migration.[73,74] Trans-endothelial migration involves P, E, and L selectins, P-selectin glycoprotein ligand, leukocyte specific integrin molecule (CD11b), CD38, intracellular adhesion molecular and vascular adhesion molecule.[73,74] Trans-epithelial migration of neutrophils encompasses sequential steps such as basolateral adhesion, trans-migration and apical adhesion.[75] This trans-epithelial migration of neutrophils instigates the production of TNF-alpha through upregulation of ADAM metalloproteinase domain 17 or a disintegrin and metalloprotease 17 (ADAM17) thereby triggering the onset of intestinal inflammation.[76] Once the neutrophils reach the intestinal lumen, they remain in close contact with the apical surface of intestinal epithelial cells and form cryptic abscesses for promptly responding to the bacterial stimuli.[75] It is important to understand that, a large number of neutrophils undergoing trans-epithelial migration might be associated with disruption of epithelial junctional proteins such as ZO-1, claudin 1, β-catenin, E-cadherin and junctional adhesion molecule-A leading to impairment of epithelial barrier integrity which can subsequently promote excess neutrophil and bacterial transmigration.[77] Enhanced bacterial entry into the intestinal mucosa can cause macrophage polarization from M2 to M1 phenotype, leading to increased pro-inflammatory cytokine secretion, a phenotype change that can provide a signal for more neutrophil recruitment to the intestinal lumen.[78] Once neutrophils reach the intestinal lumen, they usually exert their anti-inflammatory effect through reactive oxygen species (ROS) production, NET formation, phagocytosis and degranulation.[73,74] The various types of anti-microbial granules secreted by neutrophils can be classified as primary (myeloperoxidase, neutrophil elastase, cathepsin-G, and lysozyme), secondary (lactoferrin and MMP-8) and tertiary (MMP-9), all of which synergistically contribute to strengthen anti-bacterial defenses at the site of mucosal injury (Fig. 5).[73-75]

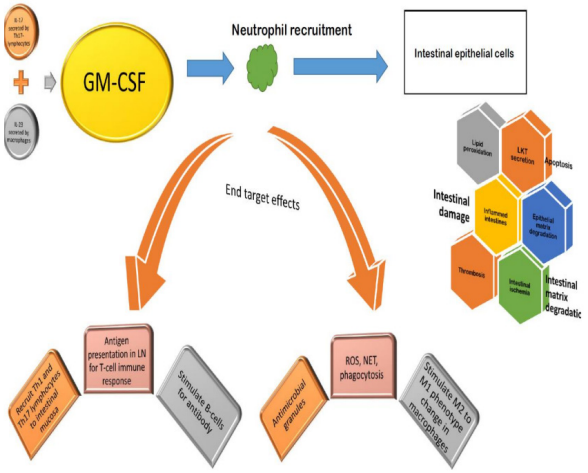

Fig. 5. Neutrophils responses towards intestinal epithelial cell death. The IL-17 and IL23 cytokines by lymphocytes and macrophages causes GM-CSF upregulation which leads to initiate neutrophils recruitment at the site of intestinal injury. This results in end target effects including recruitment of Th1 and Th17 lymphocytes to the intestinal cell mucosa, antigen response mediated by T cells activation, stimulation of B cells. These cellular events further release ROS, induce phagocytosis and conversion of macrophage from M2 to M1 phenotypes. Unfortunately, excess and uncontrolled activation of neutrophils results in multiple pathological derangements including lipid peroxidation, epithelial matrix degradation, intestinal ischemia, leukotriene secretion and intestinal damage

Activated neutrophils might induce secretion of chemokines C-X-C motif chemokine ligand 10 and CCL2, which can promote recruitment of Th1 and Th17 lymphocytes to the site of inflamed mucosa (Fig. 5). Lymphocyte recruitment to the site of intestinal inflammation leads to the production of pro-inflammatory cytokines (TNF-α, IL-12, IL-15, and IL-23) for neutralizing the bacterial threat and curbing the associated intestinal inflammation.[73,79] In addition to their effect in the intestinal lumen, neutrophils also act on the neighboring lymph nodes to initiate an immune response against the pathogens. Neutrophils can also deliver the microbial antigens to APCs (i.e. dendritic cells) which can ultimately promote proliferation of $CD4^+$ T lymphocytes in the lymph nodes to amplify the innate immune response (Fig. 5).[80] Activated neutrophils close to the B-lymphocytes in lymph nodes can also stimulate plasma cell proliferation and subsequent antibody production (Fig. 5).[80] Neutrophil recruitment to the site of intestinal inflammation is not always productive as it can be counter-productive in some instances. NET can result in release of phosphatidylserine positive micro-particles and causes microvascular thrombi through TLR4 signaling on the platelets and endothelial cells leading to microvascular ischemia. This can further exacerbating the intestinal damage during bacterial inflammation of the gut.[81] Unfortunately, excessive, and uncontrolled neutrophil activation at the inflamed mucosa can also aggravate the intestinal injury through lipid peroxidation, leukotriene secretion, amplified inflammation, epithelial matrix degradation, microvascular thrombosis, and intestinal ischemia (Fig. 5).[73,74,78]

Macrophages

Macrophages are one of most important armories of cellular defense that are recruited to the site of tissue injury for neutralizing the bacterial threat and repairing of inflamed intestinal mucosa. In response to bacterial infection, innate lymphoid cells located within the intestinal epithelium secrete (GM-CSF) which is responsible for proliferation of monocyte precursors in the bone marrow.[82] This will ultimately lead to an increase in the monocyte population in the blood stream. These monocytes will eventually migrate to the site of intestinal inflammation and transform into inflammatory macrophages.[83] In contrast to the tissue-resident-macrophages, which are non-inflammatory, these inflammatory macrophages are more responsive to the presence of microbial antigens.[83] These inflammatory macrophages secrete proteases, reactive oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radicals) and pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-8, IL-10, IL-12, and IL-18) which are helpful in promoting resolution of intestinal inflammation, enhancing tissue repair and tissue remodeling.[84] Macrophages can directly engulf the pathogens (bacteria and viruses) for direct microbial killing or presentation to the immune cells in the neighboring lymph nodes through a process called phagocytosis which involves the following steps namely; pathogen recognition, cytoskeleton remodeling, phagosome formation and phagolysosome maturation.[85] Another important function of macrophages is ingestion, degradation, transport and loading of bacterial antigens onto MHC class II molecules for presenting them to T cell receptors for initiating the lymphocyte mediated innate immune response.[84] The specific cytokine profile secreted by the macrophages can determine the appropriate T-cell mediated immune response in the inflamed intestinal tissue; with IL-12 and IL-18 promoting Th1 lymphocyte focused response whereas IL-4 and IL-6 favoring Th2 lymphocyte centered response.[86-88]

Macrophage efferocytosis

The number of intestinal epithelial cells that undergo apoptosis every day in the - human small intestine and colon is approximately $2x10^8$ and $2x10^{11}$ respectively during physiological conditions.[89] In contrast, during acute and chronic intestinal inflammatory conditions apoptosis occurs at a much higher rate, eventually leading to accumulation of dead epithelial cells at the site of intestinal injury. Timely removal of dead epithelial

cells and neutrophils is essential for rehabilitation of inflamed intestinal mucosa, regaining of dead tissue homeostasis, tissue repair and healing.[90] Macrophages are one the important immune cells that engulf dead and apoptotic cells by a process known as efferocytosis.[90,91] Defective repair of these early apoptotic cells by defective macrophages during intestinal inflammation can be detrimental to the host as it can lead to tissue necrosis, chronic inflammatory diseases (chronic obstructive pulmonary disease, asthma or atherosclerosis) and autoimmune diseases (systemic lupus erythematosus and diabetes).[92] The process of efferocytosis requires coordinated effort by a cluster of signaling molecules including find-me signals, eat-me signals, don't eat-me signals, bridging molecules and phagocytic receptors for initial interaction between dying cell and phagocytes.[90] Engagement of apoptotic cell and macrophage leads to cytoskeletal rearrangements, engulfment of the dead intestinal cell body, followed by phagosome maturation and its eventual dismantling.[91] Apart from clearance of apoptotic cells, efferocytosis also helps to dampen the tissue inflammation through secretion of anti-inflammatory mediators (IL-10, TGF-β, and pro-resolving lipid mediators) and promote adaptive immune response through antigen presentation.[90,91] In the following sections, information regarding identifying and processing of the dead intestinal epithelial cells including find-me signals, eat-me signals, bridging receptors, mechanism of macrophage efferocytosis and its anti-inflammatory effects will be presented in a detailed manner.

Find-me signals

As apoptotic cells and phagocytes are not in proximity, there should be some mechanisms set in place so that they can closely interact with one other. A chemoattractant signal would be the most logical solution to this problem so that neighboring phagocytes would get attracted towards the dying cells. Once the intestinal epithelial cells undergo apoptosis, they tend to release some chemotactic signals for attracting neighboring primary phagocytes for their removal.[93] Lyso-phosphatidylcholine, fractalkine, ATP, UTP and S1P are some examples of the chemotactic signals released by the dying intestinal cells to attract macrophages towards them (Fig. 6).[94-97]

Other find-me signals that have been studied previously include endothelial monocyte activating protein, human tyrosyl t-RNA synthase, thrombospondin-1 (TSP-1) along with its heparin binding domain, ribosomal protein, soluble IL-6 receptor and apoptotic micro-blebs.[98] In some scenarios, apoptotic cells can also secrete a "keep out" signal such as lactoferrin that mainly functions to restrict the recruitment of neutrophils to the site of apoptosis without effecting mononuclear phagocytes; which is a classic feature of non-inflammatory apoptosis.[98,99] In this regard, the presence of anti-lactoferrin antibodies has been documented in few autoimmune diseases such as rheumatoid arthritis, ulcerative colitis, Crohn's disease and systemic lupus erythematosus.[98] Find-me signals may be secreted by the same apoptotic cell and, they tend to act synergistically in the local microenvironment to attract primary phagocytes.[95] The potent chemotherapeutic drug doxorubicin was demonstrated to increase apoptosis as well as SIP expression in jurkat cells.[97] S1P, which is a bioactive lipid secreted by the apoptotic cells, has been shown to attract THP-1 monocytes, U937 leukemia cells (pro-monocytic, human myeloid leukemia cell line),

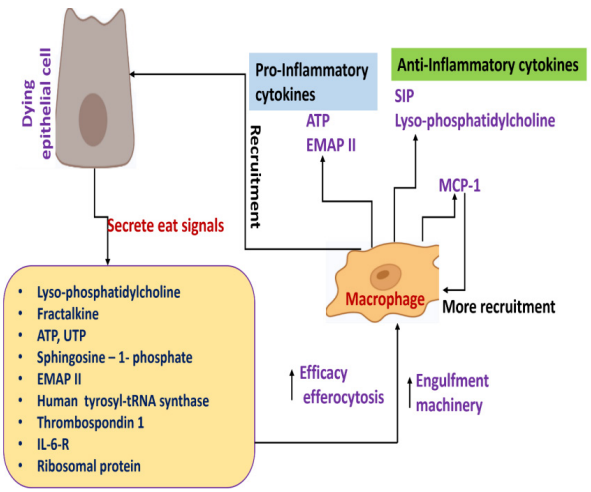

Fig. 6. Find me signals of dying epithelial cells for macrophage recruitment. Apoptotic epithelial cells release "Find me signals" for macrophage recruitment for initiation of macrophage efferocytosis. Find me signals can range from lyso-phosphatidylcholine, fractalkine, ATP, UTP, sphingosine-1-phosphate, endothelial monocyte activating polypeptide II, human tyrosynl-tRNA synthase, thrombospondin, IL-6-R to ribosomal protein. The release of these mediators causes macrophages recruitment towards the dying cells. In parallel presence of various pro-inflammatory cytokines further exaggerate the macrophage recruitment towards damaged epithelial cells resulting in their efferocytosis and heightened engulfment process through increased upregulation of efferocytosis machinery including macrophage receptors and bridging receptors

primary monocytes and macrophages.[97] Once macrophages are attracted to the apoptotic cells by these signals, they can secrete MCP-1which can attract more professional phagocytes to the apoptotic site thereby amplifying the processes of efferocytosis (Fig. 6).[100] It is possible that, the main purpose of these find-me signals is not only to attract the professional phagocytes but also to increase the expression of engulfment machinery so as to increase the efficiency of efferocytosis for enabling effective clearance of apoptotic cells (Fig.

6).[95] Furthermore, find-me signals has also been shown to stimulate pro-inflammatory cytokines (ATP and endothelial monocyte-activating polypeptide II) as well as anti-inflammatory cytokine secretion (S1P and lyso-phosphatidylcholine) from macrophages apart from their primary chemo attractive function (Fig. 6).[98,101,102]

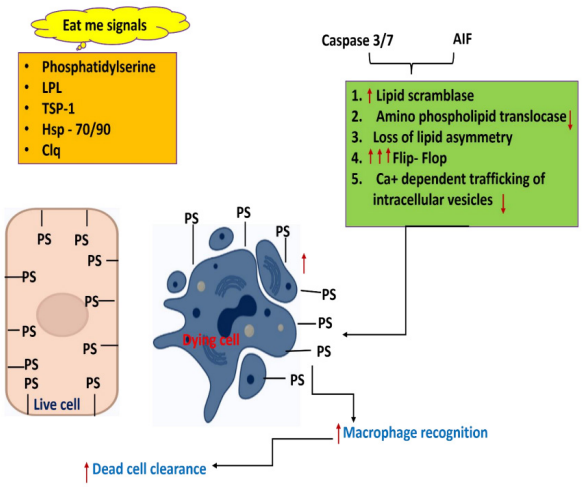

Fig. 7. Eat me signals of apoptotic cells for macrophage efferocytosis. Eat me signals such as Phosphatidyl serine (PS), lipoprotein lipase (LPL), thrombospondin-1 (TSP-1), HSP70&90 and Complement 1q (C1q) are utilized by dead epithelial cells for macrophage interaction for efferocytosis and their subsequent clearance. Primarily the phosphatidylserine expressed on the outer side of the dying cell, and it is recognized site by macrophage for engulfment and clearance. The exposure of PS can be due to number of factors such as increased activity of lipid scramblase, downregulation of amino phospholipid translocase, loss of lipid asymmetry, increased flip-flop and Ca^{2+} dependent trafficking of intracellular vesicles. The activity of these lipid degrading enzymes and calcium dependent trafficking is governed by caspase 3/7 and apoptosis inducing factors release. These events culminate in the dead cell clearance through macrophages

Eat-me signals

Once the professional phagocytes recognize the find-me signal, they migrate to the area where cells are actively undergoing apoptosis. In this scenario, apoptotic cells should display specific eat-me signals so that they can be differentiated from the nearby live cells by the professional phagocytes.[95] Display of eat-me signals indicates that the cells are in the early stage of apoptosis, and they need to be cleared before damage to the plasma membrane. Earlier removal of dying intestinal epithelial cells arrests the release of intracellular contents into the interstitium, and averts the escape of danger signals into systemic circulation and secondary inflammatory consequences.[103] A number of eat-me signals have been described in the literature so far, out of which phosphatidylserine, calreticulin, oxidized phospholipids (oxLDL), TSP-1 and complement binding sites (C1q and Cb/bi), chaperones (HSP70&90) and changes in glycocalyx are important (Fig. 7).[95,103-105]

Phosphatidylserine

One of the most studied eat-me signals is phosphatidylserine. In the resting cell, 70% phosphoryl choline, 20% phosphatidylethanolamine and 100% sphingomyelin are exposed extracellularly whereas 100% of phosphatidylserine is located intracellularly facing inner leaflet of plasma membrane.[105] During cellular activation, even though phosphatidylserine is transiently exposed extracellularly due to physiological flip-flop, it is reverted back to the intracellular location due to preserved action of amino-phospholipid translocase.[105] However, in apoptotic cells the scenario changes so that irreversible inactivated amino-phospholipid translocase, loss of lipid asymmetry and sustained increased flip-flop results in permanent exteriorization of 100% phosphatidylserine on the outer leaflet of plasma membrane (Fig. 7).[104-106] During apoptosis, caspases 3 and7's dependent activation of Xrp8 protein (lipid scramblase) can result in loss of plasma membrane lipid symmetry and subsequent phosphatidylserine exposure (Fig. 7).[106] Alternatively caspase independent mechanism of phosphatidylserine exposure has also been described through activation of scramblase by apoptosis inducing factor in T-lymphocytes undergoing apoptosis (Fig. 7).[107] Calcium dependent trafficking of intracellular vesicles formed during apoptosis from cytoplasm to plasma membrane is also proposed as another mechanism for phosphatidylserine externalization.[108] It is important to note that, deficient calcium dependent trafficking of intracellular vesicles from cytoplasm to plasma membrane in some cancer cell lines such as T98G (human glioblastoma multiforme cell line) and D32, leads to attenuated phosphatidylserine exposure and subsequent escape from efferocytosis.[108] The amount of phosphatidylserine exposed in outer leaflet of plasma membrane quantified by sensitive paramagnetic resonance method in live and apoptotic jurkat cells is <0.5 pico-moles and 240 pico-moles/million cells respectively.[109] It is important to understand that, macrophage receptors do not recognize and engulf the cells with phosphatidylserine exposure <5 pico-moles/million cells in the outer leaflet of plasma membrane.[109] This, in turn shows that apoptotic cells need to have 280-fold more phosphatidylserine exposure in the outer leaflet of the plasma membrane as compared live cells for their specific recognition and clearance by macrophage efferocytosis.[109]

Calreticulin

Calreticulin (CRT) is a protein localized to the endoplasmic reticulum and it participates in calcium homeostasis and intracellular protein folding.[105,110] Although it is mainly intracellular, it can migrate to the cell sur-

face by associating with MHC class I molecules.[111] In viable cells, the CRT present on the cell surface cannot induce phagocyte efferocytosis due to presence of co-existent inhibitory signal regulatory protein-alpha (CD47-SIRP-α) signaling pathway.[110] During apoptosis, the increased expression of CRT in the cell membrane stimulates lipoprotein receptor protein (LRP) on the phagocyte to enhance efferocytosis as the CD47-SIRP-alpha signaling pathway is inhibited.[110] Moreover, CRT has been shown to present in the cell in two forms: namely the cis- and trans-form.[105] In the cis-form, CRT interacts with LRP of the phagocyte through intermediary pattern recognition molecules such as collectins (mannose-binding lectin, complement 1q, surfactant protein D) and mediates efferocytosis.[105] Whereas in trans form, CRT can directly interact with LRP of the phagocyte without the assistance of collectins to mediate efferocytosis.[105]

Changes in glycocalyx and heat shock proteins

During homeostasis, the negative charge of the plasma cell membrane physiologically provides an electrostatic repulsive force to restrict the interaction between viable cells and phagocytes.[112] However, in apoptosis, changes in glycosylation status of the plasma membrane (increased N-acetylglucosamine-, mannose-, and fucose-containing epitopes and decreased in N-sialic acid) results in loss of negative charge and thus allows their interaction with macrophages facilitating efferocytosis.[112,113] In some instances, eat-me signal expression can be induced on tumor cell plasma membrane by chemotherapeutic drugs to promote interaction with antigen presenting cells for subsequent anti-tumor immune response and elimination. In multiple myeloma, bortezomib treatment increases the expression of eat-me signal HSP90 on dying tumor cells to facilitate their interaction with dendritic cells for generation of anti-tumor immune response and immunogenic death.[114,115]

Don't eat-me signals

Don't eat me signals function by limiting the interaction between the dying cells and macrophages and thereby preventing the occurrence of efferocytosis. In the next few paragraphs, we present information regarding four important Don't eat me signals namely CD47, plasminogen activator inhibitor (PAI-1), CD31 and CD24. All these agents function through different signaling mediators to accomplishing their task of curtailing the dead cell removal at the site of tissue injury.

CD47

CD47 is an integrin associated protein and it belongs to immunoglobulin superfamily.[116] It is a ligand for transmembrane receptors such as SIRP-α and thrombospondins and mediates physiological functions including cell motility, adhesion and phagocytosis.[116] The activation of CD47-SIRP-α on the surface of cancer cells leads to downregulation of the eat-me signals such as phosphatidyl serine, antigen-antibody complexes and CRT-LRP. This interaction is shrewdly devised by cancer cells to mask their recognition from macrophages so that it provides an avenue for escaping from innate immune mediated clearance pathways. Furthermore, interaction of CD47-SIRP-α also polarizes the macrophages towards M2 phenotype which have less efferocytosis capacity.[117] This CD47-SIRP-α signaling pathway presents an important target for chemotherapeutic drug development so that cancer cells can be made susceptible to macrophage clearance and immune mediated destruction.[117] CD47 blocking antibodies have been shown to enhance macrophage efferocytosis and prevent the atherosclerosis in animal models providing a novel therapeutic strategy to reduce the incidence of cardiovascular disease.[118]

Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is a member of serine protease family and it is implicated in microvascular thrombosis, immune cell recruitment and inflammation.[119] In viable neutrophils, PAI-1 acts as a don't eat-me signal by downregulating the CRT-LRP signal system and thereby preventing them from macrophage interaction and clearance.[119] However, in apoptotic neutrophils the levels of PAI-1 are decreased leading to elevated CRT-LRP signaling mediated clearance by macrophages.[119]

CD31

CD31 is another don't eat-me signal mainly expressed in leukocytes and neutrophils.[120] It is a member of immunoglobulin superfamily of membrane adhesion molecules.[120] During interaction between the viable cells and macrophages, activation of CD31 mediated signaling resulted in active temperature dependent disengagement of macrophages from viable cells and prevented their clearance.[120] In the contrary, CD31 signaling in apoptotic cells promoted their taut engagement with macrophages through proteo-liposomes, engulfment and their removal.[120]

CD24

CD24 expressed in cancer cells primarily interacts with Siglec-10 receptor on macrophages and resulting in decreased efferocytosis through immune-receptor tyrosine based inhibitory motif and tyrosine phosphatases 1 and 2 based signaling.[121] Accordingly, administration of CD24 blocking antibody disrupted the immune receptor tyrosine based inhibitory motif (ITIM) based signaling and promoted increased immune based macrophage mediated clearance.[121]

Bridging molecules and macrophage receptors

Intestinal epithelial cells undergoing apoptosis will upregulate eat-me signals and downregulate don't eat-me signals so that recruited macrophages interact and degrade them in a silent immunologically silent manner. Eat-me signals such as phosphatidylserine directly interacts with macrophages and indirectly interacts with phagocytic receptors and bridging molecules for initiating the macrophage induced clearance process. Macrophage phagocyte receptors involved in efferocytosis as described in the literature include brain angiogenesis inhibitor 1 and 3, T-cell immunoglobulin and mucin receptor 1, stabilin-1, 2 and CD300 -A, B and F- (immune receptors), scavenger receptor type F family member 1, tyrosine protein kinase receptor 3, tyrosine protein kinase receptor UFO, αvβ5 integrin, MER protooncogene, and tyrosine kinase (Fig. 8).[122-124]

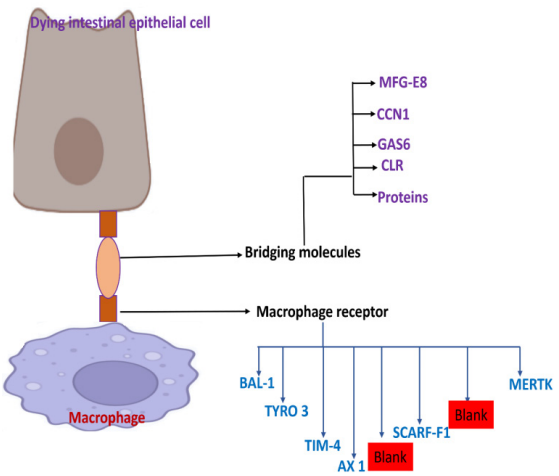

Fig. 8. Macrophage receptors and bridging molecules involved in macrophage efferocytosis. During macrophage efferocytosis, the apoptotic cells are cleared through the recognition of the major "eat-me" signal phosphatidylserine, directly through macrophage. Macrophage receptors and bridging molecules which mediate binding of phosphatidylserine to dying epithelial cells. Schematic shows some examples of macrophage receptor and bridging molecules involved in macrophage efferocytosis

The bridging molecules that mediate interaction between eat me signals on apoptotic cells and macrophage phagocyte receptors include milk fat globule-EGF factor 8 (MFG-E8), cellular communication network factor 1, growth arrest specific 6, complement 1q and protein S (Fig. 8).[122-124] During *Helicobacter pylori* infection of gastric mucosa, apoptotic gastric epithelial cells expressing phosphatidylserine are recognized by brain angiogenesis inhibitor 1 expressed gastric phagocytes resulting in engulfment, degradation, and antigen presentation.[125] Peritoneal macrophages expressing T-cell immunoglobulin and mucin receptor 1 are responsible for engulfing phosphatidylserine expressing apoptotic cells, activation of light chain 3 (LC3)-associated phagocytosis and preventing the development of autoimmunity.[126] TIM3 expressed on macrophages is responsible for mediating immune tolerance by downregulating TLR4-NF-κB mediated pro-inflammatory cytokine production.[127] CD300a receptor expressed on macrophages interacts with phosphatidylserine and phosphatidylethanolamine on apoptotic cells and inhibits engulfment via ITIM dependent manner and its blockade resulted in enhanced efferocytosis and improved neurological outcomes after ischemic stroke.[128-130] Stabilin-1and 2 receptors expressed on macrophages are responsible for clearing PS exposed apoptotic cells along with secretion of anti-inflammatory cytokines and enhancement of tissue healing.[131,132] MFG-E8 secreted by macrophages serves as bridging molecule by binding to phosphatidylserine exposed dead cells and brings them towards αvβ5 receptor expressed macrophages for their engulfment and subsequent removal.[133] Complement-3bi can bind to phosphatidylserine-exposed-apoptotic cells and interacts with CD11b/CD18 receptors on macrophages and assists in their uptake and clearance.[134] Protein C/Gas 6 protein also functions as bridging molecule by binding to phosphatidylserine exposed apoptotic cells and TIM receptors on macrophages causing tyrosine kinase mediated downstream signaling events leading to cytoskeletal rearrangement and efferocytosis.[135,136] Dying fibroblasts release TSP1, which acts as a bridging molecule by recruiting and mediating the interaction between αvβ3 receptors on macrophages and TSP1-CD36 complex on apoptotic fibroblasts for promoting their clearance.[137]

Mechanism of efferocytosis

Spingosine-1-phosphate (SIP) released from the dying intestinal epithelial cells acts on neighboring macrophages through SIP receptors (SIPR).[138] SIP-SIPR interaction on the macrophages can result in increased in erythropoietin (EPO) secretion through nuclear factor activator of T-cells (NFAT) and hypoxia inducing factor (HIF-α) (Fig. 9).[138]

Next, EPO acts through the EPO receptor on the macrophages to stimulate MAPK and PPAR-gamma signaling pathways, resulting in increased nuclear transcription of macrophage receptors and bridging molecules including MFGE8, Gas6, MerTK and CD36.[138] The interaction between the apoptotic cells and macrophages occurs due to binding of eat-me signals, bridging molecules and macrophage receptors. The process of ingesting the dead corpse by active membrane disruption occurs through a process known as micropinocytosis.[139] This is followed by formation of CRK protooncogene adaptor protein (CRKII) -dedicator of

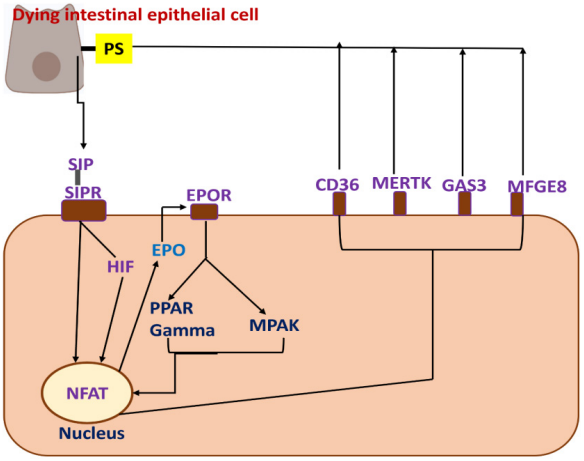

Fig. 9. Exposed PS induced downstream events in macrophages. Apoptotic cells release SIP which bind with neighboring macrophage with SIPR. The SIP-SIPR interaction induces EPO expression through NFAT and HIF. The EPO-EPOR interaction activates the MAPK and PPAR-g pathways resulting in nuclear transcription of macrophage receptors and bridging molecules such as MFGE8, Gas6, MERTK and CD36. (S1P – sphingosine-1-phosphate; NFAT – nuclear factor of activated T cells; HIF-α – hypoxia-inducible factor 1-α; EPO – erythropoietin; EPOR – erythropoietin receptor; Gas 6 – growth arrest-specific 6; MERTK – Mer tyrosine kinase; MFGE8 – milk fat globule-epidermal growth factor 8 protein; PPAR-γ –peroxisome proliferator-activated receptor gamma)

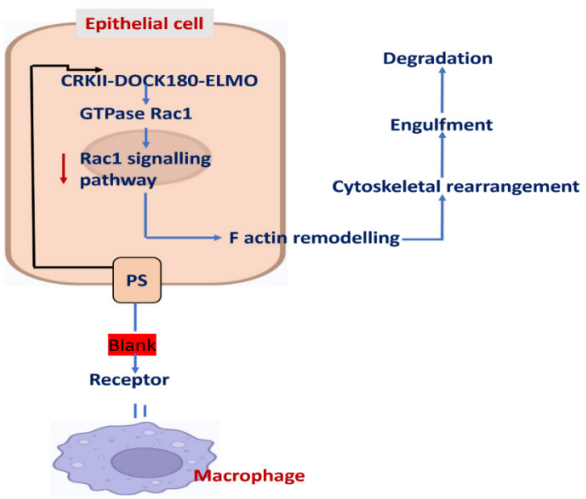

Fig. 10. Mechanism of macrophage efferocytosis. When the dying cell starts to expose PS on their surface, it is recognized by the macrophages directly or indirectly, through bridging molecules. The PS receptors stimulate CRKII-DOCK180-ELMO interaction to activate GTPase Rac1 belonging to rho family which then initiates the Rac1 mediated pathways. Rac1 signaling pathways play a crucial role in regulating the cytoskeleton through F-actin remodeling and cytoskeleton rearrangement, which is necessary for various cellular processes such as engulfment and degradation of cells in phagocytosis

cytokinesis-180 (DOCK180) - engulfment and cell mobility (ELMO) complex which activates GTPase of Rac Family small GTPase (Rac1) belonging to rho family resulting in initiation of Rac1 mediated signaling pathway (Fig. 10).[123,138]

In the final step, F1 acting remodeling leads to cytoskeletal rearrangement, engulfment, and degradation of apoptotic cell within the macrophage.[1123,138] After ingesting the dead cells, LC3 is recruited to the phagosome for ensuring complete and efficient degradation of the dying cell in an immunologically silent manner by a process known as LC3 associated phagocytosis (LAP) (Fig. 11).[140]

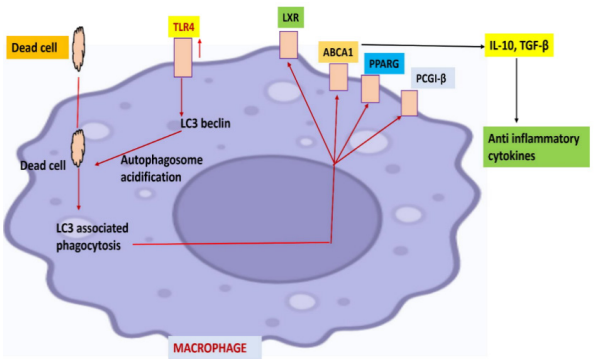

Fig. 11. LC3 associated phagocytosis and its anti-inflammatory effects. LC3-associated phagocytosis (LAP) is a type of phagocytosis that is mediated by the autophagy protein LC3. LC3 is recruited to the phagosome during engulfment, where it acts to increase the size of the phagosome and recruit lysosomal enzymes to degrade the engulfed material. In addition to its role in clearing debris and pathogens, LAP also has anti-inflammatory effects. In LAP, destruction of the phagosome containing the dead cell can result in increased secretion of anti-inflammatory cytokines such as IL-10 and TGF-beta which suppress inflammation. To further promote the degradation of the engulfed material in a non-inflammatory manner, TLR4 signaling assist in recruitment of LC3 and beclin to phagosome, autophago-lysosome formation, acidification

LAP can be differentiated from general autophagy by features such as formation of single membrane vesicles as opposed to double membrane vesicles, occurring few minutes as compared to hours after engulfment of corpse and not dependent on formation of pre-initiation complex (FAK Family interacting protein, ULK1/2 Unc-51 like autophagy activating kinase 1/2, and autophage related protein 13).[139] Destruction of the phagosome containing the dead cell without the associated LAP can result in increased secretion of pro-inflammatory cytokines and decreased secretion of anti-inflammatory cytokines with a propensity for increased occurrence of autoimmune diseases.[140] Liver X receptor (LXR), ATP binding cassette transporter, peroxisome proliferator activated receptor-gamma (PPAR-γ), per-

oxisome proliferator activated receptor gamma co-activator-1 beta and cholesterol are the important nuclear transcription factors implicated in the production of anti-inflammatory cytokines such as IL-10 and TGF-beta after ingesting the dead cells by macrophages.[139] Previous studies have demonstrated the association between deficient autophagy and auto-immune diseases such as Crohn's disease and systemic lupus erythematosus. After macrophage engulfment of apoptotic cells, activation of TLR4 signaling might result in recruitment of LC3 and beclin to phagosome, autophago-lysosome formation, acidification, and destruction in an immunologically silent manner.[141]

Fate of microorganisms after efferocytosis

During apoptosis of infected macrophages with *Mycobacterium tuberculosis* (Mtb), ingestion, and phagosome maturation by neutrophils is associated with killing of pathogens via upregulated host oxidase NADHPH oxidase-2 complex induced production of ROS and free radicals.[142] Effective compartmentalization of bacterium in the dead cells within the auto-phagosome is the key step for subsequent maturation, lysosome fusion and pathogen destruction.[143] Thus, efferocytosis represents an innate anti-bacterial immune response from the host tissues to limit bacterial replication and spread to the neighboring tissues.[143] However in the cases where virulent strains of Mtb causes necrosis of host macrophages, inhibition of phagosome maturation during neutrophil efferocytosis can cause uninhibited bacterial replication and subsequent progression of disease.[142] From the above findings, it is evident that bacterial infections are more likely to be cleared by efferocytosis during apoptosis of infected cells before progression to necrosis where pathogen induced aberrations renders the host clearance mechanisms less effective. Depending on the virulence of the microorganism, T-lymphocytes might assist in activation of efferocytosis and antibacterial defenses of macrophages through secretion of cytokines (TNF-α and IFN-γ).[143] If macrophage efferocytosis is not sufficient to curb the microbial threat, then apoptosis of macrophage laden microorganisms will be executed by T-lymphocytes followed by their removal by primary phagocytes.[143] In case of viral infection induced epithelial apoptosis, engulfment of dead corpses by the macrophages causes complete destruction and blockade of viral replication thereby leading to neutralization of viral threat.[144]

Macrophage phenotypes and immune silencing during efferocytosis

Increased ingestion of apoptotic cells by macrophages results in intracellular accumulation of large amounts of lipids, carbohydrates and proteins which might energize nuclear receptors including PPAR- γ, δ and LXR resulting in their transition into pro-resolving macrophages (Fig. 12).[122]

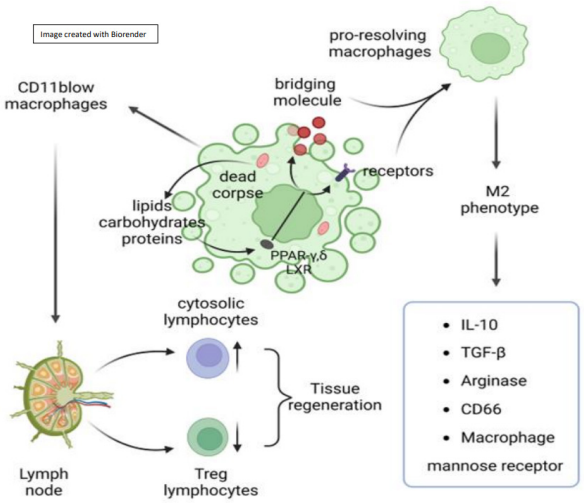

Fig. 12. Anti-inflammatory effects secondary to macrophage efferocytosis. Accumulation of large amounts of protein, lipids, and carbohydrates due to macrophage ingestion of dead cell activates the PPAR- γ, δ and LXR nuclear receptors. Activation of PPAR-γ and of LXR results in increased production of macrophage receptors and bridging molecules for efferocytosis. As efferocytosis continues, pro-resolving macrophages with M2 phenotype produce anti-inflammatory cytokines (e.g., IL-10 and TGF-beta) and express increased amounts of arginase, macrophage mannose receptor and CD36. Once macrophage engulfment reaches its capacity, macrophage transforms into CD11b low phenotype with deprived efferocytosis function. The CD11b low macrophage then migrate to neighboring lymph nodes to interact with cytotoxic and regulatory T-lymphocytes to suppress the activity of other immune cells, preventing overreaction, auto-immunity and chronic inflammation

Alternatively, these pro-resolving macrophages can be generated by cytokines such as IL-4 and IL-13.[145] Pro-resolving macrophages are tuned to perform increased efferocytosis with increased transcription of bridging molecules & receptors for binding more apoptotic cells for clearing the cellular debris from the inflammatory milieu (Fig. 12).[145] Furthermore, these pro-resolving macrophages are polarized to M2 phenotype with enhanced secretion of anti-inflammatory cytokines (IL-10 and TGF-β) and express increased amounts of arginase, macrophage mannose receptor and CD36 (Fig. 12).[145] Treg cells can also induce the production of alternative activated macrophages which are characterized by enhanced phagocytic capacity with increased expression of CD206 (macrophage mannose receptor) and CD163 (hemoglobin scavenger receptor) as well as reduced potency to secrete LPS induced pro-inflammatory markers.[146]

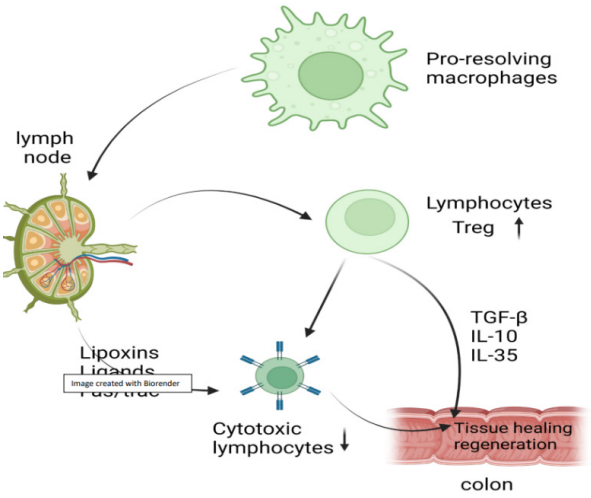

Fig. 13. Tissue healing secondary to macrophage efferocytosis. Pro-resolving macrophages migrate to the site of injury and to the lymph nodes where they interact with T cells to control inflammation. Macrophages are known to express ligands for FAS ligand/TNF-related apoptosis-inducing ligand death receptors on cytotoxic T-lymphocytes whose activation can initiate caspase dependent apoptotic signaling leading to their decreased survival. Pro-resolving macrophages can promote the recruitment of Treg cells from the lymph nodes towards the site of intestinal injury resulting in secretion of anti-inflammatory cytokines such as TGF-beta, IL-10 and IL-35. Overall, pro-resolving macrophages play a crucial role in the healing process by migrating to the lymph nodes by controlling inflammation, promoting tissue repair, modulating the immune response, and facilitating the removal of damaged cells

These resolution-phase macrophages regulated by cAMP, are mainly responsible for tissue homeostasis and repopulation of innate lymphocyte population in the resolution phase of acute inflammation.[147] Once the macrophage engulfment of apoptotic corpses reaches a saturation threshold, they transform into $CD11b^{low}$ phenotype characterized by their attenuated efferocytosis function. It is important to note that, these $CD11b^{low}$ phenotype can also be induced by pro-resolving mediators such as resolvin D1, resolvin E1 and dexamethasone.[148] As soon as these pro-resolving macrophages lose their engulfment capacity, they immediately migrate to the neighboring lymph nodes and secrete 12-lipo-oxygenase metabolites to interact with T-lymphocytes to mediate silencing of the innate immune response, which is instrumental for tissue regeneration, and healing (Fig. 13).[148] Moreover, macrophages can directly migrate and interact with T-lymphocytes in the lymph nodes to cause negative regulation of immune system via secretion of macro-molecular mediators such as interferons and complement components.[149] Activation of macrophage ligand receptors such as programmed cell death 1, cytotoxic lymphocyte associated protein 1, histocompatibility leukocyte antigen-G and histocompatibility leukocyte antigen-E) can directly suppress the cytotoxic action of T-cells, natural killer cells in the lymph nodes.[150] Macrophages are known to express ligands for FAS ligand and TNF-related apoptosis-inducing ligand death receptors on T-lymphocytes whose activation can potentiate caspase dependent apoptotic signaling in them and thereby leading to their decreased survival (Fig. 13).[150] Furthermore, macrophage induced secretion of chemokines such as CCL5, CCL20, CCL12 and CCL22 might cause Treg recruitment to the inflammatory milieu thus paving the way for tissue regeneration (Fig. 13).[150-152] Enhanced Treg recruitment might favor downregulation of immune response by inhibition of $CD4^+$ and $CD8^+$ lymphocytes through secretion of IL-10, IL-35 and TGF-β (Fig. 13).[152] Thus, macrophage engulfment of apoptotic cells along with suppression of immune response results in clearance of corpses from the inflammatory milieu in an immunologically silent manner that promotes tissue healing, regeneration, and repair.

Impaired efferocytosis and its consequences resulting in NEC intestinal injury

Any wreckage in this protective physiological phenomenon of macrophage efferocytosis can derail the clearance mechanisms of dead corpses from the inflamed tissues leading to defective tissue regeneration with subsequent progression to tissue necrosis, chronic inflammation, and autoimmunity. The physiological phenomenon of macrophage efferocytosis will be flawed when macrophages are dysfunctional or apoptotic corpuses become poor meal for engulfment of macrophages. This can occur due to variety of reasons ranging from decrease in the eat-me signals, increase in the don't eat-me signals, decreased production of bridging molecules, downregulated expression of macrophage receptors, presence of autoantibodies, NET formation, TLR4 signaling to accumulation of oxidized phospholipids Each of these abnormalities that can potentially impair macrophage efferocytosis in NEC models will be discussed individually in the next few paragraphs.

Lipid peroxidation due to accumulation of free radicals and peroxy-nitrates is implicated in the pathogenesis for transmural necrosis in the NEC models.[153,154] These free radicals can potentially oxidize the membrane phospholipids of macrophages resulting in accumulation of oxidized phospholipids in NEC.[155] These oxidized phospholipids can bind and saturate efferocytosis receptors and thereby binding capacity of macrophages for the apoptotic epithelial cells is attentuated due to competitive inhibition at the site of intestinal injury in the NEC models.[156] Moreover, oxidized phospholipids generated via lipid peroxidation might harbor

some neo-epitopes which might stimulate the production of autoantibodies by B-lymphocytes.[157] Due to their cross reactivity, these autoantibodies circulating in the blood might bind and mask the eat-me signals on the apoptotic epithelial cells.[158] This prevents their recognition by macrophages and thereby leads to their decreased clearance.

Aberrant activation of TLR4 signaling and its role in the pathogenesis of intestinal necrosis in NEC models has been extensively studied.[159,160] Enhanced TLR4 signaling in response to microbial associated molecular patterns such as LPS stimulates NF-κB mediated gene transcription of pro-inflammatory cytokines leading to epithelial apoptosis and mucosal injury in NEC. With increased presence of inflammatory markers in the intestinal tissue, macrophages are more polarized toward M1 phenotype which are more pro-inflammatory but possess less efferocytic capacity for clearing dead epithelial cells in the intestinal milieu.[161] Upregulated TLR4 signaling along with increased secretion of pro-inflammatory cytokines leads to decreased expression of lipoprotein receptor protein 1 and MerTK as well as reduced activation of LXR resulting in decreased clearance of apoptotic cells and reduced anti-inflammatory defenses.[162]

LPS stimulation of the macrophages can alter the gene expression of the transcription factors such as PPAR-γ (decreased) and IRF5 (increased) leading to attenuated expression of macrophage receptors (CD36 and CD14) and bridging molecules (Gas-6 and MFG-E8) leading to decreased efferocytosis potency in macrophages.[145,163-165] Inflammatory mediators such as LPS and TNFα can impair efferocytosis by altering the balance between RhoA and Rac in the macrophages.[145] High mobility group box1 (HMBG1) can bind and mask the macrophage receptor (αvβ3) thereby limiting its binding to MFG-E8 and subsequently to the PS exposed on apoptotic epithelial cells.[145] Receptor for advanced glycosylation end products (RAGE) and annexin V can directly bind to the phosphatidylserine exposed on apoptotic epithelial cells thereby blocking its recognition and removal by macrophages.[145]

MMPs such as streptolysin-1, implicated in the degradation of mucosal extracellular matrix, has been demonstrated in the NEC models.[166] These MMPs were also shown to degrade the eat-me signals (LRP-1) and efferocytosis receptors (MerTK) from the apoptotic cells and macrophages respectively leading to decreased efferocytosis in the intestinal tissues of NEC.[166] Furthermore, increased TNF-α might lead to NF-κB mediated increased transcription of CD47 (Don't eat-me signal) in the apoptotic epithelial cells thus concealing them from recognizing by macrophages for their timely removal.[167] The presence of pro-inflammatory cytokines such as TNF -α along with other toxins in the inflamed intestine in NEC can inhibit the binding of macrophages with apoptotic cells at the site injury through cytosolic phospholipase A2 (cPLA2) and Rho-GTPase (Rho Family of Guanosine-5'-tri-phosphatases) thereby impairing the dead cell clearance.[168]

Interferon regulatory factor 5 (IR5) levels are elevated in the macrophages and promotes M1 pro-inflammatory phenotype polarization in the murine model of NEC.[169] This increased expression of IR5 in macrophages can potentiate the disintegration of bridging molecule MFGE8 and its receptor αvβ3 ultimately leading to decreased macrophage engulfment capacity of apoptotic cells.[161]

Recruitment of neutrophils to the site of inflammation is an important part of innate immune response for combating against microbial threats in NEC.[170] Formation of NET is one of the most important mechanisms by which neutrophils neutralize the microorganisms in the inflamed gut.[170] NET is characterized by formation of web like structures, and these complexes accommodate anti-microbial proteins and DNA- like structures with histones.[170] It has been demonstrated that, NET formation impedes the process of efferocytosis through degradation of macrophage receptors, $\alpha_v\beta_3/\alpha_v\beta_5$ integrins via secretion of neutrophil elastases.[171] Moreover, histones present in the NETs can inhibit efferocytosis by competitive inhibition of macrophage receptors (MerTK and $\alpha_v\beta_5$) thereby preventing the interaction between bridging molecules Gas6 and MFG-E8 and apoptotic cells.[172]

It is important to understand that the underlying causes of decreased efferocytosis in NEC is the result of combined interplay of factors caused by imbalance of inducers and inhibitors of macrophages efferocytosis of apoptotic cells. As a result, there is an increased chance of progression from apoptosis to necrosis of intestinal tissues, which is the hallmark of NEC. Eventually, the increased lysis of necrotic intestinal cells results in spilling of intracellular contents into the extracellular milieu. The epitopes present in the extracellular nuclear DNA from the lysed cells might initiate the production of autoantibodies resulting in autoimmunity related clinical disorders. HMGB1 is nuclear protein which when released into the extracellular environment from necrotic intestinal cells can provoke inflammation leading to production of pro-inflammatory cytokines and predominance of M1 low-efferocytosis index macrophages.[173] Since there is a low population of pro-resolving high efferocytosis index macrophages, there is a lesser chance of healing and tissue repair after intestinal injury. This might lead to progression of disease with systemic complications, which is associated with high mortality and morbidity in NEC.

Pathological effects on brain and lung in NEC – relevance to perturbed efferocytosis

Brain

It is important to understand that, in order to prevent inflammation, timely clearance of apoptotic intestinal cells needs to happen so that intracellular contents containing danger signals are not spilled into the extracellular environment.[174] Apoptosis of intestinal epithelial cells occurring due to the bacterial LPS induced TLR4 signaling will gradually progress to secondary necrosis if macrophage efferocytosis mediated tissue repair is perturbed. Once secondary necrosis of intestinal epithelial cells happens, HMGB1 (danger signal) gets released into the systemic circulation leading to hyper-inflammation and sepsis with an increased production of pro-inflammatory cytokines.[174] Next, pro-inflammatory cytokines can increase the expression of MMPs which can potentially degrade the tight junctions and extracellular matrix of endothelial basement membrane of the blood brain barrier (BBB) resulting in matrix degradation and its subsequent leakiness.[175] In response with BBB injury, brain-resident microglia migrates to the site of BBB injury and produces pro-inflammatory cytokines such as IL-1 β and IL-6, further exacerbating the BBB dysfunction.[175] Moreover, in parallel to these cellular events hypoxia and ischemia which are important risk factors in the pathogenesis of NEC are associated with upregulation of inducible nitric oxide synthase leading to increase in nitric oxide and peroxy-nitrate free radicals, which are known to further aggravate the BBB dysfunction.[175-177] Furthermore, HMBG1 released into the systemic circulation can easily cross the leaky blood brain barrier and enter the brain. Once inside the brain, it can act on the TLR4 receptors of brain microglia and result in activation of NF-κB and IRF3 pathways. This results in secretion of cytokines chemokines, adhesion molecules and ROS species upregulation in the brain leading to pathological neuro-inflammation and neurological sequelae such neurodevelopmental delay.[174,178]

The pathological signs of pro-inflammatory cytokine storm inside the brain can range from impaired oligodendrocyte maturation, loss of hippocampal volume, disordered neuronal development, demyelination to axonal injury.[178-181] These pathological deficits ultimately manifest as clinical consequences ranging from cognitive impairment, psychomotor delay to neurodevelopmental delay in the NEC infants.[178]

Brain microglia engulf dead neuronal cells with the help of receptors such as C-X-3 motif chemokine receptor 3 (CX3CR1), purino receptor 6, purino receptor 12, stabilin 1, signal regulatory protein alpha, triggering receptor expressed on myeloid cells 2, MerTK, and CD11b (Integrin αM).[182] Microglia regularly perform surveillance of the brain parenchyma to remove senescent and dead neuronal cells for aiding in neuronal development.[182] Any failure in this physiological clearance mechanism in the brain parenchyma of preterm infants either due to downregulation of microglial receptors or failure to recognize apoptotic neuronal cells leads to accumulation of dead neuronal cells, secondary necrosis and amplification of inflammatory response. Allerdorf et al. reported that, addition of bacterial LPS to microglial-neuronal cultures resulted in excessive efferocytosis and neuronal loss via microglial surface de-sialylation and upregulation of engulfment complement receptor 3.[183,184] Cytokines are shown to have varying effects on the microglial efferocytosis in the brain. Increase in TNF-α in the brain causes massive upregulation of the microglial efferocytosis in both live and apoptotic neuronal cells resulting in disproportionate neuronal loss.[184,185] In the contrary, the anti-inflammatory cytokine TGF-beta was responsible for physiological pruning and neuronal development through increasing the expression of complement 1q on the neuronal synapses.[184,186] Myelin sheath and synapses are protected from excessive microglial phagocytosis through SIRP-α-CD47 interaction.[187,188] Any alteration in this regulation of microglial efferocytosis in preterm infants might result in excess microglial pruning of myelin sheath and synapses, leading to demyelination of neurons and disruption of synaptic transmission, ultimately resulting in neurodevelopmental delay. Engulfment of apoptotic neuronal cells by microglial cells can also elicit secretion of cytokines and chemokines, both of which can lead to restricted neurogenesis and cognitive decline.[189] Microglial efferocytosis primarily controls the regulation of the number of neuro-progenitor cells in the embryonic cerebral cortex, an essential structure for earlier brain development.[190] As NEC is frequently associated with presence of bacterial LPS, there might be an aggressive reduction in the number of neuro-progenitor cells, primarily through enhanced and uncontrolled microglial efferocytosis, resulting in defective cortical development in preterm infants.[190] Therefore, fine-tuning, and appropriate regulation of microglial efferocytosis is very much essential for physiological pruning of synapses as well as neurogenesis in the preterm infants. Accordingly, any downregulation and upregulation of this protective physiological mechanism and alteration of this delicate balance by inflammatory insults has been shown to be associated with increased risk of neuro-developmental delay in Necrotizing Enterocolitis as well as neurodegenerative diseases such as Parkinson disease and motor neuron disease.[191,192]

Lung

TLR4 receptor is expressed in the pulmonary epithelial cells and vascular endothelial cells.[193,194] Bacterial LPS and DAMPs has been shown to act on TLR4 receptors expressed in the lungs to stimulate pro-inflam-

matory gene expression through energizing NF-κB and MAPK signaling pathways.[195] As discussed above once the escape of danger signal HMGB1 from the necrotic intestinal epithelial cells into the systemic circulation happens, it can find its way towards pulmonary epithelial cells and act on TLR4 receptors. Activation of TLR4 receptors in the lungs by HMGB1 can cause enhanced neutrophil infiltration through production of chemoattractant factor namely CXCL5.[178] Neutrophil accumulation in the lungs can initiate lung inflammation through the release of neutrophil elastases and formation of NETs.[196].As the neutrophils try to eradicate the infection by engulfment of microorganisms by binding to their PAMPs and DAMPs, they can simultaneously undergo a change in gene expression leading to their senescence through apoptosis differentiation program.[197] In other instances, neutrophil phagocytosis of microorganisms such as *Staphylococcus aureus* can inhibit macrophage efferocytosis by upregulation of don't eat-me signals (CD47) and precipitate neutrophil death through necroptosis RIPK1 dependent manner. Moreover, apoptosis and lysis of neutrophils releases serine proteases, which can potentially stimulate the neighboring macrophages to produce pro-inflammatory cytokines such as TNF-α, IL-8 and IL-10 thereby amplifying the lung inflammation.[198]

Therefore, neutrophils that undergo apoptosis should be efficiently removed by alveolar macrophages in the pulmonary tissues before progression to necrosis for preventing the release of their toxic intracellular contents to the extracellular environment for preventing disastrous consequences. Clearance of apoptotic cells in the lung is usually performed primarily by alveolar macrophages and secondarily by dendritic cells & bronchial epithelial cells.[199] Compared to systemic tissue macrophages, lung alveolar macrophages specifically are known to possess reduced efferocytosis capability due to varied reasons such as reduced adhesion, very low expression of protein kinase CβII and inhibition of surfactant protein A and D.[199,200] Defective or lowered efferocytosis has been implicated in the pathogenesis of lung diseases such as asthma, acute lung injury and chronic obstructive pulmonary disease.[199] Engulfment of pathogenic bacteria by M2 phenotype alveolar macrophages might facilitate bacterial persistence due to their intrinsic properties ranging from enhanced oxidative metabolism, decreased antimicrobial activity to increased PGE2 production.[201]

TLR4 receptor is involved in the alveolar macrophage efferocytosis through increased expression of MerTK receptor. Moreover, analysis of blood from preterm and term infants revealed that there is decreased expression of TLR4 expression in the granulocytes and monocytes.[202] Macrophages in the preterm infants might have reduced capacity for efferocytosis due to decreased adherence receptor expression due to very low TLR4 receptor expression along with defective antigen presentation.[202-204] Pathogen-mediated TLR4 signaling has also been to implicated to alter the ratio of Th17/Treg ratio in the lung epithelium leading to increased production of cytokines along with immune cell recruitment further exacerbating the lung injury in NEC.[178]

So, defective alveolar macrophage efferocytosis along with low TLR4 receptor expression are the critical cellular abnormalities that might impair the clearance of dead apoptotic cells from the injured alveolar epithelium leading to persistent and chronic lung inflammation in preterm infants.

Novel therapeutic options for increasing efferocytosis and enhancing tissue repair in NEC

The novel therapeutic interventions that can be useful in increasing the efferocytosis and ultimately facilitating tissue repair and regeneration in NEC will be discussed in the following subheadings: anti-CD47 antibodies, blocking ADAM17 cleavage, tilting SPM:leukotriene ratio, phosphatidylserine liposomes, PPAR gamma agonists, LXR agonists, glucocorticoids and annexins (Fig. 14).[156]

Anti-CD47 antibodies

Tumor cells in multiple cancers including breast, lung, colon, and ovary are known to upregulate CD47 (don't eat me signal) and interact with SIRP-alpha receptor on macrophages to elude efferocytosis and subsequent immune mediated destruction.[205] Interaction of CD47-SIRP-alpha leads to ITIM based activation of tyrosine phosphatases (SHP-1 and 2) and down regulation of actin cytoskeleton which ultimately causes inhibition of efferocytosis by tumor associated macrophages.[206] Clinical research has shown that blocking this interaction with anti-CD47 antibodies yielded therapeutic benefit by promoting efferocytosis and, thereby allowing tumor clearance.[207] Additionally, anti-CD-47 antibody administration also resulted in the activation of dendritic cells by release of tumor cell nuclear and mitochondrial DNA into the tumor microenvironment. This dendritic cell activation results in subsequent priming of cytotoxic T-lymphocytes through release of IFN-γ. Activated cytotoxic T-lymphocytes will set in motion robust anti-tumor innate immune responses leading to enhanced tumor clearance and improved survival rates.[208]

Blocking ADAM17 induced cleavage

Studies have shown that activation of MerTK receptor signaling on macrophages leads to increased synthesis of cytoplasmic 5-lipooxygenase (5-LOX) through suppressed activity of calcium dependent protein kinase II (CaMKII).[209] 5-LOX is the key synthetic enzyme implicated in the synthesis of SPMs (specialized pro-resolving mediators) such as resolvins and lipoxins from long

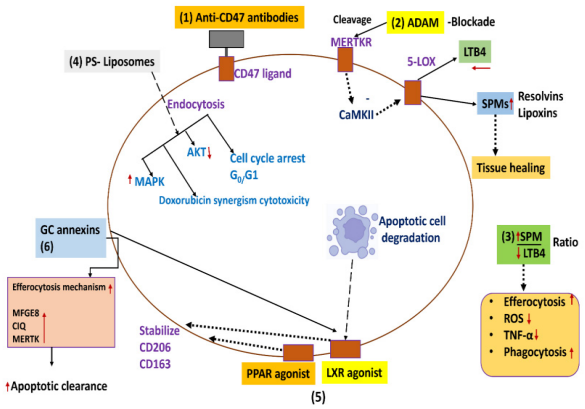

Fig. 14. Treatment modalities for alleviating cell death in NEC based on macrophage efferocytosis. (1) Anti-CD47 antibodies: Anti-CD47 antibodies are designed to bind to the CD47 protein, thus blocking its ability to act as an "Don't eat me" signal. By binding to CD47, the antibodies reduce the expression of CD47 on the surface of the dying cells, allowing macrophages to recognize and engulf them, thereby reducing inflammation and cell death in NEC. (2) Blocking ADAM17 cleavage: ADAM17 is a protease enzyme that is responsible for cleaving certain membrane-bound proteins into their active forms. MerTK (Mer protooncogene tyrosine kinase) is a receptor that is found on the surface of macrophages. MerTK activation leads to increased synthesis of 5-lipooxygenase (5-LOX). 5-LOX is an enzyme that plays a key role in the synthesis of specialized pro-resolving mediators (SPMs) such as resolvins and lipoxins which are known to have anti-inflammatory and pro-resolving properties, and their production by 5-LOX is tightly regulated. Targeting the production of SPMs by modulating the activity of enzymes such as 5-LOX by blocking ADAM17 cleavage could be a potential therapeutic approach to resolving NEC inflammation. (3) Tilting SPM: leukotriene ratio: The clearance of apoptotic cells is dependent on the delicate balance between SPMs and pro-inflammatory LTB4 (Leukotriene B4) in the inflamed tissue. By tilting the ratio of SPMs to leukotrienes, it means to increase the production of SPMs and reduce the production of leukotrienes. This can be achieved by targeting the activity of enzymes such as 5-lipooxygenase (5-LOX), which is responsible for the synthesis of SPMs and leukotrienes. This shift in the balance of pro-inflammatory and anti-inflammatory molecules would reduce inflammation and cell death in NEC. (4) Phosphatidylserine liposomes: phosphatidylserine liposomes are a potential treatment modality for alleviating cell death in NEC by attracting macrophages to the gut, promoting efferocytosis, and reducing inflammation and oxidative stress. PS-liposomes tend to enter cancer cells expressing phosphatidylserine via endocytosis and mediate malignant cell death through activation of MAPK, downregulation of AKT, and cell-cycle arrest at sub-G0/G1 phase. These mechanisms lead to cancer cell death and can be used as a potential treatment modality. This is achieved by binding to PS on the surface of the infected cells and recruiting immune cells resulting in destruction of infected cells. (5) PPAR gamma LXRs agonists: Peroxisome proliferator-activated receptor-gamma (PPAR-gamma) is a nuclear receptor that is involved in the regulation of inflammation and cell death. PPAR-gamma agonists are drugs that can bind to and activate PPAR-gamma, leading to an anti-inflammatory response and the inhibition of cell death. Liver X receptors (LXRs) are nuclear receptors that are activated by oxysterols, which are derivatives of cholesterol. Activation of LXRs has been shown to promote the resolution of inflammation by promoting the activation of anti-inflammatory macrophages. Activation of LXRs by LXR agonists can lead to the stabilization of CD206 and CD163, which are markers of anti-inflammatory macrophages. This stabilization promotes the resolution of inflammation by promoting the activation of anti-inflammatory macrophages. (6) Glucocorticoids and annexins: Glucocorticoids (GCs) have anti-inflammatory and immunosuppressive properties and may help to reduce inflammation and cell death in necrotizing enterocolitis (NEC) by decreasing the production of inflammatory mediators, upregulation of efferocytosis machinery and gene transcription (such as LXR, PPAR-gamma and RXR (Retinoid X receptor) and promoting the long-term clearance of apoptotic cells. GCs also promote macrophage and monocyte efferocytosis through increased upregulation of bridging molecules and efferocytosis receptors such as MFG-E8, C1q, stabilin, CD206, CD163 and MerTK. Annexins, a family of proteins, also play a role in cell death and inflammation in NEC by acting on the lipoxin A4 (LXA4) receptor and causing F-actin reorganization in macrophages, leading to increased engulfment of apoptotic neutrophils, and enhancing their own removal via professional phagocytes through efferocytosis

chain fatty acids.[209,210] Upregulation of SPMs has been shown to be associated with resolution of inflammation in various conditions including sterile peritonitis, ischemia-reperfusion (I/R) injury and remote organ inflammation.[210] Increase in SPMs in the tissues can be beneficial for better resolution of tissue injury as they prevent the decrease of MerTK expression on macrophages and increase efferocytosis.[211] The presence of severe hypoxia and ER stress during inflammation can precipitate the synthesis of metalloproteinase ADAM17 in the intestinal tissues.[212] This enzyme can cause proteolytic cleavage of MerTK receptor from macrophages and thereby impeding the synthesis of SPMs in the intestinal tissues required for resolution of inflammation.[210] In this regard, MerTK deficiency in cardiac macrophages after myocardial I/R injury can result in impaired car-

diomyocyte wound healing, increased infarct size and depressed cardiac pump function.[213] Accordingly, presence of cleavage resistant MerTK receptor on tissue macrophages was associated with better tissue healing and improved clinical outcomes.[209,210,213] Moreover, deletion of ADAM17 on professional phagocytes also yielded better clinical outcomes through enhancing efferocytosis, inducing anti-inflammatory cytokine profile as well as resolution of inflammation.[214] These above findings add credence to the concept that therapeutic agents aimed at cleavage and inactivation of enhanced ADAM17 generated during hypoxia and oxidative stress might be beneficial in enhancing efferocytosis as well as subsiding tissue inflammation via MerTK induced production of pro-resolving mediators.

Tilting SPM-leukotriene ratio

The clearance of apoptotic cells is dependent on the delicate balance between SPMs and pro-inflammatory leukotriene B4 (LTB4) in the inflamed tissue. In atherosclerosis, downregulation of SPMs and predominance of LTB4 can be detrimental and leads to enhanced atherosclerotic plaque instability[215]. Accordingly, supplementation of SPMs had shown clinical benefit in atherosclerosis and hepatic diseases by rectifying this abnormal SPM: LTB4 ratio.[215,216] SPMs that can be used to enhance efferocytosis and promote resolution of inflammation can range from lipoxins, resolvins, protectins, maresins cysteinyl-conjugated SPMs (CTRs) to 13-series resolvins.[217] Resolvin D1 acts through its receptor ALX/FRP2 (Formyl peptide receptors 2) and increases efferocytosis by making the MerTK receptor resistant to cleavage due to senescence, increased M2 polarization and enhanced production of pro-resolving mediators through feed forward mechanism.[216-218] Other mechanisms proposed for action of RD1 in resolution of inflammation include downregulation of ROS, decreased production of TNF-α, ER mediated phagocytosis, release of calreticulin from macrophages, epigenetic mechanisms, and increased metabolism of macrophages (fatty acid oxidation and oxidative phosphorylation).[217] Lipoxin A4 (LXA4) is another SPM that has been studied extensively and it has been shown to increase efferocytosis by promoting cytoskeletal rearrangement and engulfment through signaling molecules such as MYH9 and CDC42.[219]

Phosphatidylserine liposomes

Phosphatidylserine dependent recognition and signaling is a key event in recognition and clearance of apoptotic cells by macrophages as well as resolution of inflammation.[220] In chronic granulomatous disease (CGD), impairment of macrophage efferocytosis was normalized by injection of phosphatidylserine through IL-4 dependent macrophage reprogramming and increase in apoptotic cell uptake.[221] Cancer cells are known to exhibit unusually high amount of phosphatidylserine on their surface (approximately 3-7-fold more than phosphatidylserine expressed by non-tumor cells).[222] Research studies utilizing phosphatidylserine -liposomes have yielded encouraging results by enhanced killing as well as inhibiting the growth of phosphatidylserine expressing cancer cells in animal models of cancer.[223,224] Phosphatidylserine -liposomes tends to enter the cancer cells expressing phosphatidylserine via endocytosis and mediate malignant cell death through activation of MAPK, downregulation of AKT and cell-cycle arrest at sub-G0/G1 phase.[224] Additionally, incorporation of anti-cancer drugs like doxorubicin and gemcitabine into the phosphatidylserine -liposomes have demonstrated synergism in killing effects of the cancer cells as along with inhibiting metastasis in mice models of cancer.[223-225] Furthermore, administration of chimeric antibodies (bavituximab) which are developed to recognize the phosphatidylserine expressed on virus infected cells are known to promote viral destruction and clearance through antibody dependent cytotoxicity in guinea pigs infected with *Pichinde* virus.[226]

LXR agonists and PPAR ɣ agonists

Both PPAR and LXR regulate apoptotic cell uptake as well as anti-inflammatory cytokine profile of macrophage thereby playing a central role on tissue repair and resolution of inflammation.[227-229] Accumulation of fatty acids and oxysterols released from the nuclear membrane of dead cells stimulate PPAR and LXR nuclear receptors respectively in the macrophages and bolsters the process of apoptotic cell removal.[230] Upregulated PPAR and LXR genes are known to further promote the clearance of apoptotic cells by macrophages via increased production of MFG-E8/C1q and MerTK respectively in feed-forward manner.[230] Application of PPAR and LXR agonists had yielded encouraging results in the clinical diseases by increasing macrophage efferocytosis of dead cells. In CGD, neutrophils treated with pioglitazone (PPAR agonists) displayed abundant eat-me signals upon apoptosis via oxidant dependent manner resulting in enhanced efferocytosis and resolution of sterile inflammation.[228] In CGD, macrophages exhibited significant impairment in the efferocytosis of carboxylated beads which was reversed by overnight incubation with pioglitazone (PPARγ receptor agonist).[231] Treatment of macrophages isolated from *Trypanosoma cruzi* infected mice with PPAR ligands caused predominance of M2 polarization with increased expression of arginase-1, TGF-β and mannose receptors which are primarily responsible for efferocytosis, tissue repair as well as resolution of inflammation.[232,233] On the contrary, administration of PPAR antagonists resulted in downregulation of genes responsible for macrophage-induced

uptake of apoptotic cells: namely CD36 (membrane glycoprotein), TG2 (tissue transglutaminase), AXL receptor tyrosine kinase and pentraxin related protein 3.[234] It has been documented that TLR4 receptor activation might inhibit the LXR mediated signaling pathways leading to decreased M2 macrophage phenotype and impaired resolution of inflammation.[235] So, usage of LXR agonists might overcome this inhibition along with prompt resolution of inflammatory response after intestinal tissue injury.[235]

Glucocorticoids and annexins

Glucocorticoids (GCs) and annexins have previously been shown to be efficacious in resolving inflammation and enhancing efferocytosis in some research studies.[156] GCs enhance macrophage efferocytosis by acting through various modes of action including secretion of anti-inflammatory mediators, upregulation of efferocytosis machinery and gene transcription.

GC-treatment-induced increased expression of annexin D-1 in macrophages and monocytes is one of the important underlying mechanisms for increased uptake of apoptotic cells.[236] Moreover, GCs promote macrophage and monocyte efferocytosis due to increased upregulation of bridging molecules & receptors such as MFG-E8, C1q, stabilin, CD206, CD163 and MerTK receptors.[236-238] In lungs of chronic obstructive pulmonary disease (COPD) patients, GCs treatment resulted in increased efferocytosis of apoptotic neutrophils due to upregulation of CD163, CD64 and MerTK receptors on the alveolar macrophages.[239] Furthermore, GCs act at the nuclear level and increase the transcription of lipid sensing receptor genes such as LXR, PPAR-γ and retinoid X receptor thereby promoting the long-term clearance of apoptotic cells.[240]

According to a report by Scannell M et al., apoptotic neutrophils, lymphocytes and thymocytes secrete annexin and related peptide derivates into the conditioned medium which can potentially enhance their own removal by professional phagocytes such as macrophages through efferocytosis.[241] Mechanistically, annexin acts on LXA_4 receptor and causes F-actin reorganization in macrophages leading to increased engulfment of apoptotic neutrophils.[242] In mycobacterial infections, the presence of annexins on the apoptotic cells promotes their engulfment by professional phagocytes such as dendritic cells (DCs) leading to MHC-class I antigen presentation, CD8+T cell activation, immune response and control of bacterial infection.[243]

Necrotizing enterocolitis is a multifactorial disease accounting for approximately 1-5% of NICU admissions.[244] Specifically, it occurs in the preterm newborns who are born at less than 32 weeks pregnancy and weigh < 1500g. Some of the important predisposing factors implicated include sepsis, asphyxia, meconium aspiration syndrome, prolonged parenteral feeding, and immature intestinal immunity. NEC infants usually present with non-specific signs and symptoms. Most of the NEC infants are referred to surgical management due to late detection and for management of complications such as pneumo-peritoneum, ascites, portal venous gas and fixed persistent intestinal loop. Unfortunately, the case fatality rate of NEC infants referred to surgical management is very high (30%-50%).[245] Therefore, earlier detection and prompt management is the key for improving clinical outcomes as well as for preventing mortality and morbidity. As there are no specific therapeutic interventions to arrest the disease progression after its instigation by microbial insult, disease pathogenic mechanisms surrounding intestinal injury in NEC need to be comprehended and fathomed in a meticulous manner. As a result, clinical researchers have started to characterize and grasp the underlying downstream signaling events that are responsible for NEC tissue injury.

Conclusion

Intestinal cell death is one of the critical cellular events that occurs in the acute phase of NEC. Its occurrence is perceived as a forewarning for subsequent derangements of disease progression. It is regarded as a harbinger of pathological events such as increased intestinal permeability, local inflammation, and systemic inflammation. Bacterial LPS-TLR4 signaling with the resultant release of pro-inflammatory cytokines, chemokines and free radicals is postulated to be an important mechanism for inciting intestinal cell death in NEC. The different types of intestinal cell death that are encountered in NEC include apoptosis, necrosis, necroptosis, pyroptosis and autophagy. Upon intestinal epithelial cell death, there will be compensatory response provoked by cellular battalion comprising epithelial cells, Paneth cells, neutrophils, T-lymphocytes, B-lymphocytes, and macrophages for neutralizing the infectious threat, limiting the spread of inflammation as well as for regenerating the injured intestinal epithelium. This review mainly discusses innate cellular defense mechanisms that occur post intestinal cell death in NEC in a scrupulous manner. Particularly, macrophage efferocytosis is explained in an efficient and structured manner with a special emphasis on find-me signals, eat-me signals, macrophage receptors, bridging molecules, mechanism, and anti-inflammatory responses. Macrophage efferocytosis is a principal mechanism of ingesting dead intestinal epithelial cells. This physiological process is of paramount importance as its successful materialization in the intestinal milieu results in efficient dead cell clearance and facilitates mounting of robust anti-inflammatory cytokine responses for promoting tissue healing and regeneration.

Epithelial cells dying in the inflamed gut should be cleared in a timely manner so that tissue repair and healing can occur by replacement of dead cells with new cells. Engulfment of dead cells occurs by a process known as efferocytosis performed primarily by professional phagocytes (macrophages). This physiological mechanism is very important to resolution of intestinal inflammation and any impairment of this phenomenon can cause the dying epithelial cells to undergo necrosis. The release of inflammatory mediators by necrotic intestinal epithelial cells into systemic circulation leads to further cell death, inflammation, and autoimmune diseases.

Dying intestinal epithelial cells secrete chemotactic signals known as find-me signals which facilitates the migration of macrophages towards them. In some instances, cancer cells express specific don't eat-me signals such as CD-47 that prevents their recognition, removal, and subsequent immune response. Epithelial cells in earlier phase of apoptosis express specific eat-me signals such as phosphatidylserine which facilitates their recognition by migrating macrophages. Macrophage phagocyte receptors (BAI-1, TIM-4, CD300, αvβ5 and Mer-TK) as well as bridging molecules (MFG-E8, GAS6 and protein S) compromise efferocytosis machinery which are utilized for engulfment and removal of dying epithelial cells. The interaction of dead cells and macrophages causes formation of CRKII-DOCK180-ELMO complex, Rac1 activation and cytoskeletal rearrangement leading engulfment and destruction of dead cells. Successful elimination of dead epithelial cells by macrophage efferocytosis is associated with tissue healing, repair, and resolution of inflammation due to secretion of anti-inflammatory cytokines such as IL-4 and IL-13.

Decreased efferocytosis in NEC can result from combined interplay of factors such as increased oxidized phospholipids, enhanced TLR4 signaling, increased pro-inflammation cytokines, M1 macrophage phenotype predominance, NET formation, and increased matrix metalloproteinases. Macrophages in the preterm infants might have reduced capacity for efferocytosis due to decreased adherence receptor expression, attenuated antigen presentation as well as very low membrane bound TLR4 receptor. As a result, decreased efferocytosis capability in the preterm infants predisposes them to develop lung and brain complications in NEC due to disrupted removal of dead cells and resulting pathological consequences. Novel therapeutic interventions for increasing efferocytosis and preventing disease progression in NEC can be classified into following categories such as a) anti-CD-47 antibodies, b) blocking ADAM-17 cleavage, c) phosphatidylserine liposomes, d) PPAR γ agonists, e) correcting SPM-leukotriene ratio and f) annexins. Basic science and clinical research studies are warranted for probing the mechanisms of defective efferocytosis in preterm infants susceptible to NEC. Such studies might unveil new molecular targets that can counteract deficiencies for facilitating macrophage efferocytosis. Development of disease specific therapeutic interventions based on these uncovered molecular targets might be beneficial in facilitating dead cell removal and improving tissue repair in earlier stages of NEC. Rectifying the decreased efferocytosis during intestinal inflammation in NEC models might be valuable for reversing disease progression, optimizing clinical outcomes as well as decreasing mortality and morbidity.

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Author contributions

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Conflicts of interest

None of the authors has any conflict of interest.

Data availability

No data is available. Data sharing is not relevant because no datasets were created and/or analyzed for this study.

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REVIEW PAPER

Progestins and combined oral contraceptives in the hormonal treatment of endometriosis – a review

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ABSTRACT

Introduction and aim. Endometriosis is a common inflammatory disease affecting 6-10% of women of reproductive age. It is defined as the growth of endometrial-like tissue outside the uterine cavity. Dysmenorrhea, pelvic pain, dyspareunia and infertility are the main symptoms of endometriosis patients. Endometriosis treatment methods can be broadly divided into surgical and pharmacological. Currently, hormonal drugs are often used for women with endometriosis to relieve bothersome symptoms. The aim of this article is to review new publications presenting the effectiveness as well as side effects of the use of progestins and combined oral contraceptives in the hormonal treatment of endometriosis.

Material and methods. A review of the literature regarding progestins and combined oral contraceptives in the treatment of endometriosis was performed using the PubMed database. In the end, 67 articles were included in this review.

Analysis of the literature. Progestins and combined oral contraceptives significantly reduce dysmenorrhea, dyspareunia and pelvic pain in women with endometriosis. However, there is a risk of potential side effects, which should be taken into account when choosing a therapy for each patient individually.

Conclusion. Endometriosis is a chronic disease that has a significant impact on the health-related quality of life of patients. When choosing a treatment, many aspects should be considered, primarily the patient's preferences, drug tolerance and safety. Further drug research is needed to determine the most effective treatment for endometriosis.

Keywords. combined oral contraceptives, endometriosis, health-related quality of life, infertility, pelvic pain, progestins

Introduction

Endometriosis is a chronic inflammatory disease, that affects about 6-10% of women of reproductive age and is characterized by endometrial tissue outside the uterus. It is a common cause of infertility, pelvic pain and dyspareunia. The clinical picture is often non-specific, which leads to diagnostic difficulties and delayed diagnosis.[1,2] Currently, three subtypes of endometriosis are presented: superficial peritoneal, deep and ovarian.[3] Laparoscopy is the preferred method in diagnosing endometriosis.[4] Although causal treatment is not possible, clinical trials have provided many strategies for managing the symptoms of this disease - combating pain, improving fertility and treating complications. They can be divided into surgical removal of lesions and drug treatment.[1] Currently, the role of hormone therapy in endometriosis is emphasized, which effectively relieves the symptoms of the disease and improves the health-related quality of life (HRQoL).[5]

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Aim

The aim of this article is to review the latest publications mostly from 2015-2022, which present the medical approach with progestins and combined oral contraceptives in the hormonal treatment of endometriosis, the advantages and potential side effects of this pharmacological treatment.

Material and methods

Using the PubMed database, a literature review on progestins and combined oral contraceptives in the hormonal treatment of endometriosis was performed; search terms "*endometriosis, endometriosis treatment, progestins, dienogest, dydrogesterone, norethindrone acetate, medroxyprogesterone acetate, levonorgestrel intrauterine device, etonogestrel-releasing subdermal implant, combined oral contraceptives, ethinylestradiol, drospirenone, levonorgestrel, desogestrel*" were applied. Ultimately, 67 articles were used for this review.

Analysis of the literature

Estrogens stimulate the proliferation of the endometrial mucosa and increase the number of receptors for progesterone. In turn, progesterone induces cyclical secretory changes in the endometrium in preparation for implantation.[6] Ectopic endometrial lesions respond to the cyclical secretion of ovarian steroids, mainly estrogen and show significant resistance to progesterone.[7] The aim of hormonal therapy is to block menstruation by inducing a state of iatrogenic menopause or pseudopregnancy. Medical hormone therapy can control pain symptoms to prevent or postpone surgery.[5] Commonly used drugs are Progestins, Combined Oral Contraceptives Pills, Gonadotropin Releasing Hormones (GnRH) agonists, Gonadotropin Releasing Hormones (GnRH) antagonists, Aromatase inhibitors, Danazol, Gestrinone, Selective estrogen receptor modulators (SERM), Selective progesterone receptor modulators (SPRM). In addition, Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are used in the symptomatic treatment of pelvic pain and dysmenorrhea.[8] Surgical interventions are aimed at removing endometriosis lesions. Resection of endometriosis helps in restoring fertility and reduces pelvic pain symptoms.[9,10]

Progestins

Progestins are synthetic compounds that have a similar effect to progesterone. They differ in potency and profile in the hypothalamic-pituitary axis, genitals and breast tissue. Progestins reduce the frequency and increase the amplitude of the pulsatile release of gonadotropin-releasing hormone (GnRH), which causes a decrease in the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Administration of progestins in continuous therapy leads to inhibition of ovarian steroidogenesis and anovulation.[11] Progestins binding to progesterone receptors (PRs) may induce anti-estrogenic, anti-inflammatory and pro-apoptotic effects. They help to relieve pain and suppress endometriosis.[12] Side effects of progestins are included in Table 1. According to the guidelines, progestins are the first-line therapy for the treatment of pain in endometriosis.[13] There are various forms of progestins available to treat endometriosis. They can be administered by an oral, intramuscular, subcutaneous, patch or intrauterine route.[14] Below is a description of a few selected representatives from this group.

Table 1. Major side effects and complications of endometriosis treatment with progestins and COCs

Medical treatment	Side effects and complications
Progestins	unscheduled bleeding, breast tenderness, bloating, mood changes, weight gain,[15] hot flushes, acne, loss of libido, headache, fatigue,[14] possible reduction in bone density (long-term use of depot medroxyprogesterone acetate and dienogest)[16]
Combined Oral Contraceptives (COCs)	weight gain, water retention, leg swelling, cellulite, breast tenderness, nausea, headaches, spotting, negative impact on carbohydrate metabolism, lipid profile and liver function, risk of venous thromboembolism[17]

Dienogest

Dienogest is a derivative of 19-nortestosterone with a high specificity towards PR.[8] Dienogest inhibits the systemic secretion of gonadotropins and exerts local anti-inflammatory and anti-proliferative effects.[18] This progestin is administered orally, mainly at a dose of 2 mg per day.[19] Dienogest has been shown to improve endometriosis-related symptoms such as chronic pelvic pain, dysmenorrhea, dyspareunia and thereby improve HRQoL.[20] Randomized controlled trials showed dienogest efficacy comparable to GnRH analogues but with better tolerability.[21,22] Dienogest has also been shown to be superior to NSAIDs in improving pain relief and quality of life in long-term therapy.[23] The study proved that long-term dienogest treatment (24 months at a dose of 2 mg once a day) in women with endometriosis has a positive effect on the quality of life and sexual function. A slight improvement in dysmenorrhea, chronic pelvic pain and dyspareunia was observed in the study group after 3 months of treatment with dienogest, and a greater improvement from 6 to 24 months. The Female Sexual Function Index and the Female Sexual Distress Scale did not change after 3 months but improved from 6 to 24 months. Quality of life from 6 to 24 months improved in all categories. No changes were observed in the control group receiving NSAIDs.[24] Another study showed a delay in regaining fertility during the first three cycles after discontinuation of dienogest. Subsequently, the cumulative conception rate was no different from that observed in fertile women who were not using contraception.[25] The most common side effects of dienogest are abnormal uterine bleeding, weight gain, headache, acne and

depressed mood.[26,27] There is a possibility of bone loss in some women, therefore bone mineral density (BMD) should be checked in patients on long-term treatment.[28]

Dydrogesterone

Dydrogesterone (6-dehydro-retroprogesterone) is a retroprogesterone that is similar in structure and pharmacology to endogenous progesterone. It is a selective progesterone receptor agonist and has good oral bioavailability.[29] Dydrogesterone is used as a postmenopausal replacement hormone, in endometriosis and the treatment of menstrual disorders. It has been shown to improve endometriosis symptoms and reverse lesions. Meta-analysis showed the advantage of dydrogesterone over gestrinone in relieving dysmenorrhea and increasing pregnancy rates. Compared to GnRH-a, dydrogesterone reduced the risk of endometriosis recurrence.[30] In a 6-month cohort study, it was proven that prolonged cyclical and continuous regimens of dydrogesterone treatment (dydrogesterone 10 mg 2 or 3 times a day, between days 5 and 25 of the menstrual cycle as an extended cyclic regimen or continuously as a continuous regimen) showed a significant reduction in chronic pelvic pain, dysmenorrhea, improved HRQoL and sexual well-being.[31] Dydrogesterone is preferred in cases where the patient is planning a pregnancy because when taken in a cyclical regimen, it does not inhibit ovulation. Abnormal bleeding from the uterus is the most common side effect.[32]

Norethindrone acetate

Norethindrone acetate is effective in the long-term treatment of endometriosis. By inhibiting gonadotropins, it causes hypoestrogenism, inhibition of ovulation, development of amenorrhea and endometrial tissue atrophy.[33] Norethindrone acetate has progestogenic and androgenic effects. It can cause weight gain, seborrhea and acne.[34] There was a study that looked at the percentage of patients who were satisfied with their treatment of norethindrone acetate (2.5 mg/day) and dienogest (2 mg/day). The percentage of women satisfied with the treatment with norethindrone acetate was 71% and with dienogest 72%. Switching from norethindrone acetate to dienogest was not associated with significant improvements in pain relief and HRQoL. Dienogest should be suggested in women intolerant to norethindrone acetate.[35]

Medroxyprogesterone acetate

Medroxyprogesterone acetate is a 17-OH derivative of progesterone. It is available as an oral or depot preparation (DMPA) that can be administered intramuscularly or subcutaneously every 3 months. The standard dose of DMPA is 150 mg/ml intramuscularly.[36,37] In a clinical trial, DMPA-SC (104 mg/0.65 ml given by subcutaneous injection) was shown to be as effective as leuprolide (11.25 mg by intramuscular injection). However, DMPA-SC has fewer hypoestrogenic side effects and less impact on BMD, but more bleeding.[38] This therapy should not be used in women who want to get pregnant in the near future, due to long-term inhibition of ovulation and its delayed return. In a clinical study evaluating the pharmacokinetics and pharmacodynamics of medroxyprogesterone acetate, none of the women ovulated before 7.5 months.[39]

Levonorgestrel intrauterine device (LNG-IUS)

LNG-IUS can be used as an alternative therapy in women with endometriosis. The LNG-IUS is a small device that is placed in the uterus. LNG progestin is a 19-nortestosterone derivative. The LNG-IUS delivers LNG directly to the endometrium at a rate of 20 μg per day and can stay in place for five years or more.[40] The main mechanisms of LNG-IUS in the treatment of endometriosis are an intensification of apoptotic activity, induction of endometrial gland atrophy and stroma transformation.[8] LNG-IUS is also used for contraception, treatment of heavy menstrual bleeding, and protection of the endometrium in women with breast cancer receiving tamoxifen.[41-44] This method of treatment also comes with some potential side effects. The risks associated with the device, such as expulsion, pelvic inflammatory disease and perforation, are suggested to be low. Breakthrough bleeding and spotting may occur during the first few months of use. Other side effects include pelvic pain, ovarian cysts, breast tenderness, acne and weight gain.[40]

Etonogestrel-releasing subdermal implant (ENG-implant)

ENG is a subcutaneous implant that systematically releases a synthetic substance similar to progesterone.[45] In a study evaluating the effectiveness of the ENG-implant compared to the 52-mg LNG-IUS in the control of endometriosis-related pelvic pain, both contraceptives were shown to significantly improve dysmenorrhea, pelvic pain and HRQoL.[46] Subdermal implants (ENG-implant 68 mg with a life span of 3 years) have been found to be as effective in pain relief over 12 months of use as DMPA. ENG-implant is safe, well tolerated and achieving contraception.[11]

Combined oral contraceptives

COCs inhibit LH and FSH, resulting in the inhibition of ovulation. They significantly reduce pelvic pain, dysmenorrhea, profound dyspareunia and dyschezia in women with endometriosis.[47] A clinical study was conducted in women with recurrent dysmenorrhea after conservative surgery for endometriosis, which demonstrated that continuous and long-term use of COCs reduces the frequency and severity of pain symptoms of patients.[48] Studies have shown that treatment with

COCs, compared to placebo, relieves dysmenorrhea, dyschezia, dyspareunia and reduces the size of endometriosis.[49,50] Another study evaluating the effects of oral contraceptives and dienogest on endometriosis-related chronic pelvic pain, sexual function, and quality of life found comparable efficacy for both treatments.[51] A systematic review reported that the effectiveness of COCs in reducing pain was similar to or less than that of GnRH agonists and oral progestogens.[52] There is evidence that COCs taken for six months reduce heavy menstrual bleeding (HMB) in women. However, compared to LNG-IUS, combined oral contraceptives were less effective.[41] The use of COCs causes a 4 to 7-fold increase in the risk of thromboembolic disease.[53] The risk factors for thromboembolic events are mainly age, positive family history, genetic thrombophilias, smoking, obesity, hypertension, atrial fibrillation, prolonged immobilization, major surgery or trauma.[54] The most common side effects of COCs are listed in Table 1.

Ethinylestradiol/Drospirenone

Ethinylestradiol(EE)/drospirenone is a new generation COC that has antimineralocorticoid and antiandrogenic effects. This combined formulation contains 3 mg of drospirenone and 20 μg of EE. The 24/4 regimen causes less fluctuation in hormone levels compared to the conventional 21/7 regimen. This COC provides good cycle control and reduces estrogen-related side effects. EE/drospirenone has a good safety profile and good tolerability. There is a low risk of thrombosis, water retention and weight gain. Side effects are mainly headache, nausea and chest pain.[55] An observational study has shown that HRQoL is significantly improved after treatment with EE 20 μg/drospirenone 3mg in a 24/4 cycle in patients with dysmenorrhea.[56] Menstrual pain can be severe during withdrawal bleeding, which is why an extended EE 20 μg/drospirenone 3mg regimen has been developed. It is taken for a maximum of 120 days, followed by a 4-day tablet-free period. This COC has been used successfully in the treatment of dysmenorrhea and pelvic pain associated with endometriosis.[57] Another study showed that a flexible extended regimen of EE 20 μg/drospirenone 3mg (one tablet daily for 24–120 days followed by a 4-day rest period) compared with a 28-day cyclical regimen (one tablet daily for 24 days followed by 4 days of a placebo tablet for six cycles) reduced the number of days with dysmenorrhea in women and was well tolerated.[58]

Ethinylestradiol/Levonorgestrel

LNG is a second-generation synthetic progestin that, in combination with estrogen, is a long-term contraceptive.[59] A study was conducted to evaluate the 28-day cyclic and 84-day extended regimen of EE 0,02 mg/levonorgestrel 0,09 mg in patients with dysmenorrhea. Extended and cyclic regimens significantly reduced the severity of dysmenorrhea compared to placebo. However, the extended regimen was superior to the cyclic regimen in reducing these symptoms. The use of this COC carries the risk of side effects. Cyclic regimens are associated with symptoms such as headache, bloating, nausea, and breast tenderness. Prolonged and continuous regimens may cause breakthrough bleeding.[60] The use of low dose (<50 μg ethinylestradiol) COCs containing cyproterone acetate, desogestrel, dienogest, drospirenone or gestodene was associated with an increased risk of venous thromboembolism compared to COCs containing levonorgestrel.[61]

Ethinylestradiol/ Desogestrel

Long-term continuous use of a COC containing EE 0.02 mg and desogestrel 0.15 mg is effective in relieving menstrual pain in women with endometriosis. Side effects include intermenstrual bleeding, bloating, weight gain, headache, breast tightness, and decreased libido.[48] COCs containing 0.03 mg of EE and 0.15 mg of desogestrel have been proven to influence the immune mechanisms in endometriosis. They cause an increase in the level of NK cells and Tregs, as well as a significant decrease in the number of macrophages.[62] In a randomized controlled trial, cyproterone acetate 12.5 mg and a monophasic COC with continuous action containing EE 0.02 mg and desogestrel 0.15 mg have been shown to be an effective and safe option in the treatment of recurrent pelvic pain associated with endometriosis.[63]

Risks and benefits for both types of treatment

Endometriosis is an estrogen-dependent disease, which means that estrogen is the key hormone that is responsible for the growth and development of ectopic endometriosis lesions. In a clinical study, the effects of COCs (combination of EE 0.03 mg and desogestrel 0.15 mg) and desogestrel alone on cell proliferation and apoptosis in ectopic endometrial tissue were evaluated. Patients treated daily with desogestrel alone or COC (EE/desogestrel) for 28-35 days were compared with patients not receiving treatment. This study proved that desogestrel alone increased cell apoptosis in the ectopic endometrium. In turn, COCs increased proliferation and caused a greater apoptotic effect in endometriosis lesions.[64] Progestins do not cause endometrial proliferation, but they also have side effects, which are included in *Table 1*. There is a potential for bone loss in some patients with long-term use. Therefore, it suggests that the addition of a small amount of estrogen may be beneficial for patients requiring long-term use of dienogest.[65] There are concerns about BMD with ultra-low-dose oral contraceptives (<20 μg EE), while COCs containing 20 to 30 μg EE may protect against bone loss.[66] Low-estrogen COCs cause more frequent spotting and breakthrough bleeding.[67] There is an increased risk of venous

thromboembolism with the use of COCs. To reduce the risk of thrombosis, COCs with the lowest possible dose of estrogen should be chosen. Both COCs and progestins have been shown to be effective in the treatment of symptomatic endometriosis. However, progestins do not increase the risk of thrombosis and can be used in women with contraindications to estrogens, as well as in those who do not tolerate estrogens.[34] Pre-operative hormonal treatment is not recommended in patients scheduled for surgery. However, in the postoperative period, in women who do not have procreative plans, hormonal treatment can be introduced in order to reduce the risk of recurrence of pain and endometriosis.[13]

Conclusion

Endometriosis is a chronic disease and treatment should focus on drugs that can be used long-term with minimal side effects. The nature of this disease makes progestins and COCs often the first-line treatment. These drugs relieve troublesome symptoms and improve the HRQoL of patients suffering from endometriosis. When choosing a specific treatment regimen, the type of ailment, the patient's preferences regarding the method of drug administration, drug tolerance and safety, procreation plans and treatment costs should be considered. Endometriosis is still an under-researched disease that is often underdiagnosed. Due to the high prevalence in society, actions should be taken to raise awareness and educate in this regard. This disease requires a holistic therapeutic approach as it can affect many different organ systems. Patients should be educated about the multitude of therapeutic methods in the treatment of endometriosis, their effectiveness and potential side effects. Despite the widespread use of COCs and progestins in clinical practice, there is still little current literature on the treatment of endometriosis with them. Further research is needed to determine the length of treatment, choice of preferred drugs and their dose, intermittent therapy and combinations with other drugs that are most effective in the treatment of endometriosis.

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Author contributions

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Conflict of interest

The authors declare no conflict of interest.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

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REVIEW PAPER

Global risks of endometriosis in women – an appraisal

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ABSTRACT

Introduction and aim. Endometriosis is a complex condition in which endometrium, tissue that resembles the uterine lining, develops outside the uterus. It is considered to be a chronic, estrogen-dependent, inflammatory gynecological disorder having multi-factorial origins. This review paper aims to consolidate recent information on ethnic differences, endometriosis risks, and the disease's etiology in the global context.

Material and methods. A systematic search was performed using a variety of international electronic databases, including "PubMed" and "DOAJ", using the terms endometriosis, endometriosis and infertility, endometriosis and cancer, and treatment of endometriosis.

Analysis of the literature. Endometriosis can appear anywhere in the body, including the umbilicus, the cecum and ileum of the digestive tract, the breast, the lungs, and the genitourinary organs. It is typically clinically asymptomatic with no obvious clinical manifestation and expensive treatment, which makes the diagnosis late. There is a complex interplay between socio-economic status, family history, societal beliefs and laws, personal habits, reproductive and gynaecological conditions, and environmental influences in the development of endometriosis.

Conclusion. Women with endometriosis should be given more attention, and specific resources in the healthcare system should be utilized to provide more efficient multidisciplinary healthcare and treatment.

Keywords. endometriosis, hormonal imbalance, infertility

Introduction

Endometriosis is a complex condition in which endometrium, tissue that resembles the uterine lining, develops outside the uterus, which leads to chronic inflammation, pain, and infertility.[1–9] It affects 190 million women globally who are in reproductive period.[4,8–12] Endometriosis affects around 70 percent of reproductive women who have dysmenorrhea and dyspareunia, and adolescents may have more severe symptoms[13]. However, this figure can be significantly higher because it poses a significant challenge to diagnose the illness correctly.[1,4,7,9,14–17] The schematic presentation of different factors affecting the development of endometriosis is shown in Figure 1. Endometriosis is considered to be a chronic, estrogen-dependent, inflammatory gynecological disorders[1,4,7–9,14–18] that may cause severe, life-altering pain during menstruation, inter-menstrual bleeding, painful intercourse, painful bowel movements and urination, as well as persistent pelvic pain, stomach bloating, nausea, exhaustion, depression and anxiety (Fig. 1).[2,4,9,10,13,19–22]

One in nine to ten women experiences endometriosis during their reproductive period.[12,16,21] The symptoms are diverse, but yet many reproductive women are unaware of the disease progression, which makes diagnosis difficulties.[3,9,11,19] Most of the time it is clinically asymptomatic with no obvious clinical manifestation and

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needs expensive diagnostic tests and treatments.[1,18,19] Endometriosis symptoms are non-specific and can overlap with gastrointestinal and pelvic pain diseases,[2,9,12] and it is associated with a variety of gynecological and non-gynecological co-morbidities such as autoimmune disease, bowel syndrome, migraine, cardiovascular disease, mental disorder, and overall morbidity in reproductive women (Fig. 1).[6,7,9,12,23] Endometriosis is a complex inflammatory condition that affects reproductive women all over the world, from menarche to menopause, regardless of ethnicity or socio-economic status, causing serious reproductive and health consequences.[11,14,24,25] Because endometriosis symptoms typically appear in adolescence, early detection, diagnosis, and treatment of the conditions may reduce pain, halt disease progression, and preserve fertility.[19] Endometriosis can appear anywhere in the body, including the umbilicus, the cecum and ileum of the digestive tract, the breast, the lungs, and genitourinary organs such as the bladder, ureters, vagina, cervix, or the recto-vaginal septum, excluding the spleen.[2,12,15,17,24,26,27] Endometriosis is classified into three types based on where it appears: peritoneal, ovarian, and deeply infiltrating endometriosis.[2,6,7,9,15,18,24,26,27]

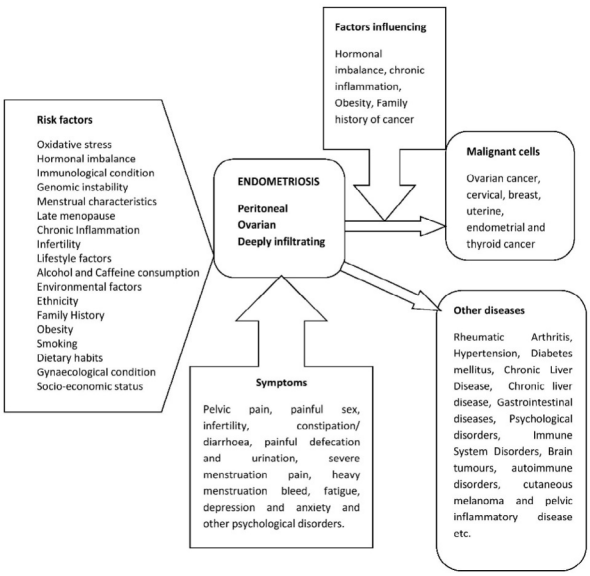

Fig. 1. Risks factors and association of endometriosis with malignancy and non-communicable diseases

Endometriosis is considered to have multi-factorial origins, which implies that it may have appeared by a combination of factors.[1,9,11,17,22,26,28,29] Several studies have consistently connected a rise in endometriosis incidence to a variety of causes.[19,24] It is hypothesized that endometriosis may arise as a result of the intricate interactions and combined effects of both inherited risks and environmental factors.[8,15,17] Dietary, immunological, and environmental conditions have a significant impact on the development of endometriosis.[8,15,17,28] Endometriosis risk factors include age, ethnicity, alcohol usage, body mass index, smoking, infertility, hormonal fluctuations, and menstrual characteristics such as early menarche, a shorter and less regular menstrual cycle, dysmenorrhea, menstrual flow intensity, and prolonged estrogen exposure from early menarche to late menopause.[10,16,17,19,21,23,24] Additionally, there is an association between early adult body mass index and endometriosis that is unfavorable.[19,23,27] Several studies have shown possible risk factors such as greater height and low birth weight.[19,27] Notably, although smoking has been proven to either raise or lessen the risk of endometriosis, the association is still not clear.[1,8,19,27]

Dietary factors associated with endometriosis risk may be confounding variables that are amenable to ongoing lifestyle changes. Women are more likely to develop endometriosis than women without first-degree relatives who have the condition to have endometriosis.[27,30] It has been observed that the genetic makeup, hormonal activity, inflammatory state, and immunological environment all have a substantial impact on endometriosis expression and progression.[9,15,19,22,27,28] However, there is a complex interplay between socioeconomic status, family history, societal beliefs, laws, personal habits, reproductive and gynaecological conditions, and environmental influences in the development of endometriosis.[1,8,17,23,27] An increase in pollution and perfluoroalkyl exposure may have an impact on the pathogenesis of endometriosis.[1,27] Experimental investigations revealed that perfluoroalkyl compounds have a detrimental effect on the woman's reproductive system in experimental animals, and are associated with endometriosis.[31] Synthetic fluorinated chemicals are known as Perfluoroalkyl substances and these compounds have been utilized in surfactants, household cleaning goods, textiles, paints, fire-fighting foams, and food packaging because of their hydrophobic and lipophobic qualities.[31] Studies have found that women with higher levels of dioxins and polychlorinated biphenyls in their bodies are more likely to develop endometriosis.[27,31] Despite the fact that these pollutants contribute to the development of the illness, including the production of oxidative stress, further research is required to determine the precise pathways.[1,27]

Exercise and diet are two risk factors for endometriosis that can be addressed.[32,33] Food intervention is one of the most effective self-management strategies for managing endometriosis symptoms, though it is unclear which dietary changes may have an effect on specific types of endometriosis.[29,32] Women suffering from endometriosis may benefit from anti-inflammatory and anti-estrogenic foods by experiencing fewer symptoms.[29,33] Despite the fact that moderate and regular physical activity can help prevent endometriosis, dietary preferences, alcohol and caffeine consumption, smoking, and

intense physical activity have been associated with significantly increased risk factors.[8,16,23,27] Endometriosis risk may be influenced by dietary choices and steroidal hormones.[28,32] Fresh fruits and vegetables have been shown to reduce this risk, but other dietary choices, particularly those involving red meat consumption, have been associated with an increased risk of endometriosis.[19,23,24,27,32] However, eating a lot of cruciferous vegetables and taking excess beta-carotene supplements may increase the risk of getting endometriosis.[29] Caffeine and alcohol use can hinder a woman's ability to conceive, which can then affect her reproductive hormones and cause endometriosis to appear.[19,27,29] While severe exercise may promote endometrial proliferation by increasing estrogen levels and insulin-like growth factor-1, moderate exercise may prevent endometrial cancer by lowering inflammation and oxidative stress.[27] In terms of gynaecological and reproductive concerns, the preponderance of endometriosis risk factors are related to parity, menstrual cycle length, flow duration, and age of menarche.[19,24,27] Early menarche and lengthy, intense menstrual cycles are associated with increased risk due to greater levels of estradiol and estrone, whereas parity and oral contraceptive use are associated with protective status.[15,27] Oral contraceptives work by lowering follicular stimulating hormone and stabilizing the endometrium, which relieve endometriosis-related pain by regulating the menstrual cycle and reducing endometrial tissue growth.[12,19,24,34] Thus, women who use oral contraceptives, tubal ligation, and parity are at low risk of developing endometriosis.[12,19,34]

Further, endometriosis alters gene expression in the brain, causing pain sensitization and mood disorders, as well as influencing metabolism in the liver and adipose tissue and promoting systemic inflammation.[35] Furthermore, endometriosis symptoms frequently can also have a negative psychological and social impact on how women act, putting their mental health, sexuality, and interpersonal relationships at risk.[18,24,36] Endometriosis symptoms are exacerbated by psychological factors, and endometriosis women frequently report high levels of anxiety, depression, and other psychological disorders.[3,4,6,7,9,12,36,37] Endometriosis can cause depression and anxiety owing to persistent pain, and the inability to conceive in women.[7,9,12] Their income is negatively impacted by their low productivity, and they exhibit minimal engagement in daily life, relationships, personal affairs, and social activities.[3,6,12,18,24] Endometriosis was identified more than 160 years ago, yet significant scientific gaps remain, including evidence of the disease's etiology.[12] Endometriosis research funding is inadequate, with the National Institutes of Health committing only 0.038% of the health budget for 2022 to a condition that affects nearly 190 million women worldwide, including 6.5 million in the United States.[11,12]

Endometriosis has serious social, public health, and economic consequences. It may reduce women's quality of life owing to extreme pain, exhaustion, melancholy, stress, and infertility.[9,11] Endometriosis discomfort can be so severe that it stops some women from being productive in their daily lives.[12] Hence, it has a significant impact on the woman's mental and socio-emotional health.[9] Endometriosis treatments can minimize absenteeism in the classroom or improve a woman's capacity to work in certain situations.[11] Endometriosis can cause people to stop or avoid sexual activity, impact on their own and their partners' sexual health. Endometriosis treatment and care will empower women by assisting their human right to optimal sexual and reproductive health, quality of life, and overall well-being. Increased awareness, followed by early detection and treatment, may reduce and/or prevent the disease's natural course and lessen the long-term burden of its symptoms, including the risk of increased central nervous system pain sensitivity, but there will be no cure by that time.[9]

Aim

Therefore, it is imperative to recognize that this ongoing adverse endometriosis condition is a serious public health concern with severe consequences for women's quality of life and a substantial economic burden. Future research investigation is needed to look into various factors that influence global endometriosis prevalence, which could help with the development of future strategies and intervention programmes. This review manuscript aims to consolidate recent information on ethnic differences, endometriosis risks and the disease's etiology in the global context. In addition, the review manuscript emphasized on how genetic, epigenetic, ethnic differences, environmental and immunological disorders play a part in endometriosis in a broader context.

Material and methods

A systematic search was performed using a variety of international electronic databases, including "PubMed", "DOAJ", "Google Scholar", "Scopus", "Research Gate", "Web of Science". The primary search terms included endometriosis, endometriosis and infertility, endometriosis and cancer, determinants of endometriosis, endometriosis and hormones, and treatment of endometriosis to find the pertinent literature. All papers relevant to the objective of the present investigation were identified and reviewed after the necessary search. According to a set of criteria, the search papers were evaluated to determine whether they should be included in the current investigation. These criteria included descriptive studies, review articles, cross-sectional studies, original survey studies, etc. This manuscript is a systematic review of the literature published between 2000 and 2022. The search results were limited to full articles

that were published in English. There were no publication date criteria, but new studies were included in this review paper during the manuscript's preparation and final revision. Following the present study keywords criteria and study objectives, a total of 168 publications, including titles, abstracts, and full-texts, were identified. The details of the literature search, inclusion, and exclusion are summarized in Figure 2.

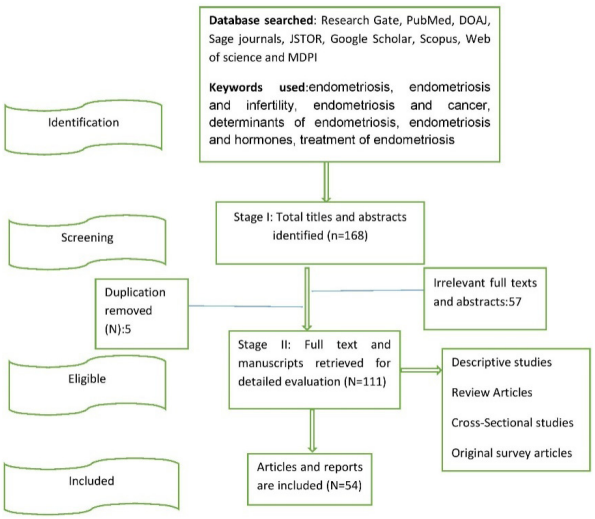

Fig. 2. Manuscript identification and selection procedures of the present review study

Manuscripts (N=111) were identified and retrieved for detailed evaluation during the short-listing of published literature and manuscripts. Because a few of the papers were found to be cited in duplicate (N=5), such manuscript duplications were eliminated from the list of publications. Following a thorough review of the published literature, N=54 research papers were identified as appropriate and were considered for the current manuscript. The finalized manuscripts both abstracts and full-length manuscripts, were downloaded in order to interpret the present study, revise, and complete this review manuscript.

Analysis of the literature

Ethnic differences and endometriosis risks

Endometriosis affects between 10 and 15 percent of women worldwide, according to estimates.[4,8,9,11–13] Endometriosis affected 3,430,094 individuals worldwide in 1990, and 3,785,955 people in 2019, an increase of approximately 10 percent.[38] Endometriosis affects 5-15 percent of women in reproductive age and 2-5 percent of postmenopausal women in Canada and the United States, respectively.[25] It is estimated that more than 4 million women of reproductive age have been diagnosed with endometriosis, according to population-based investigations.[6] Nevertheless, some cases are misdiagnosed, suggesting that endometriosis may affect a greater number of reproductive women.[6] Approximately, 11 percent of American women found to have endometriosis at some point in their lives.[12] In the United States and Australia one out of nine women in their reproductive age has endometriosis.[12] After pelvic inflammatory illness, endometriosis is the third-leading reason for gynecologic hospitalization in the United States.[5,10,32] Endometrial cancer is the most common gynecologic cancer in the United States. According to data, it is also the fourth most common type of cancer among American women.[39] It was predicted that 63,230 women would be diagnosed with this cancer in 2018, with 11,350 dying as a consequence.[39] Endometriosis affects Asian women more than African-American and Caucasian women; medical treatment may make a difference.[5,26,40] Endometriosis was found to be extremely common in developing countries, and it is often underestimated and estimated to affect approximately 42 million women in India.[25] Around 20-25 percent of endometriosis patients are asymptomatic, with only 6-10 percent reporting pelvic pain and 30-50 percent suffering from chronic pain and infertility.[1,4,13,24.38,41] With 10-15 percent of women in reproductive age affected, pelvic endometriosis appears to be one of the most common premenopausal benign gynaecological proliferations.[22,24,42] The symptoms of this chronic gynaecological disorders include persistent pelvic discomfort, unpleasant bowel movements, lower back pain, dyspareunia, infertility, and dysmenorrhea, these symptoms may significantly increase the economic burden and impact socio-economic status and morbidity.[4,7,9,12,13,25,27,41] Endometriosis frequently causes pelvic pain, which has a significant impact on a woman's emotional well-being, general symptoms, and the protracted course of this condition lowers quality of life.[4,12,36,37] Furthermore, there is little evidence that ethnicity influences endometriosis risks.[5,20] It should be noted, however, that these conclusions are based on limited evidence and could be influenced by factors such as healthcare access, diagnostic equipment, and cultural perceptions of women's health issues.[13,38] Ethnicity, along with socio-economic, cultural, and genetic factors, may affect a person's ability to access healthcare and receive proper endometriosis treatment.[5,11]

Endometriosis and infertility

Endometriosis should be taken into account due to its effects on women of reproductive age, has an effect on the reproductive system and decreases their ability to conceive and their fertility.[43] Endometriosis can make pregnancy difficult for women because the growths can interfere with the regular functioning of the ovaries, fallopian tubes, and uterus.[24,43] Endometriosis can cause inflammation, disruption of the normal hormonal balance, and oxidative stress, all of which can interfere with the normal functioning of the reproductive system (e.g., lowering egg quality, interfering with ovulation, and implan-

tation of a fertilized egg) and lead to infertility.[15,24,26,27,43] As a result, even if not related to infertility, treatment and counseling should always be considered and work to preserve patients' chances of pregnancy.[4,43] In terms of female fertility, it has been reported that 30–50 percent of endometriosis patients are infertile due to diminished ovarian reserve, while 25–50 percent of infertile women have endometriosis.[10,12,18,24,26,27,41,43] Endometriosis symptoms and diagnostic probability worsen with age, and incidence is rising among women in the age group of 25–40 years and older.[24,41] The process of oxidative stress contributes significantly by causing chronic inflammation, leading to the deterioration and dysfunction of the reproductive system in endometriosis patients.[43] The connection between infertility and endometriosis is still being researched. Endometriosis-related infertility is now recognized as a multifaceted health problem involving altered immunity and genetics affecting not only the fallopian tubes and embryo transport but also the normal endometrium.[27,43] Medical therapy has been shown to be effective in slowing disease progression.[43] Endometriosis-related infertility is treated with medical and/or surgical intervention, as well as assisted reproduction technology, by reducing or removing the ectopic endometrial implant and restoring normal pelvic anatomy.[27] One of the best remedies for infertility caused by endometriosis has been proven to be in vitro fertilization.[27] Two methods for preserving fertility in women are cryopreservation of embryos and oocytes and cryopreservation of ovarian tissue. At the moment, it appears that oocyte vitrification is the most viable option.[43] Endometriosis treatment may not be beneficial to patients who want to become pregnant. The two suggested treatments in this case are surgery or in vitro fertilization and embryo transfer.[41] Endometriosis patients should have a yearly check-up to determine their ovarian reserve (e.g., anti-mullerian hormone levels, antral follicle count, and cyst size).[43] When endometriosis is first diagnosed, the woman's overall care must be planned, taking the disease's potential impact on her reproductive life into account. Furthermore, modern women are delaying their first pregnancies, which must be considered.[43] The right care must be given, patients must be closely watched, and those who can gain from fertility preservation must be found as soon as possible.[43]

Endometriosis and cancer

Endometriosis may raise a woman's risk of certain cancers, and immune system disorders have also been linked.[9] It raises the risk of ovarian, cervical, breast, uterine, and thyroid cancer (Fig. 1).[2,9,40,44,45] Endometriosis-affected women are more likely to develop ovarian cancer, which is known as endometriosis linked ovarian cancer, especially if they have a prolonged history of the condition and have undergone several operations.[12,17,40,44,45] It is thought that these changes in hormones and inflammation in the body are the causes of the increased risk of ovarian cancer caused by endometriosis, even if the precise mechanisms are still not entirely known. Compared to women without endometriosis, women with endometriosis have a 2–3 percent higher risk of having ovarian cancer.[44-46] Endometriosis-related hormonal imbalances and ongoing inflammation may be to blame for this, which may promote the growth of abnormal cells.[44,45] It's important to remember that the overall risk of ovarian cancer remains low, particularly for women with endometriosis. Furthermore, the relationship between ovarian cancer and endometriosis is still poorly understood. Endometriosis is also associated with an increased risk of endometrial cancer, though this risk is still considered low.[9,45] Ovarian cysts and abnormal cells may be more likely to form in endometriosis-affected women if they also have chronic inflammation, hormone imbalance, obesity, or a family history of endometrial cancer.[4,5,9,47] Endometriosis-associated ovarian cancer development is significant not only because of the danger of infertility, but also because of the possibility of fatal outcomes.[40]

Endometriosis cells eventually transform into malignant cells, and endometriosis risk factors and outcomes, including late menopause and infertility, are observed to be similar for both ovarian cancer and endometriosis.[44] Endometriosis and endometrial cancer are two distinct diseases that affect women and are both regulated by estrogen.[39,46] Endometrial exposure, elevated estrogen production, and low progesterone levels can all cause endometrial hyperplasia, which, if ignored, can turn into endometrial cancer.[39] Endometrial cancer is also more likely to occur in women who have an estrogen-dominant hormonal environment, such as those who are obese, go through menopause later in life, or use estrogen-only hormone replacement therapy. This is because these women's endometrial cells are exposed to high levels of estrogen.[47] Endometriosis is characterized by the formation of tissues that resemble endometrium outside the uterus, whereas endometrial cancer or uterine cancer is made up of carcinoma cells that line the uterus[46], and pelvic discomfort is the main symptom in both conditions.[46] Additionally, investigations have revealed the association between endometriosis, endometrial cancer, and breast cancer.[45,48] Breast cancer and endometriosis are both chronic, estrogen-dependent gynaecological illnesses that are predisposed by factors related to reproduction and hormone replacement therapy.[46] Endometriosis slightly increases a woman's risk of breast cancer in adulthood, defined as age 50 years or older by twofold compared to a woman without endometriosis.[45,46]

Endometriosis and thyroid issues are pathogenic and can result in hypothyroidism or hyperthyroid-

ism.[7] There is an association between thyroid cancer and endometriosis too, endometriosis patients have a 39 percent increased risk of developing thyroid cancer.[45] Thyroid dysfunction with endometriosis-related infertility was also common in endometriosis.[7,10] Several studies have reported that the uterus has the highest cancer risk, followed by the ovary, cervix, breast, and thyroid. In contrast to other women, endometriosis patients have higher rates of other illnesses including cutaneous melanoma, non-Hodgkin lymphoma, brain tumours, asthma, autoimmune diseases, gastrointestinal disorders such as irritable bowel syndrome, chronic liver disease, diabetes mellitus, hypertension, rheumatoid arthritis, and pelvic inflammatory diseases (Fig. 1).[2,7,9,10,23,26,40]

Socio-economic, demographic, and lifestyle determinants of endometriosis

Regardless of ethnicity and/or socioeconomic status, endometriosis is a complex disorder that affects reproductive women worldwide from menarche to menopause. It is believed that endometriosis has multifactorial causes, which implies that it is brought on by a number of variables.[1,9,11,29] Endometriosis becomes more common with age, and the incidence of endometriosis is inversely related to body mass index.[5,49] Menstrual cycle length, early life factors including preterm delivery, low birth weight and feeding pattern, vitamin D levels, body mass index, specific types of endocrine disruptor chemicals including polychlorinated biphenyls, oral contraceptive pills, mono-2-ethyl-5- hydroxyhexyl phthalate, and ethnicity characteristics are associated with endometriosis.[8] Certain chemicals have been associated with an increased risk of endometriosis in female fetuses during pregnancy; exposure to toxins during pregnancy may also increase their daughters' risk of endometriosis.[1] Endocrine disruptor chemicals, which interfere with the function of the endocrine system, which is a prevalent environmental risk factor found in the environment and food chains.[8] It enters the human body via food, drink, dust, and cosmetics, and its metabolites have been associated with endometriosis and infertility.[1,8,10] Healthy consumption of vitamin D, citrus fruits, dairy products, and long-chain omega-3 fatty acids has been associated with a lower risk of endometriosis.[28,29,33] But, it's unclear whether vitamin D is related to endometriosis symptoms.[29] The risk factors associated with the development of endometriosis are also summarized in Fig. 1. Exercise and omega-3 fatty acids may help prevent endometriosis by lowering inflammatory signs and symptoms, even if there is no direct link between them and the condition.[2,19] Regular exercise is anticipated to lower menstrual flow, stimulate the ovaries, have an estrogen effect, relax the muscles, and lessen pain in endometriosis patients. Moreover, consistent exercise provides anti-inflammatory benefits.[24,33] Endometriosis is positively associated with premenstrual syndrome, dysmenorrhea, and heavy menstrual flow, which are also the symptoms caused by alcoholism.[8]

Dietary factors have been connected to the pathophysiology of endometriosis because of their influence on the steroid hormone metabolism, muscular contraction, inflammatory control, oxidative stress, and menstrual cycle.[28,29] With dietary changes, many women get relief from endometriosis-related symptoms.[29,33] Women with laparoscopically confirmed endometriosis consumed less fresh fruit and green vegetables than women without endometriosis.[1,28] Fresh fruits, particularly citrus fruits, were found to reduce the risk of developing this condition. A diet high in fruits and vegetables is high in provitamin A, which has been linked to lower levels in endometriosis patients.[1,23] The proinflammatory cytokine interleukin-6, which is inhibited by vitamin A, is found in high concentrations in the amniotic fluid of endometriotic women. Citrus fruits are also high in vitamin C, which has anti-inflammatory and antioxidant properties. Fruit consumption has been linked to an increased risk of endometriosis in American women. This could be related to the large amounts of pesticides used during cultivation in the United States.[1] Furthermore, Asian women are more likely than black women to develop endometriosis, and women in industrialized countries with a higher socio-economic level have a higher incidence of this condition.[8,23,24,26] This could be because they have improved healthcare facilities and can thus be diagnosed faster.[8,23,26,38] Endometriosis prevalence and incidence data are scarce or non-existent in many low-income countries and locations.[38]

There was an insignificant association found between eating chicken, fish, shellfish, or eggs and the risk of endometriosis, but the consumption of red meat may increase the risk of endometriosis.[1,19,23,24,28] This effect could be due to the pro-oxidant effects of haemoglobin produced by red meat.[1,19,28] Low levels of sex hormone binding globulin (SHBG) are associated with endometriosis symptoms, while vegetarian women have higher SHBG levels.[1,19,24,28,32] Red meat consumption has been associated with a number of chronic illnesses, such as diabetes, heart disease, and several types of cancer. Red meat consumption has more recently been linked to overall cardio-vascular disease, and cancer mortality.[28,32] The only saturated fatty acid that was positively associated with a higher incidence of endometriosis was palmitic acid.[1,19,32] However, eating a lot of trans-unsaturated fat increased the prevalence of endometriosis, even if eating long-chain omega-3 fatty acids did not.[1,19,28,32] Endogenous estrogen is present in animal fat, which can be decreased by consuming fewer fats.[28,32] Increased estrogen levels are linked to high-fat diets; female bodies create more estrogen in proportion to fat

consumption.[28,33] Eating more red meat is associated with an increased risk of endometriosis,[28,33] independent of heme iron, while heme iron itself is linked to an increased risk of endometriosis.[32] Heme iron is present in myoglobin and hemoglobin derived from animal sources. Non-heme iron is primarily obtained from plants. Although heme iron accounts for a smaller proportion of total iron intake, due to its rapid absorption rate, it can make up to 40 percent of all iron that is absorbed.[32] During retrograde menstruation, erythrocytes are known to discharge haemoglobin and its metabolites, iron and heme, into the peritoneal cavity. If iron and heme are not chelated, they can produce damaging reactive oxygen species, a type of oxidative stress.[28,32] Fish oil has been associated with lower prostaglandin levels, fewer inflammatory symptoms, and a reduction in dysmenorrhea.[28,32] Higher prostaglandin levels are associated with a rise in estrogen production, which may have an impact on how endometrial tissue develops.[28,33] Furthermore, the essential heme iron-carrying protein hemopexin is present at lower levels in the bodies of endometriosis patients.[32] Despite the fact that the physiological mechanism by which nutrition affects endometriosis is not entirely understood, it has been suggested that circulating steroid hormones are involved.[32] Moreover, different foods and nutrients exert different effects on different stages of endometriosis.[29] It is well recognized that endometriosis is brought on by disturbances in the pro-antioxidant balance, which lead to oxidative stress.[1,23]

Due to hormonal changes, pregnancy has a beneficial influence on endometriosis and its related discomfort.[50] Endometriosis is influenced by physiological mechanisms such as breastfeeding; and the length and type of breastfeeding are considered as important factors.[50] Breastfeeding may reduce the risk of endometriosis or be found to have protective effects.[8,50] Benefits of breastfeeding may result from how it influences pituitary hormonal activity, which reduces circulating estrogen levels. In particular, estrogen is crucial for the maintenance and growth of endometriotic lesions.[50] Breastfeeding has been shown to have anti-inflammatory properties that may help in endometriosis prevention and minimize uterine estrogen exposure by slowing ovulation, despite the fact that this topic hasn't received much attention.[8,50] Patients with endometriosis may benefit from prolonged and exclusive breastfeeding, since it can reduce pain feelings and prevent recurrences. The duration and intensity of breastfeeding, in particular, significantly reduces the intensity of any discomfort symptoms.[50] Few studies have examined the impact of breastfeeding on endometriosis, but some have found an opposite association between breastfeeding and the probability of developing endometriosis.[8,50]

Hormonal imbalance and endometriosis

Hormonal fluctuation, especially steroid hormones, has a significant impact on the development and maintenance of endometriosis.[19,26] Younger menstrual women and nulliparous women have higher levels of estradiol and estrone in their blood, which supports the growth of ectopic and eutopic endometrial tissue.[19,51] In both eutopic and ectopic endometrium, estrogen is considered to be the most effective and upstream activator of endometrial tissue survival and inflammation.[15,26,51] Ectopic is the phenotype, and eutopic endometrium is a region of tissue that mimics the uterine lining.[2] Several studies have reported that higher estrogen levels such as estradiol and estrone are associated with an increased risk of endometriosis.[15,43,51] Endometriosis risk in obese women may be explained by the anovulatory cycle, and the retrograde of menstrual blood theories.[19,49] The growth and maintenance of endometriotic tissue, as well as the pain and inflammation brought on by endometriosis, depend on the estrogen 17-estradiol.[15] Patients with endometriosis may have hormonal abnormalities that elevate estrogen levels and raise the possibility of endometrial tissue growth and proliferation, thereby hastening the beginning and development of the condition.[22,43] Patients with endometriosis who have high aromatase activity create more estrogen, which stimulates endometrial tissue growth and multiplication and increases symptoms.[8,15] Aromatase, a cytochrome P450 superfamily enzyme, converts androstenedione and testosterone into estrogens, which is the final step in the formation of the estrogen 17-estradiol.[15] Consumption of alcohol may make the enzyme aromatase more active, which turns testosterone into estrogen, resulting in a drop in testosterone levels and an increase in estrogen levels.[8,23] Additionally, drinking alcohol may prevent the pituitary gland from producing luteinizing hormone, which would increase the ovaries' ability to synthesize estradiol.[8] Aromatase inhibitors significantly lower estrogen levels by preventing the production of estrogen in endometriosis foci and the ovaries.[24] An increase in endogenous estrogen can trigger the development of aromatase P450, which increases endogenous estrogen and prostaglandin levels and exacerbates inflammation in endometriosis.[32] Endometriosis is one of the diseases that can result from hormonal imbalances and significantly affect a woman's reproductive health.[15,19]

Additionally, women with endometriosis may experience pain and discomfort from the hormonal changes brought on by the menstrual cycle.[51] High estrogen levels can hasten the development of endometriosis by causing endometrial tissue to grow and multiply.[51] Luteinizing hormone, androgens, endocrine disruptors and genes influencing sex hormone metabolism are also potential candidate genes for influencing endometriosis onset and progression.[51] Furthermore, high amounts

of androgens may encourage the development of androgen-sensitive endometrial tissue, which would enhance endometriosis growth and proliferation.[27,52] As a result, regulating and treating endometriosis symptoms are dependent on maintaining a balance between these hormones.[15] Hormone therapy allows for both hormone level management and the prevention of endometrial tissue formation, and hormonal contraception and gonadotropin-releasing hormone agonists are two examples.[27] Estrogen is required for the regulation of cyclic gonadotropin release and folliculogenesis.[51,53] Endometriosis is frequently triggered by endocrine disruptor substances, which are abundant in the environment and food chain. However, a few medications interfere with hormonal balance and contribute to the pathophysiology of endometriosis.[1,8]

Diagnosis and treatment of endometriosis

Despite 100 years of research, there is no known cause of endometriosis, nor is there any permanent cure, and treatments mainly focus on managing symptoms.[2,12,18,22,25] Knowing about the inflammatory immune response would aid in a better understanding of this complex condition which could lead to the creation of biomarkers for endometriosis diagnosis because it is implicated in endometriosis pathogenesis.[25] Biomarkers include cell growth, cell survival, high energy requirements, oxidative stress, and fatty acid levels.[16,24] Several biomarkers, including angiogenesis indicators, carcinoma antigen 125 (Ca125), brain derived neurotrophic factor, stem cell markers, steroids, hormones, cytokines, and growth factors, have been examined in relation to endometriosis, but none have shown to be reliable diagnostic tools.[24,27] However, surgical visibility is required for a conclusive and clear diagnosis, although it is difficult to determine the disease's prevalence and incidence.[27] The quality of life of endometriosis patients can be improved by specific combinations of medicinal, surgical, and psychological treatments.[11] To change the hormonal conditions that favour endometriosis, medical treatments concentrate on either reducing estrogen or raising progesterone.[11] Along with medicine, yoga and lifestyle changes significantly affect endometriosis.[21,33] Women with endometriosis who regularly exercise claim it provides them with more energy by increasing the metabolic rate of serotonin neurons in the brain, which may encourage the release of chemicals that improve mood. Exercise is therefore one of the best strategies to raise serotonin levels. Swimming and walking are two exercises that more efficiently raise serotonin levels. These workouts help the body's blood circulation and muscle strength.[24,33]

Endometriosis can recur in postmenopausal women, especially those taking hormone replacement therapy or after a bilateral oophorectomy.[21] There is little evidence that combining medical and surgical treatment improves fertility, and it may cause unnecessary delays in subsequent reproductive care.[22] Modern endometriosis care should be tailored to the individual patient using an interdisciplinary, multimodal, and patient-centered approach.[22] Further, to identify hazards and provide a diagnosis and appropriate treatment, it is essential to comprehend the magnitude of endometriosis in a given community. The requirement for clinical and surgical expertise to appropriately diagnose clinical symptoms, and recognize the presence of ectopic endometrial implants, also known as lesions on the pelvic organs and in the peritoneal cavity, is one of the challenges in reaching a diagnosis.[22] Histological evaluation of lesions removed during laparoscopic surgery continues to be the most accurate way to identify endometriosis, despite the prominence of imaging techniques like transvaginal ultrasonography with intestinal preparation and magnetic resonance imaging.[6,16,21,22,24,43] Due to the vast range of sonographic characteristics, endometriosis lesions may resemble various disorders, such as dermoid cysts, hemorrhagic cysts, neoplasms, ovarian abscesses, and ectopic pregnancies.[27] Consequently, a clear differential diagnosis is necessary to reduce the possibility of misdiagnosis.[27] Endometriosis-related adhesions are most commonly found in the ovaries, uterus, bladder, and fallopian tubes.[2,12,15,17,26] Dysmenorrhea, dyspareunia, pelvic pain, back pain, or weariness are symptoms of early or light stages of the disease, but painful urination, micturition, and blood in the urine are symptoms of more severe stages of impairment.[27,54]

Endometriosis can also manifest clinically as bladder involvement, which can result in cyclic microscopic hematuria in the ureters, recurrent dysuria, and supra-pubic discomfort.[27] Symptoms of gastrointestinal endometriosis can include cycles of abdominal pain, meteorism, tenesmus, constipation, malena, diarrhoea, vomiting, and hematochezia.[27] When endometriosis manifests in unusual anatomical sites like the thoracic cavity, it can result in hemoptysis, pneumothorax, hemothorax, and chest pain.[27] This disease is categorized into three types based on where the endometrial implants are located: peritoneal endometriosis, ovarian endometriosis, and profoundly infiltrative endometriosis.[12,27,43] Endometriosis has the capacity to mimic many other illnesses. Consequently, a complete medical history must be used in conjunction with other specific diagnostic instruments for a correct diagnosis.[27] To diagnose endometriosis, a variety of instruments are required, including surgical methods for direct observation as well as non-invasive imaging methods like ultrasonography and biological cues. Assessing the presence of symptoms and doing a physical examination are the first steps in the diagnosis of endometriosis. Endometriosis cannot be diagnosed with a standard clinical examination alone; further testing, such as laboratory

and imaging procedures, is required to assess the severity and consequences of the disorder.[16,27]

Ultrasound is one of the least expensive, most accessible, and most noninvasive diagnostic methods for identifying endometriosis.[16,27] Transvaginal ultrasonography is the primary imaging modality for detecting ovarian endometriomas due to its high sensitivity and specificity for this purpose.[5,6,27,43] Nonetheless, the gold standard for identifying endometriosis remains laparoscopic examination with histological confirmations.[5,6,12,18,22,27,43] Unfortunately, 10 percent of patients discontinue their medication because of the adverse effects, which include exhaustion, dry vagina, reduced libido, hot flashes, and liver damage.[2,4,17] Along with medication, endometriosis patients also use a wide range of self-management techniques, including self-care, complementary therapies, and dietary adjustments. Relaxation, exercise, meditation, and dietary changes are all part of lifestyle therapy.[29,33] Hence, endometriosis must be managed for the rest of a person's life in order to receive the best medical care and avoid the need for further surgery. The key elements influencing endometriosis treatment technique are the symptoms, age, and fertility of the affected woman.[41] Nowadays, the most prevalent types of treatment are medicinal, surgical, or a combination of these.[41] To improve the current status of endometriosis, a multidisciplinary strategy for early diagnosis, effective treatment, and mental health is required.[26]

Conclusion

Endometriosis is a multifaceted disorder with numerous forms, sites, and symptoms. Early identification of these conditions have the potential to improve patient response to therapy, reduce complications, and improve women's health by lowering the social, economic, and overall health burden of endometriosis. The period elapsing between being exposed to the substance of interest and developing endometriosis is still unknown and being difficult to pinpoint the exact onset. Endometriosis is poly-etiological, and the reasons are unknown, so it is impossible to rule out the involvement of determinant factors in its pathogenesis. Women with endometriosis should be given more attention, and specific resources or healthcare delivery systems should be utilized to provide efficient multidisciplinary healthcare and treatment. Low-cost, simple early diagnosis screening techniques and effective, non-invasive treatments must be offered in healthcare institutes. It is imperative to limit possible risk factor exposures, identify those who are at risk, and conduct the proper screening by identifying effective disease risk factors, and educating society.

Declarations

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Author contributions

Conceptualization, C.R. and N.M; Methodology, C.R.; Software, C.R.; Formal Analysis, C.R. and N.M.; Resources, C.R.; Writing – Original Draft Preparation, C.R.; Writing – Review & Editing, N.M.; Visualization, N.M.; Supervision, N.M.; Funding Acquisition, C.R.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

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REVIEW PAPER

Anemia – a scourge to maternal and child development in Bihar, India

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ABSTRACT

Introduction and aim. Anemia remains a leading contributor to years lived with disability (YLDs), being responsible for 50.3 million (5.82%) YLDs worldwide and 19.3 million (12.03%) YLDs in India, respectively. Results of the National Family Health Survey 2019-2021 (NFHS-5) suggest a high burden of anemia in India among women of reproductive age and children aged 6-59 months at the national level (57%, 67.1%), and in the state of Bihar, India (63.5%, 69.4%). Iron deficiency is the leading cause, accounting for more than half the cases. Anemia bodes harmful implications for both the mother and child, with long-lasting consequences for the latter. Anemia control programs have yielded little benefit despite efforts stretching over five decades. This narrative review aims to highlight the burden of anemia and the probable factors behind it among under-5 children and women of reproductive age in the Indian state of Bihar.

Material and methods. The paper is a narrative review. The following databases were used to search and select literature: PubMed, Web of Science, Scopus, and Google Scholar. In addition, the websites of relevant government departments and national health programs were searched for pertinent material.

Analysis of the literature. A multitude of reasons seem to be behind the unabated high prevalence in Bihar: low socioeconomic status, gender disparities, traditional customs and practices, food insecurity, lack of diverse diets, poor consumption, and no adherence to iron and folic acid (IFA) supplements, groundwater contamination with arsenic and fluoride, and supply chain mismanagement, all playing roles of varying degree.

Conclusion. An all-encompassing approach and not merely the provision of IFA supplements are necessary to unravel the intricate web of factors that lead to anemia.

Keywords. anemia, diet habits, heavy metal toxicity, iron deficiency anemia, maternal child health services, socioeconomic factors

Introduction

Anemia remains a major unresolved public health problem, affecting around 1.8 billion people and accounting for nearly 50.3 million or 5.82% of years lived with disability (YLDs) worldwide, with the burden concentrated in low and middle-income countries.[1,2]

As of 2019, The World Health Organization (WHO) estimates that one in every three women in the reproductive age group (15-49 years), amounting to over half a billion women worldwide are anemic. It also estimates that 39.8% or 269 million children aged 6-59 months are anaemic.[3] The Indian context is much grimmer and anemia is one of the leading contributors to YLDs [19.3 million or 12.03%].[2] Findings of the National Family Health Survey 2019-2021 (NFHS-5) have revealed that one in every two women in the reproductive age group

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and two in every three children in the age group of 6-59 months in India are anemic, with an estimated prevalence of 57% for the former and 67.1% for the latter group (Fig.1).[4]

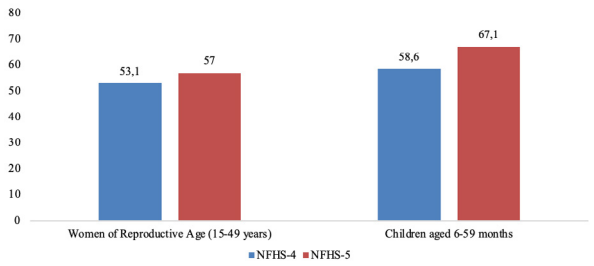

Fig. 1. Prevalence of anemia among women of reproductive age and children aged 6-59 months in India (Source: NFHS-5 India Report)[4]

It is a matter of great concern that despite the introduction and implementation of several programs over the years to curb anemia, these figures have only risen over the previous estimates of the National Family Health Survey 2015-16 (NFHS-4), a prevalence of 53.1% and 58.6% for anemia in women of reproductive age group (15-49 years) and children in the age group of 6-59 months respectively.

Aim

This narrative review aims to highlight the burden of anemia and the probable factors behind it among under-5 children and women of reproductive age group in the Indian state of Bihar.

Material and methods

The paper is a narrative review. The following databases were used to search and select literature: PubMed. Web of Science, Scopus, Google Scholar. In addition, the websites of relevant government departments and national health programs were searched for pertinent material.

Analysis of the literature

Etiological determinants

Anemia is a disorder of red blood cells, characterized by a decrease in the oxygen-carrying capacity either due to a reduction in the number or a decrease in the amount of hemoglobin of red blood cells. The WHO defines anemia in pregnant women and children aged 6-59 months as a hemoglobin level of <11g/dl.[5]

The etiology of anemia is varied but most commonly nutritional in origin. Nutritional causes include Iron deficiency (ID) and micro-nutrient deficiencies like folate, vitamin B12 & vitamin A. ID is the predominant cause, with more than half the cases in South Asia & Africa attributable to it.[1,6] Blood loss, either gynecological or due to parasitic infestations like hookworms commonly encountered in South-East Asia, and Africa is the leading cause of ID. Other common causes of ID include insufficient intake during a period of increased requirement (Infancy, adolescence in girls, pregnancy), poor absorption (Celiac disease, H. Pylori infection) and anemia of Chronic Disease (ACD) (chronic kidney disease, chronic heart failure, inflammatory bowel diseases).[6]

Haemoglobinopathies (Thalassemia, sickle cell disease), hemolytic anemias and infections (HIV, Tuberculosis, and Malaria) comprise some of the common non-nutritional causes.[7]

Consequences of anaemia

Anemia, irrespective of its etiology, causes fatigue and weakness, hindering work capacity and productivity.[8] Haas et al. found that ID states require a higher energy cost to perform the same amount of work.[9] A diminished capacity to work has not only economic implications but also social consequences in the form of a decreased ability to perform household activities and childcare,[8] which has special emphasis in a conservative country like India where women are the primary caretakers of households.

Generally viewed as a benign condition, anemia can turn fatal. Anemia during gestation predisposes the woman to a higher chance of developing post-partum hemorrhage and higher mortality.[10] A multi-level analysis by Daru et al showed that the odds of mortality are twice as high in severe anemia (Hemoglobin level <7g/dl) than in those without.[11]

The health of the mother and her child are closely intertwined. A woman's health status, even before pregnancy, has a bearing on the development and health of her future offspring. Birth outcomes and chances of neonatal and infant survival are adversely affected by maternal ill-health. Pre-term births, stillbirths, low birth weight and higher neonatal and perinatal mortality rates are some of the complications seen more frequently in anemic pregnant women.[10,12] Children born to anemic mothers have lower hemoglobin levels and are at an increased risk of anaemia.[13] A cohort study in rural Bihar found the hemoglobin level to be 0.2g/dl lower than children born to non-anemic women.[14]

Iron is also essential for early brain development due to its involvement in several biochemical processes, a deficiency of which could impede cognition and neurodevelopment.[15] Evidence, however, is equivocal. A Costa Rican study concluded that infants with chronic ID anemia (IDA) had lower cognitive scores and failed to catch up with their non-anemic peers, even when followed up to adulthood.[16] Some studies have also found an association between low hemoglobin levels and post-partum depression,[17] further compromising the well-being and health of both mother and child.

The phase spanning between 6-59 months of life is a vital and vulnerable period. The young child's nutritional needs begin to outgrow the nourishment provided by breast milk, a gap to be bridged by complementary feeding. Improper feeding practices compromise the child's nutritional status with long-term consequences. ID in these children has been linked with poor cognitive and motor development and long-lasting behavioral changes. They are also prone to recurrent respiratory and intestinal infections and struggle to gain weight.[18]

India's fightback

India's programmatic efforts to tackle anemia stretch back over five decades, commencing with the National Nutritional Anemia Prophylaxis Programme (NNAPP) in 1970, which focused mainly on prophylactic supplementation of iron and folic acid (IFA) in pregnant women, nursing mothers, women with intra-uterine devices and children aged 1-5 years.[19] The focus eventually shifted from prevention to management, with the NNAPP rechristened as the National Nutritional Anemia Control Program (NNACP) in 1991.[20] An ICMR evaluation of the program in 1992 saw an increase in the dose of elemental iron for adults to 100mg from 60mg.[21] A policy review concerning IFA supplementation expanded the scope of beneficiaries in 2007.[22] Recognizing the high susceptibility of adolescents and their importance as future functioning members of society, the Ministry of Health and Family Welfare (MoHFW) launched the Weekly Iron Folate Supplementation (WIFS) program in 2012.[23] The year 2013 saw the convergence of the existing IFA supplementation program under the umbrella of the National Iron Plus Initiative (NIPI), which adopted a life-cycle approach (Table 1).[24]

The Government of India launched the POSHAN Abhiyaan in 2018, an overarching scheme aimed at holistically viewing and enriching nutritional outcomes in children, pregnant, and lactating women.[25] Anemia Mukt Bharat (AMB),[26] an intensified version of the NIPI, was formulated and implemented as a part of POSHAN. AMB follows a 6×6×6 strategy, consisting of six beneficiaries, six interventions and six institutional mechanisms.

A non-governmental initiative worth noting is the Reduction in Anemia through Normative Innovations (RANI) project funded by the Bill & Melinda Gates Foundation. It aims at reducing anemia among Women of Reproductive Age (15-49 years) in Odisha, India.[27]

It is also pertinent to mention programs like the Integrated Child Development Services (ICDS), one of the world's largest for early childhood nutrition and development since its inception in 1975. Although the program doesn't directly target anemia, it adopts a comprehensive outlook, striving to improve nutritional outcomes, reducing the burden of malnutrition, and other nutritional

Table 1. List of anemia programs, salient features, beneficiaries, and dose of elemental iron

Year	Program	Salient features	Beneficiaries	Dose of elemental iron
1970	**National Nutritional Anemia Prophylaxis Program (NNAPP)**	----------	Children (1-5 years)	20 mg
			Pregnant women	60 mg
			Lactating women & IUD acceptors	60 mg
1991	**National Nutritional Anemia Control Program (NNACP)**	Three-pronged strategy: i. Promotion of consumption of iron-rich foods. ii. Promotion of IFA supplement consumption in vulnerable groups. iii. Detection and treatment of Hb <7g/dl.	Children (1-5 years)	20 mg
			Pregnant & Lactating women	100 mg
2007	**National Nutritional Anemia Control Program (NNACP)**	The scope of beneficiaries expanded – Children of age 6-12months, 6-10 years and adolescents included	Children (6 to 60 months)	20 mg (Syrup) 30 mg
			Children (6-10 years)	100 mg
			Adolescents (11-18years) Pregnant & lactating women	100 mg
2012	**Weekly Iron Folate Supplementation (WIFS)**	6th to 12th class adolescent boys and girls aged 10-19 years enrolled in government, government-aided/municipal schools. Out-of-school adolescent girls are also covered Weekly IFA supplementation using a fixed-day approach & Biannual deworming	School-going adolescent boys and girls	100 mg
			Out-of-school adolescent girls	100 mg
2013	**National Iron Plus Initiative (NIPI)**	Life-cycle approach Comprehensive coverage of vulnerable age groups Weekly/biweekly supplementation of iron and folic acid.	Children (6-60 months)	20 mg (Syrup)
			Children (5-10 years)	45 mg
			Children (10-19 years)	100 mg
			Pregnant & lactating women	100 mg
			Women of Reproductive Age (WRA)	100 mg
2018	**Anemia Mukt Bharat (AMB)**	6x6x6 Strategy Reduce anemia prevalence by three percentage points between the years 2016 and 2022. Switch from 100mg to 60mg of elemental iron	Children (6-60 months)	20 mg (Syrup)
			Children (5-10 years)	45 mg
			Children (10-19 years)	60 mg
			Pregnant & lactating women	60 mg
			Women of Reproductive Age (WRA)	60 mg

diseases in children aged 0-6 years, pregnant, and lactating mothers via supplementary nutrition.[28] Another nutritional intervention is providing one hot cooked meal to every school-going child studying in Classes I-VIII in Government & Government-Aided Schools under the PM POSHAN (POshan SHAkti Nirman) Scheme. Earlier known as the 'National Program for Mid-Day Meal in Schools, the program mainly aims to improve the nutritional status of school-going children and encourage children from disadvantaged backgrounds to attend school regularly to minimize dropout rates.[29]

The scenario in Bihar, India

Bihar in Eastern India, the third most populous state in the country[30] and a socio-economically backward region, is amongst the worst performers in terms of health indicators, especially infant mortality, under-5 mortality, malnutrition and anemia in women and children. While the infant mortality rate (IMR) and under-5 mortality rate (U5MR) of Bihar have shown a downward trend, The NFHS-5 factsheet shows that this progress has been unsatisfactory. At an IMR of 46.8 (per 1,000 live births) and an under-5 mortality rate of 56.4 (per 1,000 live births) against a national average of 35.2 and 41.9, respectively, Bihar stands as the second worst performer in India concerning these indices. The state also witnesses a high burden of child undernutrition. Stunting has been observed in 43% of under-5 children, and 41% are underweight, the second highest and the highest levels in the country. An estimated 63.5% of women in the reproductive age group (15-49 years) and 69.4% of children in the age group of 6-59 months are anemic (Fig.2), a rise of 3.2% and 5.9%, respectively over the previous estimates of the NFHS-4.[4]

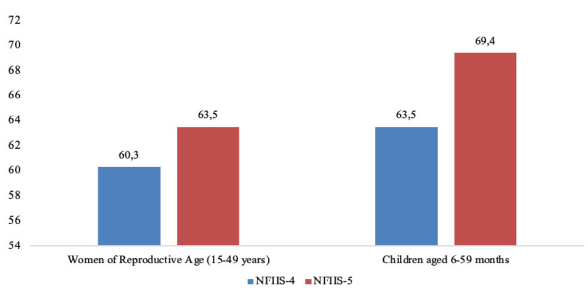

Fig. 2. Prevalence of anemia among women of reproductive age and children aged 6-59 months in Bihar (Source: NFHS-5 India Report)[4]

Anemia – is it socioeconomic or nutritional?

Anemia is often erroneously viewed as a nutritional disease when it is as much a result of socioeconomic determinants as of nutrition. Poor women and children are less likely to obtain hygienic, adequate, and balanced nutrition and often live in poor sanitary conditions, leading to higher rates of infectious diseases. Helminthic infections like hookworm result in blood loss and IDA. A cross-sectional survey in four districts of Bihar found that 42% of school-going children were infected with hookworms,[31] indicating a need for periodic deworming in this population. A poor socioeconomic status also results in a lower level of education, which is associated with a greater risk of anemia in women.[7] Higher education levels among mothers result in a better understanding of nutrition and dietary practices, consequently lowering rates of anaemia in young children.[32] In countries like India, the predilection of anemia in women can be due to the difference in opportunities afforded to male and female children. On average, girls are given less childcare, nutrition, and education, have lower participation levels in the workforce and are married off at a younger age compared to boys.[33] A common phenomenon, seen particularly in rural India, is that women consume leftover food after the rest of the household has eaten. The resultant compromise in nutritional status renders them vulnerable to nutritional disorders, including but not limited to anemia. The NFHS-5 data reveals that only 55% of women in Bihar are literate compared to 76.4% of men.[4] This figure is well below the national average of 71.5% literacy in women and could be one of the factors contributing to the high prevalence of anemia in the state.

Dietary diversity and food security

Dietary diversity refers to the number of food groups consumed over a period. A diverse diet helps meet daily nutritional requirements, especially those of micro-nutrients. A poorly diversified diet is associated with an increased risk of anaemia,[34] and found to be a strong predictor of stunting in children aged 6-59 months.[35] The diet in India is predominantly plant-based and cereal-rich, with most of the iron derived from non-heme sources such as legumes, millets, soybean, nuts, dried fruits and green leafy vegetables.[36]

Results of a baseline survey in Purnia district, Bihar, as a part of the SWABHIMAAN project, found that approximately 75% of pregnant women lived in food-insecure households and their diet mainly consisted of a mix of grains, pulses, and vegetables. Minimum dietary diversity was met by only 33.5% of pregnant women, and an astonishingly low percentage (3.3%) of them incorporated micronutrient-rich nuts & seeds in their diets. Consumption of heme-rich food like meat, poultry and fish (32.7%), vitamin A-rich fruits and vegetables (22.6%), and other fruits (13%) that could stave off anemia was low.[37] Although half the women were consuming green leafy vegetables, there is a definite need to boost these numbers.

The young child is dependent on its mother for its nutritional needs, either breastfeeding in the initial six months or complementary feeding later. A formative

study in Bihar showed that women lacked awareness about the frequency and quantity of feeding. They also succumbed to myths and indulged in harmful practices like withholding certain key food groups from children, negatively impacting dietary diversity.[38]

Findings from the Comprehensive National Nutritional Survey (CNNS 2016-18) reveal that a dismal 13.2% of children aged 6-23 months in Bihar achieved minimum dietary diversity.[39] The situation wasn't much different in children aged 2-4 years as few ate flesh foods (11.4%), legumes and nuts (27.5%), and fruits and vegetables (35.8%).

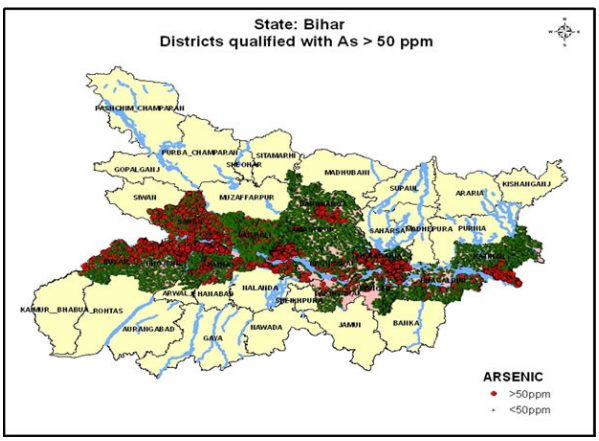

Fig. 3. State map of Bihar depicting Arsenic hotspots (Source: http://phedbihar.gov.in/WaterQuality.aspx)[45]

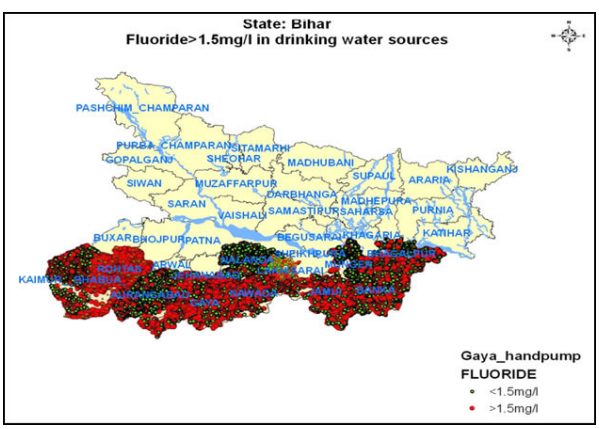

Fig. 4. State map of Bihar depicting Fluoride hotspots (Source: http://phedbihar.gov.in/WaterQuality.aspx)[45]

IFA consumption and adherence

Wendt et al concluded that IFA consumption was associated with higher educational levels and socioeconomic status. They also found that the likelihood of consuming IFA tablets for more than 90 days was higher if they had at least four antenatal (ANC) visits.[40] Only 1 in 4 (25%) of pregnant women in Bihar attended at least 4 ANC check-ups,[4] which would explain the low proportion of expecting mothers consuming IFA supplements for more than 100 days (18%) and more than 180 days (9.3%). A study in Southern India assessing compliance to IFA supplements among pregnant women determined that forgetfulness and adverse effects were the major factors contributing to non-compliance.[41]

The menace of arsenic and fluoride in water

Bihar is also afflicted with the menace of groundwater contamination. Elements like Arsenic and Fluorine enter the human body via drinking water and produce grown using contaminated water. Arsenic is known to change erythrocyte morphology, resulting in their death. It also diminishes bone marrow activity leading to anemia, thrombocytopenia, and leukopenia.[42] Fluoride, on the other hand, is a peculiar entity. Some amount of fluoride in drinking water is essential for optimum dental health due to its enamel-protecting property. However, excess levels can wreak havoc on the skeletal system and teeth, and lower hemoglobin levels and other red blood cell indices.[43] Of the 38 districts in Bihar, 22 report groundwater arsenic levels higher than the WHO provisional guideline of 10 µg/L,[44] 13 of them have levels more than 50ppm (Fig.3), and 11 have groundwater fluoride levels over 1.5mg/L (Fig.4).[45] These numbers portray a lamentable situation wherein many, to this day, lack access to safe drinking water in Bihar, rendering them vulnerable to ill health.

Iron folate coverage and supply chain issues

The primary strategy of anemia control program in India is the periodic provision of IFA supplements to the beneficiaries. Coverage of beneficiaries and stocks for distribution should be adequate. The Anemia Mukt Bharat Scorecard 2019-20 (Q4) placed Bihar at rank 14 with a coverage of 12.2%, 15.7%, 47.6%, and 77.6%, respectively, among children aged 6-59 months, children aged 5-9 years, adolescents (10-19 years) and pregnant women.[46] Additionally, IFA coverage saw a steep fall in the subsequent year (2020-21), with Bihar slumping to rank 21. This decline could be the effect of the COVID-19 pandemic, which extensively disrupted routine life and led to widespread lockdowns.

The supply chain is a vital cog in the machinery of any intervention-based program and a failure in any link deprives the target population of its intended benefits. An assessment of the supply chain for AMB conducted in two aspirational districts in each state (August 2019) found the lead time to be 41 weeks in Bihar.[47] The lead time is the time interval between placing an order and its delivery to the recipient. The slowest activities were the tender/purchase order (13 weeks), procurement and delivery (10 weeks) and block-level distribution (11 weeks).

The assessment also found significant gaps in the indent, ranging from 68% for IFA-red to 87% for IFA syrup. Procurement gaps were huge, with little receipt of IFA supplements. A long procurement time of almost four months resulted in the unavailability of IFA supple-

ments at health facilities, evidenced by the fact that only three districts in Bihar had IFA-Red available in all four quarters of the year. None met the criterion for IFA syrup, IFA-blue and IFA-pink.

The state warehouses lacked space for appropriate storage of the supplements. They were also short-staffed, with the existing staff ill-equipped to handle the inventory, increasing the chances of drug damage and expiration. Insufficient transport vehicles and the lack of a distribution plan compounded the delay of supplies.

Another concerning aspect is the absence of an integrated Management Information System (MIS) in the IFA supply chain. The DVDMS (Drug & Vaccine Distribution System) exists only at the district level in most states, and the levels below follow a manual system of reporting. This is not strictly adhered to, affecting the procurement process as there is no feedback available from the lower-level facilities.

Conclusion

Anemia rages relatively unabated in India, especially in a socio-economically backward state like Bihar. Tackling these surging levels requires a comprehensive approach that involves uplifting the socio-economic status of the weaker strata of society, and improving education levels, particularly among women. This will improve child-rearing and curb harmful practices like withholding certain food groups in young children. Expecting mothers and those with young children should be counselled on appropriate feeding practices and the importance of dietary diversity.

Measures to mobilize expecting mothers to the ANC clinics and steps to counsel them regarding nutrition and IFA supplements are needed to address the low consumption of IFA supplements for the recommended duration. A directly observed IFA supplementation program akin to the Directly Observed Treatment, Short-course (DOTS) strategy for Tuberculosis (TB) could be experimented with to combat the issue of forgetfulness. Parenteral iron options can be explored for women unable to tolerate oral iron supplements.

Access to potable drinking water is a human right, and it is disheartening that people lack access to it even today. Swift action is needed to ensure access in areas that bear the brunt of contamination, as prolonged exposure is known to cause irreparable damage.

Unlike its predecessors that focused predominantly on information, education & communication (IEC) activities, the AMB program incorporates a novel intensified year-round Behavior Change Communication (BCC) campaign among its interventions. It has also expanded the list of beneficiaries and strengthened institutional mechanisms for its implementation. These suggest that the program holds great potential in overcoming the barriers surrounding anemia. However, an assessment of the program indicates that coverage has been poor in Bihar. Intensified efforts need to be undertaken to address the same. The DVDMS should be extended to the lower-level facilities and the staff trained on proper reporting, and monitoring for timeliness and accuracy of reported data. Further operational research is thus, of paramount importance, to address the long procurement time and other deficiencies.

Declarations

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Author contributions

Conceptualization, S.K.N., C.M.S. and Sa.P.; Methodology, Sh.P., R.R. and B.N.N.; Writing – Original Draft Preparation, R.R., Sh.P. and BNN.; Writing – Review & Editing, S.K.N., R.R., B.N.N., Sh.P., M.V., CM.S. and Sa.P.; Visualization, Sh.P.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

Data supporting the results of this study shall, upon appropriate request, be available from the corresponding author.

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CASUISTIC PAPER

Parathyroid adenoma in a 15-year-old girl with recurrent urolithiasis

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ABSTRACT

Introduction and aim. The incidence of urolithiasis in children has been growing steadily for several decades, and it accounts for an increasing percentage of hospitalizations. Kidney stones are deposits of various mineral salts. Most of them are composed of calcium, favored by hypercalcemia and hypercalciuria. Primary hyperparathyroidism is one of the reasons for increased calcium levels in the blood.

Description of the case. A 15-year-old girl was hospitalized due to recurrent urolithiasis. Investigations revealed hypercalcemia with elevated parathyroid hormone. Ultrasound of the thyroid gland showed a local change near the lower pole of its right lobe, and Sestamibi nuclear scan confirmed the presence of the adenoma of the lower right parathyroid gland. Surgical removal of the parathyroid gland with the present adenoma was performed. Calcium and phosphate homeostasis parameters and the kidneys' ultrasound image were without any significant deviations from the norm.

Conclusion. After finding the cause of recurrent urolithiasis, the applied surgical treatment resolved all disease manifestations.

Keywords. primary hyperparathyroidism, urolithiasis, pediatrics, hypercalcemia, kidney stones

The list of abbreviations:

PHPT – primary hyperparathyroidism, **PTH** – parathyroid hormone, **SPECT** - single-photon emission computed tomography, **FHH** – familial hypocalciuric hypercalcemia, **HPT-JT** – hyperparathyroidism jaw tumor syndrome, **XLHP** – X-linked hypophosphatemia

Introduction

The incidence of urolithiasis in children has been growing steadily for several decades and accounts for an increasing percentage of hospitalizations.[1-8] The etiology of the formation of kidney stones is not fully understood. The risk of urolithiasis seems to be higher among boys in the first decade of life and girls in the second decade, i.e., during puberty, which suggests the role of reproductive hormones.[3,4] Kidney stones are deposits of various mineral salts, most of which are composed of calcium, favored by hypercalcemia and hypercalciuria.[8] Ultrasound is the initial imaging method in diagnosing urolithiasis disease in children.[4,9]

Aim

We present a case of a 15-year-old girl hospitalized due to hypercalcemia in the course of recurrent urolithiasis.

Description of the case

Within a year and a half, the girl was hospitalized three times due to periodic symptoms of renal colic. Previously

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used antispasmodics and analgesic drugs resulted in rapid resolution of colic, and the girl was discharged home.

According to anamnesis, she has not reported any problems with the digestive system, denied pain in the bone and joint system, fatigue, and hyperhidrosis. In the family history, the patient's uncle had two episodes of urolithiasis disease, patient's brother is healthy. The parents of our patient performed the blood test with the parameters of the calcium phosphate homeostasis - both of them were found to have elevated levels of parathyroid hormone (PTH) (mother: 90.8 pg/mL, father: 123 pg/mL) with slightly lowered vitamin D level (mother: 24 ng/mL, father: 20 ng/mL). Both parents had normal calcium (mother: 9.2 mg/dL; father: 9.7 mg/dL) and phosphorus (mother: 3.1 mg/dL; father: 2.7 mg/dL) levels and did not agree to extend the diagnostics.

The girl was repeatedly admitted to the hospital with abdominal pain and vomiting. In physical examination, she presented moderate abdominal tenderness with right-sided positive Goldflam's sign.

Laboratory tests showed an increased level of calcium and creatinine in the blood, with a slightly elevated level of these parameters in the daily urine sample. Abdominal ultrasound showed deposits in both kidneys with size 3-4 mm. Additionally, in the right kidney, dilatation of the pyelocalyceal system with urinary retention and dilation of the right ureter was seen. The patient was consulted by a urologist who did not find indications for surgical intervention.

After the applied treatment (antispasmodics and analgesic drugs), the deposits in the kidneys were excreted.

Due to the recurrent nature of the ailments, the diagnostics were extended – high levels of parathyroid hormone were found with simultaneous normal thyroid hormone levels (TSH, FT4), vitamin D deficiency, persistently elevated levels of calcium and creatinine in the blood, phosphorus and cystatin C levels were normal. In the daily collection of urine, there was hypercalciuria and low tubular phosphate reabsorption which proved hyperphosphaturia. The most relevant patients' blood and urine test results are shown in Table 1.

Table 1. Patients' blood and urine test results

	Patient's test results	Reference value
Serum creatinine	1.33 mg/dL	0.24–0.73 mg/dL
Serum calcium	12.84 mg/dL	9.12–10.2 mg/dL
Serum phosphates	3.3 mg/dL	2.9–5.1 mg/dL
Vitamin D	18.62 ng/mL	30–100 ng/mL
Parathyroid hormone	351 pg/mL	18.5–88.0 pg/mL
Ionized calcium	1.53 mmol/L	1.15–1.33 mmol/L
Alkaline phosphatase	180 U/L	70–370 U/L
Creatinine in 24-hr urine collection	25.52 mg/kg/24h	16–30 mg/kg/24h
Calcium in 24-hr urine collection	4.98 mg/kg/24h	1–4 mg/kg/24h
Tubular Reabsortion of Phosphate (TRP)	83.64%	85–89%
Cystatin C	1.11 mg/L	0.64–1.23 mg/L

Ultrasound examination of the thyroid gland revealed a focal change near the lower pole of its right lobe. Due to the ambiguous image, a reactive lymph node or parathyroid adenoma was suspected. The performed parathyroid single-photon emission computed tomography (SPECT) confirmed the presence of an adenoma of the lower right parathyroid gland (Fig. 1)

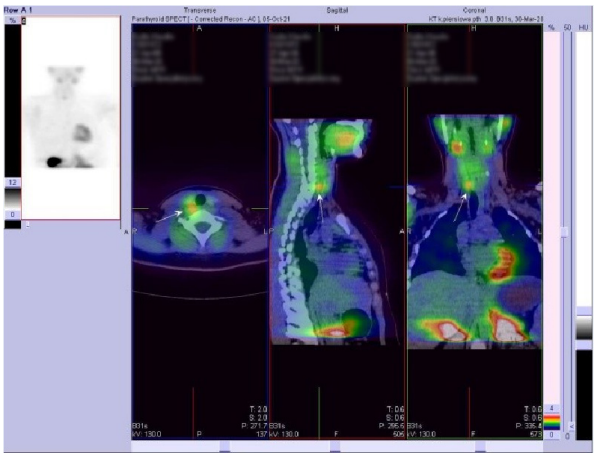

Fig. 1. Scintigraphic examination of the parathyroid glands (planar + SPECT/CT). The arrow marks the focus of intense Technetium (99mTc) sestamibi uptake in contact with the lower pole of the right thyroid lobe

In order to exclude the presence of MEN1 and MEN2a syndromes, in subsequent studies, the levels of prolactin, CEA, blood chromogranin A, urinary catecholamines, and methoxy-catecholamines have been measured, and no abnormalities were found. The circadian rhythm of cortisol was maintained. Slightly elevated levels of insulin and C-peptide were found with a normal glycemic profile. A genetic test for the syndromes mentioned above was not performed in our patient.

The patient was administered for parathyroidectomy (Fig. 2A-B).

Intraoperatively, the level of parathyroid hormone was measured twice (before – 317.9 pg/mL and after the right lower parathyroidectomy) – about 20 minutes after the removal of the gland, the level significantly decreased (34.8 pg/mL), which confirmed the excision of the right parathyroid gland. The course of the operation and the postoperative period were uneventful. Control blood levels of calcium and parathyroid hormone were normal. Histopathological examination of the sample confirmed the presence of an entirely removed parathyroid adenoma (Fig. 2C).

Bone densitometry, performed one month after the operation, revealed the results within limits expected for age and gender. During the follow-up visit, approximately two months after the operation, calcium and phosphate homeostasis were checked: normocalcemia (10 mg/dL), normophosphatemia (3.4 mg/dL), and decreased vitamin D levels (18 ng/mL) were found. Slightly elevated parathyroid hormone (120.6 pg/mL), renal parameters, and

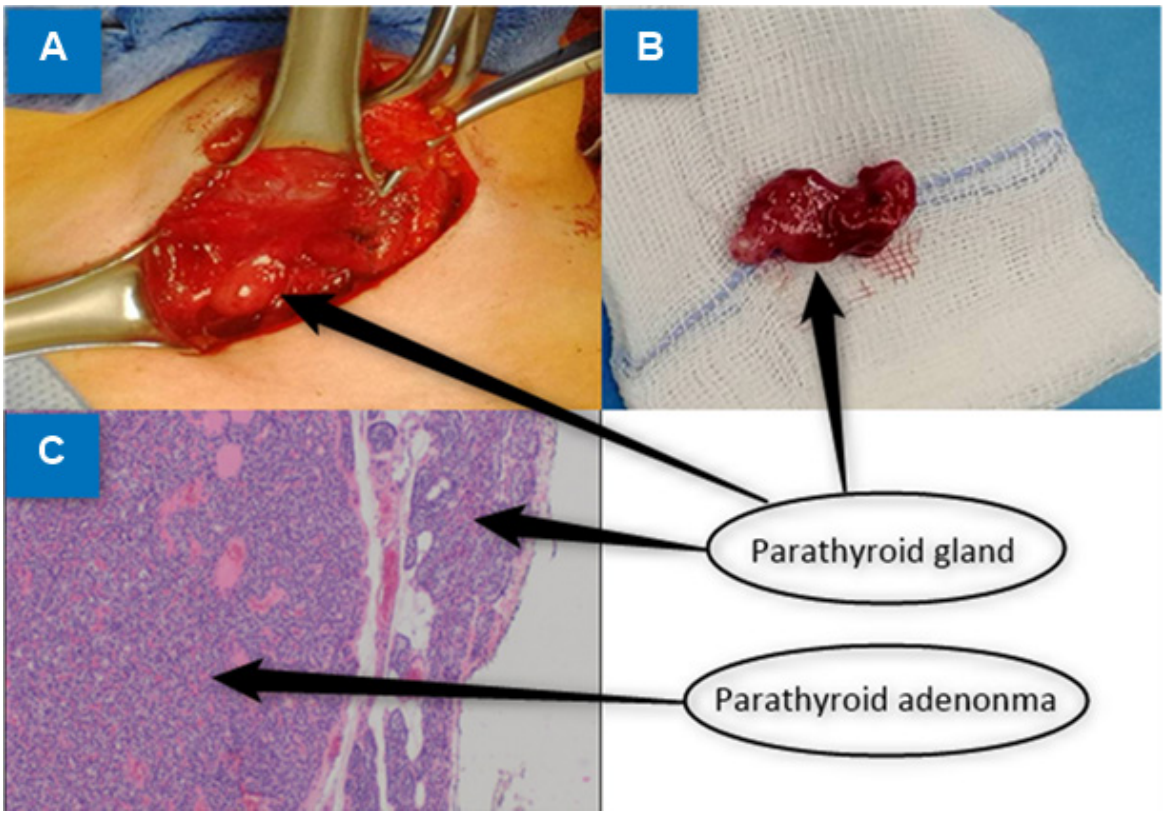

Fig. 2. (A) Intraoperative picture of adenoma and parathyroid removal from the cervical access; (B) Removed right inferior parathyroid gland with adenoma; (C) Histopathological picture of the normal parathyroid gland with adenoma at 40 times magnification

ions in a single urine portion were within normal limits. The ultrasound image of the kidneys revealed no deposits, urine retention or dilatation of the pyelocalyceal system. In the postoperative field, no pathological structures (except the fibrous scar) were visualized within the thyroid gland. The patient started vitamin D supplementation and currently remains under the supervision of the Endocrinology and Nephrology Clinic.

Discussion

Primary hyperparathyroidism (PHPT) in young patients is much less common than in adults, with an estimated incidence of 2–5 per 100,000.[10] Its most common cause is a single parathyroid adenoma (65-70%). More rarely, hyperparathyroidism may be a manifestation of multiple neoplasms of the endocrine glands (MEN 1 or 2a) or rare syndromes of familial isolated hyperparathyroidism. So far, parathyroid carcinoma has been described in 20 patients in the pediatric population.[11]

All clinical symptoms of PHPT are associated with the excessive production of parathyroid hormone, which leads to disturbances in calcium and phosphate homeostasis.[12,13] The laboratory indicator of the changes taking place in the organism is hypercalcemia. Clinical manifestations of persistently elevated blood calcium levels may be atypical to the underlying disease, i.e., eating disorders, weight loss, apathy, chronic abdominal pain, nausea, vomiting, bone and joint pain, muscle weakness, polyuria, polydipsia, depressed mood, difficulty concentrating, headaches. The above issues cause a delay in making a correct diagnosis, leading to the consolidation of complications in internal organs. Usually, the patient has abnormalities in one system (60%).[10]

Primary hyperparathyroidism can lead to urolithiasis (like in the case of our patient), as well as to nephrocalcinosis, intensifies bone metabolism, which may result in early osteoporosis, increased susceptibility to fractures and bone deformities (such as valgus knee or osteitis fibrosa cystica).[10,13,14] Hypercalcemia can affect the heart muscle, causing vascular, myocardial, and valvular calcifications, left ventricular hypertrophy, and arrhythmia. It also leads to arterial hypertension and increases the risk of myocardial infarction and stroke.[15,16] The essential element in diagnostics is looking for complications, which most often concern the osteoarticular and excretory systems. In order to determine the condition of the skeleton, the level of alkaline phosphatase is determined, and the bone morphology is assessed using the densitometric method. It is also important to assess kidney function with the blood markers such as creatinine, cystatin C and estimated glomerular filtration rate. In the case of symptoms of hyperparathyroidism in the form of urolithiasis, an ultrasound examination of the urinary system and 24 hour urine collection should be

performed to determine the excretion of calcium, phosphate, uric acid, oxalate, citrate, and cysteine.[17] Ultrasound examination of the urinary system also makes it possible to assess nephrocalcinosis. Nephrocalcinosis refers to abnormal calcium deposits within the tubulointerstitial tissue of the kidney.[18]

Considering that PHPT is the most characteristic feature of the MEN1 syndrome and may be the first related pathology, the presence of other syndrome components should be excluded, including mainly tumors of the anterior pituitary gland and pancreatic islets. For this purpose, the levels of prolactin, chromogranin A, insulin, and peptide C were measured in our patient. Less often, primary hyperparathyroidism may appear as an element of MEN2a manifestation, including medullary thyroid carcinoma and pheochromocytoma. Therefore, the level of catecholamines and methoxy-catecholamines in urine and CEA in blood serum were determined.[19] Since both parents had elevated levels of PTH, genetically determined disorders i.e., familial hypocalciuric hypercalcemia (FHH), hyperparathyroidism jaw tumor syndrome (HPT-JT), or X-linked hypophosphatemia (XLHP) should be considered. FHH was excluded because the patient's Calcium-Creatinine-Clearance-Ratio was 0.014 (<0.01: FHH is likely). HPT-JT is a rare genetic disease characterized by ossifying fibroma of the maxilla and/or mandible. XLHP is less likely because of the X-linked inheritance and normal level of phosphorus in both parents blood. In addition, its initial clinical symptoms usually appear in the first two years of the patient's life. Predominant manifestations include deformation, usually of lower extremities, and growth disturbances.[20] As the deficiency of the vitamin D is the most common cause of disrupted parathyroid hormone levels, it was considered to be the reason of the patient's parents abnormal blood test results.

In diagnosing hyperparathyroidism, imaging tests are aimed at locating the lesion before surgery. The standard methods include neck ultrasound and single-photon emission computed tomography.[14,17,21]

The surgical treatment is the 'gold standard' therapy of primary hyperparathyroidism in the pediatric population in the case of symptomatic course and the presence of organ lesions in this group of patients.[13,14] Currently, minimally invasive parathyroidectomy techniques are used in most cases. A helpful tool for assessing the procedures' effectiveness is the Miami criterion: if the concentration of PTH in the blood collected 10-20 minutes after the lesion removal decreases by 50% of the baseline value, it proves the success of the surgery.[11,17] After surgery, most patients (75%) may experience transient hypocalcemia and hypophosphatemia – the so-called hungry bone syndrome.[11] In specialized centers, the effectiveness of surgical treatment is 90%.[22]

Conclusion

In the described case, after finding the cause of recurrent urolithiasis, the applied surgical treatment resolved all disease manifestations.

Declarations

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Author contributions

Conceptualization, P.K., K.DG., R.P., P.B. and B.K.; Methodology, P.K. and K.DG.; Formal Analysis, P.K. and K.DG.; Investigation, P.K., K.DG., and B.K.; Resources, R.P., and P.B.; Writing – Original Draft Preparation, P.K., K.DG.; Writing – Review & Editing, P.K., K.DG, B.K.; Visualization, K.DG..; Supervision, B.K.

Conflicts of interest

Authors declare no competing interests.

Data availability

The data may be made available to interested persons at the request of the corresponding author via e-mail.

Ethics approval

All subjects gave informed consent to the inclusion prior to participating in the study.

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— 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.

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.

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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.

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— **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.

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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.

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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.

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