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VITAMIN D AND VITAMIN D RECEPTOR GENE IN OSTEOARTHRITIS

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ABSTRACT

Osteoarthritis is a degenerative, painful and irreversible disease that affects millions of people worldwide. The causes and mechanisms of osteoarthritis have not been fully understood. Vitamin D is an essential factor in bone metabolism. Its actions are mediated by the vitamin D receptor, a transcription factor that controls gene expression, thus maintaining calcium and phosphate homeostasis. Vitamin D has been hypothesized to play essential role in a number of musculoskeletal diseases including osteoarthritis, and its deficiency is prevalent among osteoarthritis patients. A large number of studies have been done regarding the effects of vitamin D in pathogenesis and progression of osteoarthritis, as well as its use a therapeutic agent. Up to date, studies have provided controversial results, and no consensus concerning this matter was achieved. With this review, we aim to explore current data on the possible role of vitamin D and its receptor in pathogenesis of osteoarthritis and assess the efficiency of vitamin D supplementation as a therapeutic strategy.

Keywords: *Osteoarthritis, vitamin D, vitamin D receptor, inflammation.*

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INRODUCTION

Osteoarthritis (OA) is progressive and degenerative joint disease that affects and causes disability in about 3.3% of global population (1). It starts as an inflammation of the synovium, followed by degradation and loss of articular cartilage, and abnormal subchondral bone growth. The most commonly involved joints are on the fingers, at the base of the thumb, neck, lower back, knee, and hips. The symptoms include joint pain, stiffness and swelling, that primarily appear sporadically in the morning, progress over the years, causing loss of mobility. The etiology, and pathogenesis of OA are not completely understood. However, there are known factors that, when combined, cause damage to the cartilage and subsequent bone remodeling. Based on the risk factors OA is classified as primary and secondary, both of which have the same clinical appearance. Primary OA is caused by, genetic predisposition, age (incidence increases among people over 60), gender (more prevalent in postmenopausal women), obesity (affecting large, weight bearing joints such as knee and hip), physically demanding work, joint imobility or joint injury. Secondary OA is not age related, and represents 20% of the cases. It is associated with congenital diseases such as Ehlers-Danlos syndrome, Marfan syndrome, alkaptonuria, Wilson's disease, with joint infections, injuries and inflammatory diseases (2). Diagnosis is made based on history and clinical examination. On radiography, the severity of joint damage can be graded by evaluating typical radiological changes: subchondral bone sclerosis, joint space narrowing, osteophyte formation, subchondral cyst formation and loss of bone contours (3).

Vitamin D is a steroidal hormone primarily involved in calcium homeostasis and bone metabolism. Through binding to the vitamin D receptor (VDR), vitamin D regulates absorption of calcium and phosphate from kidney and the gastrointestinal system, thus maintaining blood calcium levels. Along with parathyroid hormone (PTH) and calcitonin, vitamin D plays the fundamental role in bone mineralization and resorption (4). However, vitamin D has a wide range of effects on various cell types and has a potential key role in a number of common diseases, including OA, various cancer types, and autoimmune diseases (3). As a consequence of crucial role of vitamin D on bone metabolism and inflammation, number of studies have been conducted to investigate potential role of this vitamin in various degenerative conditions of the bones and joints. Since no treatment or specific diet has been proven for effectively treating or preventing OA, various studies explores the vitamin D suplementation as potential treatment.

Vitamin D Biology

Vitamin D in humans has two forms, dietary form vitamin D2 or ergocalciferol, and endogenous form vitamin D3 or cholecalciferol. Major dietary sources of vitamin D2 are fatty fish, such as salmon and tuna, egg yolks, cheese, as well as food fortified with vitamin D. Cholecalciferol is endogenously synthesized by the skin upon to sunlight exposure. Biosynthesis begins with the photoisomerisation of 7-dehydrocholesterol by UVB radiation to form previtamin D in the skin. Subsequently, previtamin D undergoes thermal-dependent isomerization to form vitamin D3 (5). After two hydroxylations biologically inactive lipophilic cholecalciferol reach its active form. First hydroxylation occurs in the liver, creating calcidiol (25-hydroxyvitamin D_3, 25(OH)D_3), which can be measured in the serum. An additional hydroxylation occurs in tubules of the kidney, osteoblasts and osteoclasts, generating the most active form dihydroxyvitamin D3 calcitriol (1,25(OH)2D_3) (3,6).

Vitamin D Receptor

Vitamin D receptor (VDR) is a transcription factor located in the nuclei of target cells in various organs, including bones, brain, heart, skin, gonads, prostate, and breast. The binding of vitamin D to the VDR allows the VDR's rapid binding to regulatory regions of target genes, including the transport proteins involved in calcium absorption in the intestine (3). VDR also regulates gene networks involved in bile acid metabolism in the colon, the differentiation of keratinocytes in the skin, the development and cycling of dermal hair follicles, and the functions of key cell types involved in both innate and adaptive immunity (7). Chondrocytes, osteoblasts and osteocytes express VDR, indicating that these cells could be potential targets for vitamin D signalling and suplementation in OA.

Activation of VDR in osteoblasts, leads to synthesis of non-collagen proteins, osteocalcin and osteopontin, that are components of the bone organic matrix. Binding of active form 1,25$(OH)_2D_3$ stimulates osteoblasts and osteocytes to produce Fibroblast growth factor 23 (FGF23) (7) FGF23 together with PTH from the thyroid glands, and vitamin D, regulate the intestinal absorption and renal excretion of calcium and phosphate, as well as the storage and removal of these key minerals from the skeleton. (8) FGF23 produced in osteocytes has systemic as well as local effects that influence mineralization. Osteocytes directly affect bone remodeling by inhibiting or stimulating osteoblast and osteoclast activity in order to maintain bone homeostasis, therefore all of these functions are partly affected by 1,25(OH)2D3 (3). FGF23 lowers phosphate levels by downregulating phosphate co-transporters in the kidney, thus reducing the phosphate reabsorption. It also downregulates the hydroxylase, an enzyme in the kidney which converts the precursor of 25-hydroxyvitamin D_3 into its active form 1,25(OH)2D3. FGF23 also induces the expression of 24-hydroxylase, an enzyme that catabolizes 1,25(OH)2D3 and reduces its action. 1,25(OH)2D3 is the primary factor for increasing FGF23 production and FGF23 self-regulates its production with this negative feedback. Other factors, such as PTH and serum phosphate may stimulate FGF23, but vitamin D_3 is its most significant regulator (7,9).

The physiological function of VDR in osteocytes is questionable under the normal dietary calcium intake. In one study, VDR-ablated mice were fed diet enriched with calcium and phosphorus, and did not show alterations in bone mineral density or bone histology until the old age (10). In another study, genetic ablation of the VDR in osteocytes showed no effect on bone and mineral homeostasis. However, 1,25(OH)2D3-induced inhibition of bone mineralization in osteoblasts /osteocytes was shown to be important for maintaining calcium homeostasis when vitamin D levels are upregulated due to increased calcium demand (11).

Due to its crucial role in vitamin D signalling, the VDR gene and its corresponding protein have been investigated in many diseases including OA. Genetic polymorphisms in the VDR gene have been investigated using restriction fragment length polymorphism, which employs restriction enzymes to cut DNA at specific sequence sites, and polymorphisms are named by the restriction enzymes used to identify them. Among others, three single nucleotide polymorphisms (SNPs) located near the 3' un-translated region (3′UTR) TaqI, BsmI, and ApaI have been investigated for possible involvement in the OA (3,12). Although not functional, these SNPs are associated with a poly (A) repeat in the 3′UTR that could influence the stability of the VDR mRNA. Another SNP, FokI is a functional polymorphism of the VDR gene which results in different translation initiation sites on VDR. Finnish study discovered that genetic polymorphisms may be associated with an increased susceptibility to bilateral hand OA in females (13). In 2009, Lee et al. Conducted a meta-analysis of 10 studies into the association of the TaqI, BsmI, and ApaI polymorphisms with OA (14). The analyyed studies had investigated various OA sites: knee, lumbar spine, hand and hip. No evidence was found to support an association between any of the polymorphisms and susceptibility to OA. This conflicts with a more recent meta-analysis in which a statistically significant association was found between the ApaI polymorphism and OA in Asian populations, but the association was not observed in Europeans (15). Investigations into relationships between VDR polymorphisms and different aspects of OA, like joint space narrowing, osteophyte formation or inflammation will be valuable in the future.

Role of Vitamin D in OA

Tissue changes in OA like aberrant bone remodeling, sclerosis, and osteophyte formation are caused by three main cell types: osteoblasts, osteoclasts and chondrocytes. Vitamin D has a range of effects on these cells in osteoarthritis affected joints.

Vitamin D, specifically $1,25(OH)2D_3$ stimulates osteoblastic bone mineralization by activating the VDR. Study with cell culture of OA osteoblasts showed increased cell proliferation after vitamin D treatment compared to healthy osteoblasts. After vitamin D treatment, production of osteocalcin and alkaline phosphatase, two proteins crucial for bone mineralization was also significantly higher in OA osteoblasts compared to healthy and osteoporotic ones (16). Another study found that $1,25(OH)_2D_3$ induced a significantly higher production of osteocalcin in OA osteoblast-like cells compared to controls (17). These findings propose that OA osteoblasts, when induced by vitamin D, increase bone formation. That could lead to an explanation of subchondral sclerosis and osteophyte formation in advanced OA.

During the pathogenesis of OA, an extensive vascular network develops in synovium, menisci, pannus, osteophytes, and osteochondral junction within affected joints. Vascular growth is associated with the loss of structural integrity of the cartilage, and progression of pain through the extension of sensory nerves (18). The level of vascular endothelial growth factor (VEGF), main angiogenic cytokine, is associated with the severity of OA. The expression of VEGF is partially regulated by $1,25(OH)^2D_3$, thus linking vitamin D to the progression of the disease (19).

Osteoclasts are specialized macrophages, involved in bone resorption. However, they do not express VDR, and vitamin D is thought to affect them indirectly through osteoblastic activity (20).

Chondrocytes are responsible for the production and maintenance of the extracellular matrix in cartilage. In OA there is an imbalance between anabolic and catabolic processes that leads to the destruction and loss of articular cartilage. Osteoarthritic chondrocytes have a higher VDR expression than healthy cells. The VDR expression is associated with the expression of matrix metaloproteinases, specifically MMP-1, MMP-3, and MMP-9, enzymes that degrade bone and cartilage, which worsens OA (21). $1,25(OH)_2D_3$ and inorganic phosphate are involved in the hypertrophy and defective minrealization in osteoarthritic chondrocytes through FGF-23 signalling (22) suggesting that vitamin D has a negative effect on cartilage in OA.

Deficiency of Vitamin D in OA

Hypovitaminosis is defined as 25(OH)D3, levels under 20ng/mL. It is prevalent among older population since, the capacity of skin to produce vitamin D decreases with old age. It frequently coexists with OA, and a large procent of rheumatology patients are vitamin D deficient (23, 24). However, the effect of hypovitaminosis D on the development and progression of OA is still unclear. Up to date, there are many studies with conflicting results.

Felson et al. used 2 different longitudinal studies of knee OA, to reevaluate $25(OH)D_3$ levels and their association with knee OA worsening (consisting of disease incidence and progression). In one study the association of 25(OH)D levels with joint space loss over time was examined in cohort selected without regard to the presence of knee OA. In the second study, they examined the cohort of patients who had symptomatic knee OA. They evaluated the association of 25(OH)D levels with loss of joint space seen on radiographs and with cartilage loss as seen on magnetic resonance imaging (MRI). Examining knee OA, no association was found

between vitamin D levels and structural disease worsening, defined as joint space loss on radiography or cartilage loss on MRI (25). These findings were supported by large cohort study of 5,274 OA-free participants in Finland. The study found no association between low serum 25 $(OH)D_3$ levels and increased risk of developing hip or knee OA over a 10-year period (26). Neither of these studies support the hypothesis that high levels of 25(OH)D protect against knee and hip OA (27). Contrary to these studies, there are those that demonstrate an association between low 25 $(OH)D_3$ levels and OA. A prospective cohort study of 5,995 men in the US found a high prevalence of vitamin D insufficiency or deficiency in hip OA patients and found that these patients were twice as likely to have radiographic hip OA than the men with normal 25$(OH)D_3$ levels (28). Bergink et al. conducted prospective cohort study of 1248 cases of knee OA. They concluded that low dietary vitamin D intake increases the risk of progression of knee OA. Improving the vitamin D status in the elderly could protect against the development and worsening of knee OA, especially in those with low bone mineral density (29). In Egypt, research into postmenopausal women found lower serum 25$(OH)D_3$ was associated with knee OA, when compared to healthy males (30). A systematic review performed by Cao et al. analyzed 15 studies. They found strong evidence for an association between 25$(OH)D_3$ and cartilage loss in knee joints. The authors also observed moderate evidence to support a positive connection between low levels of vitamin D and radiographic knee OA (31).

The differences in study results may be related to differing effects of serum vitamin D in bone and cartilage metabolism. Vitamin D influences the mineralization of bone matrix, and low serum levels of vitamin D may result in poorly mineralized bone that might alter forces across the joint and reduce joint deterioration (28). However, low levels of vitamin D may also alter chondrocyte metabolism and augment degeneration (32). Additional studies are warranted to determine what role vitamin D might have on the development and progression of OA.

The most recent overview of research published over the last 50 years in English language found no consensus on this topic (33). Clinical studies, as well as laboratory based studies were reviewed. In sum, among the various laboratory studies examining the association between vitamin D and articular cartilage in diverse models of OA most imply some relationship between vitamin D and cartilage integrity. While vitamin D may be considered beneficial in some instances, in others it may prove to facilitate cartilage erosion (34). With no definitive model, or study approach, the observed outcomes may depend on the substrate employed, the measurement approaches employed, and durations of exposure. Animal models of osteoarthritis may also lack validity if they do not replicate the clinical conditions: if muscle quality is normal, obesity is not evident, comorbid conditions are not apparent. Reviewing clinical studies, Marks found that there is no consensus regarding vitamin D and OA due to many factors. Studies have different results since they also have different durations, age cut-off or inclusion points, sample inclusion criteria, type and extent of disease, numbers and types of affected joints, presence, number, and type of other chronic conditions and interventions, and unknown medication usage rates. Optimal versus insufficient serum levels of vitamin D are not uniform, nor standardized as well (33).

Therapeuthic possibility of Vitamin D in OA

Since there is no consistent data on the role of vitamin D as a precursor of OA, there is no consensus on its use as a therapeutic agent. In a double-blind, randomized placebo-controlled trial, Arden et al. showed that there was no significant difference in the rate of joint space narrowing in the medial compartment of knees due to vitamin D supplementation (35). While vitamin D may have little effect on cartilage loss, it is possible that vitamin D deficiency could importantly affect other elements of disease, including pain and weakness, which are critical to a patient's experience with OA. Low vitamin D levels have been shown to be associated with muscle weakness and with an increased risk of pain (36,37). In a double-blind study with 103 knee OA patients, patients receiving vitamin D oral supplements had slightly less pain compared with those receiving placebos after a 1-year follow-up. These patients, however, were not as physically capable as their placebo counterparts (38). Additionally, 787 members of the Hertfordshire Cohort Study in the United Kingdom took part in a cross-sectional study that found no association between vitamin D levels and radiographic knee OA, but did suggest a significant association between vitamin D and knee pain (39). On the other hand, in a 2-year study in which symptomatic knee OA patients were given oral doses of cholecaliciferol, to raise serum levels of vitamin D, there was no reduction in knee pain scores (40).

Despite efforts for several decades to reduce the osteoarthritis burden, no true progress in more than 30 years can be detected except in the realm of surgical replacements (41). Evidence shows that patients undergoing joint revision surgery for end-stage OA, who have low vitamin D levels, are more likely to experience 90-day complications as well as periprosthetic joint infections after surgery, than those with adequate levels, and this finding may affect up to 84% of surgical cases (42,43). Because of that, Traven et al. (42) suggest considering preoperative vitamin D levels as a risk factor for complications, and to treat it accordingly. Various authors concluded that, either directly or indirectly through its influence on muscle and bone, inflammation and obesity (44,45), the presence of 'sufficient' vitamin D serum levels may help reduce the pain and joint dysfunction in cases of osteoarthritis, regardless of disease stage (30,36,37). Even if the disease progression remains static in the presence of vitamin D supplementation, this alone could be regarded as a positive result in the context of this progressive degenerative disease.

CONCLUSION

Vitamin D through its receptor, is one of the most important factors involved in the function, development as well as the pathology of the musculoskeletal system. Further

studies are required to settle the debate over the role of Vitamin D in the development and progression of OA. Randomized-controlled trial is considered to be the most rigorous way of determining the effects of vitamin D supplementation on the development of osteoarthritis. Even if it has not been truly examined, vitamin D supplementation in OA patients, as well as in OA "risk groups" may prove to be of value. Thoughtful application of vitamin D may impact OA correlates such as inflammation, pain, joint dysfunction and low quality of life. Identifying the presence of vitamin D deficiencies and correcting them across the lifespan earlier rather than later, may prove to be an efficacious cost-effective form of minimizing joint disability in later life.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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IN VITRO HYPOGLYCEMIC AND RADICAL SCAVENGING ACTIVITIES OF CERTAIN MEDICINAL PLANTS

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ABSTRACT

The purpose of this study is to investigate in vitro hypoglycemic and free radical scavenging activities of some medicinal plants including Ficus glomerata (FG), Pandanus amaryllifolia (PaA), Artocarpus altilis (AA), Gomphrena celosioides (GC) and Gynostemma pentaphyllum (GP). Alpha-amylase inhibitory assay was examined by dinitrosalicylic acid reaction. Glucose uptake assay was investigated by LO-2 cell model. DPPH and $ABTS^{+}$ scavenging assays were performed by spectrophotometry. Cell viability was determined by MTT method. It was found that the extracts including FG, PaA, AA, GC and GP were able to inhibit alpha-amylase activity up to 38.4 ± 4.2%, 47.8 ± 4.3%, 49.3 ± 3.5%, 40.1 ± 4.4% and 38.5 ± 3.8%, respectively. Moreover, glucose adsorption and glucose uptake capacity of these extracts were evidenced. In addition, free radical scavenging activity of these extracts was indicated in a range of 30.6-54.5% for DPPH radical and 31.8-51.1% for $ABTS^{+}$ radical. Especially, these extracts exhibited no cytotoxicity effect on human hepatic LO-2 cells and human gastric BGC-823 cells at the concentration of 100 µg/ml. The results indicated that A. altilis leaves were effective in inhibiting alpha-amylase activity, increasing glucose adsorption and glucose uptake and scavenging free radicals. Therefore, it could be suggested to be a promising hypoglycemic agent for managing type 2 diabetes.

Keywords: *Alpha-amylase, anti-diabetes, antioxidant, medicinal plants, ischemia treatment.*

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INTRODUCTION

Diabetes is one of the most frequent non-communicable lifestyle-related diseases in the world. According to World Health Organization projections, around 300 million people will be affected by diabetes by the year 2025 (1). Especially, a major metabolic disorder prevalence of diabetes mellitus is increasing daily. It is characterized by the relative or absolute deficiency of insulin secretion and/or insulin action that causes glucose intolerance and impairs carbohydrate, lipid and protein metabolisms (2, 3). Patients affected by diabetes mellitus develop different diabetes mellitus-related complications such as retinopathy, nephropathy and peripheral neuropathy (4). Currently, the oral anti-diabetic drugs, such as sulfonylureas, biguanides, α-glucosidase inhibitors, thiazolidinediones and dipeptidyl peptidase-4 inhibitors are the approved medications for type 2 diabetes mellitus (5). However, these drugs are less effective in long-term regulation of the glycemic level and exhibit many undesirable side effects. Therefore, the discovery of safer and more effective anti-diabetic agents is still necessary.

Plants produce a huge array of natural products with high diversity of structures (6, 7). Hundreds of active compounds have been identified in plants including terpenoids, alkaloids, flavonoids and phenolic compounds (8). However, 90% useful natural lead compounds are still waiting for the discovery in the future (9). Since ancient times, numerous plants have been used as herbal medicines to treat various diseases due to their secondary metabolites (10, 11). Up to now, the plant-based medicines are still a common tendency for primary health care as these are affordable and safer to use (12). Notably, herbal medicines have played an important role in the prevention and treatment of type 2 diabetes via prophylactic and therapeutic management (13, 14). Prophylactic action of herbal medicines may be attributed to healthy organs and their cellular tissue, especially beta cells of pancreas and hepatic tissue. Meanwhile, therapeutic action of herbal medicines may be due to curative action on affected tissue of pancreas, liver and diabetes-related organs (15). Hence, medicinal plants are considered as a bright future in the therapy and management of diabetes. Especially, medicinal plants such as *Ficus glomerata, Pandanus amaryllifolia, Artocarpus altilis, Gomphrena celosioides and Gynostemma pentaphyllum* have been known as useful herbs for the treatment of various diseases. *F. glomerata* is an evergreen tree of Moraceae family widely distributed throughout warmer parts of Asia, Africa, America, and Australia. It has been used for the treatment of biliary disorders, jaundice, dysentery, diabetes, diarrhoea and inflammatory conditions (16). *P. amaryllifolia* is a tropical plant from Pandanaceae family which is widely used in South and Southeast Asia for cooking. *P. amaryllifolius* leaves have a number of local medicinal uses in reducing fever, relieving indigestion and flatulence, and decreasing postprandial blood sugar (17, 18). *A. altilis* belongs to Moraceae family and is grown throughout Southeast Asia, Pacific Ocean islands, Senegal, Ghana, Liberia and India (19). The medicinal values of *A. altilis* have been known due to the treatment of tongue thrush, skin infections, sciatica, diarrhoea, low blood pressure, asthma and diabetes (20). *G. celoisiodes* is a sprawling herb which belongs to Amaranthaceae family and spreads throughout the whole tropical world, especially Brazil, Paraguay, Uruguay and Argentina (21). It has been recognized in different traditional systems of medicines for the treatment of various diseases such oliguria, heat and empacho, hypertension, cough, diabetes, kidney problems, jaundice, high cholesterol, bronchial asthma and fever (22). *G. pentaphyllum* is a perennial creeping herb of Cucurbitaceae family. It disperses throughout India, Nepal, Bangladesh, Sri Lanka, Laos, Myanmar, China, Korea and Japan. *G. pentaphyllum* is applied as herbal medicine for the treatment of haematuria, oedema and pain of the pharynx, heat and oedema of the neck, tumours and trauma, haematuria, hyperlipidaemia, palpitation and shortness of breath, chest congestion, dizziness, headache, forgetfulness, tinnitus and spontaneous perspiration (23). Notably, these plants have received a lot of attention due to lowering blood glucose in type 2 diabetes. However, the scientific reports regarding anti-diabetic activity of these medicinal plants are still limited. Accordingly, these five medicinal plants have been subjected and screened for their potential anti-diabetic effect via inhibiting alpha-amylase activity and scavenging free radicals.

MATERIALS AND METHODS

Materials

Leaves of *Ficus glomerata*, *Pandanus amaryllifolia* and *Artocarpus altilis* were collected from Tay Ninh province, Vietnam (April 2018), while all parts of *Gomphrena celosioides* and *Gynostemma pentaphyllum* were bought from the local market in Vietnam (District 5, Ho Chi Minh city). Acarbose and Metformin were purchased from the pharmacy store at district 7, Ho Chi Minh city, Vietnam. Alpha-amylase from *Bacillus licheniformis* (A4582) was purchased from Sigma-Aldrich (USA). Solvent was purchased from Xilong (China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction

Materials were air-dried under shade and powdered by a grinder. The powder was soaked with ethanol under the extract conditions: ratio (1/4, w/v), time (4 h) and temperature (60 ^{0}C). The ethanol extracts of these plants were kept at 4 ^{0}C for further investigation. The concentration of 100 µg/ml of the extracts was used for all assays.

Alpha-amylase inhibitory assay

The determination of alpha-amylase inhibition was carried out by dinitrosalicylic acid (DNS) method (24). Briefly, 1ml of the extract (100 µg/ml) was pre-incubated with 1 ml of alpha-amylase (diluted for 10000 in 20 mM sodium phosphate buffer, pH 6.9) for 30 min and 1 ml (1% w/v) of starch solution was then added into the mixture. The mixture was further incubated at 37 ^{0}C for 10 min. The reaction was then

stopped by adding 1 ml of DNS reagent (12.0 g of sodium potassium tartrate tetrahydrate in 8 mL of 2 M NaOH and 96 mM 3,5-dinitrosalicylic acid solution), followed by the heating of contents in a boiling water bath for 5 min. The control (C) was prepared without plant extracts and the blank (B) was without the alpha-amylase enzyme. The absorbance was measured at 540 nm. Acarbose (100 µg/ml) was used as reference. The inhibition of alpha-amylase in percentage was calculated by the following equation (OD_B is the absorbance of blank, while OD_C is the absorbance of control):

$$\text{Inhibition (\%)} = \frac{(OD_C - OD_B) - (OD_{sample} - OD_B)}{(OD_C - OD_B)} \times 100\%$$

Glucose adsorption capacity assay

Glucose adsorption capacity of the extracts was determined *in vitro* (25). The extracts (1%) were added to 25 ml of glucose solution (50 mM). The mixture was well mixed and incubated at 37 ^{0}C for 6 h, centrifuged at 4000g for 20 min and the glucose content in the supernatant was determined. The bound glucose was calculated using the following formula (G1 is the glucose concentration of original solution; G2 is the glucose concentration after 6 h incubation):

The glucose adsorption (mM glucose/g extract) = [(G1 – G2)*Volume of solution]/Weight of sample

Glucose uptake capacity assay

Human hepatic LO-2 cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ^{0}C using Dulbecco's modified eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES buffer, 100 U/ml of penicillin G, and 100 mg/ml of streptomycin. The glucose uptake into LO-2 cells was determined *in vitro* (26). Briefly, the cells ($1x10^4$ cells/ml) were incubated with the extracts (100 µg/ml) or metformin (20 µg/ml) for 48 h. The spent culture medium was then removed and replaced with 50 µl incubation buffer (0.1% BSA and 8 mM glucose) and further incubated for 3 h at 37 ^{0}C. Afterward, glucose concentration in supernatant was measured using Contour™ Plus Meter (Ascensia Diabetes Care, Switzerland). The percentage of glucose uptake was calculated as a percentage compared to control C (The untreated cell group). The percentage of glucose uptake was calculated by the following formula (T is glucose concentration in supernatant of the treated cell group, while C is glucose concentration in supernatant of the untreated cell group):

Glucose uptake (%) = [(8 – T)/(8 – C)]*100

1,1-Diphenyl-2-picryl-hydrazyl assay

The antioxidant activity of the extracts was determined by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay (27). Briefly, 100 µl of the extracts (100 µg/ml) was mixed with 100 µl of DPPH (3 mM) solution and incubated in the dark at room temperature for 30 min. The absorbance of the mixture was then measured at 490 nm by using Genova Nano (Jenway, UK). Vitamin C (20 µg/ml) was used as a reference. DPPH scavenging ability was determined by the following formula (OD: absorbance or optical density):

DPPH scavenging ability (%) = $[(OD_{control} - OD_{sample})/OD_{control}] \times 100\%$

2,2-Azinobis-3-Ethyl benzothiazoline-6-sulfonic acid assay

The antioxidant activity of the extract was also determined by 2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid assay (27). Briefly, the photometric assay was conducted on 0.9 ml of $ABTS^+$ solution and 0.1 ml of the extracts (100 µg/ml), mixing for 45 sec. Measurement was taken immediately at 734 nm after 15 min incubation. Vitamin C (20 µg/ml) was used as a reference. The $ABTS^+$ scavenging ability was determined by the following formula (OD: absorbance or optical density):

ABTS scavenging ability (%) = $[(OD_{control} - OD_{sample})/OD_{control}] \times 100\%$

Cell viability assay

The percentage of viable cells (LO-2 and BGC-823) was determined after 24h treatment with investigated agents using MTT assay (28). Briefly, the cells ($1x10^5$ cells/ml) were incubated with 100 µg/ml of the extracts for 24 h. The medium was removed, and the cells were incubated with a solution of 1 mg/ml MTT for 4 h. Finally, supernatant was removed, and DMSO was added to solubilize the formed formazan salt. Amount of formazan salt was determined by measuring absorbance at 540 nm using a microplate reader. The cell viability was calculated as a percentage compared to blank.

Statistical analysis

Data were analysed using a one-way analysis of variance test of the statistical package for social sciences (SPSS). The statistical differences among groups were assessed using Duncan tests. The differences were considered significant at $p < 0.05$.

RESULTS

Alpha-amylase inhibitory activity of the extracts

The extracts of various medicinal plants including FG, PaA, AA, GC and GP were investigated for their capability against alpha-amylase activity. The results showed that these extracts exhibited inhibitory activity on alpha-amylase at the concentration of 100 µg/ml (Figure 1). Among them, AA and PaA extracts possessed the highest inhibition on alpha-

amylase activity. The inhibitory effects of AA and PaA extracts were observed up to 49.3 ± 3.5% and 47.8 ± 4.3%, respectively, followed by GC - 40.1 ± 4.4%, GP - 38.5 ± 3.8% and FG - 38.4 ± 4.2%. Meanwhile, the inhibitory activity of acarbose (59 ± 5.3 %) was higher than that of these extracts.

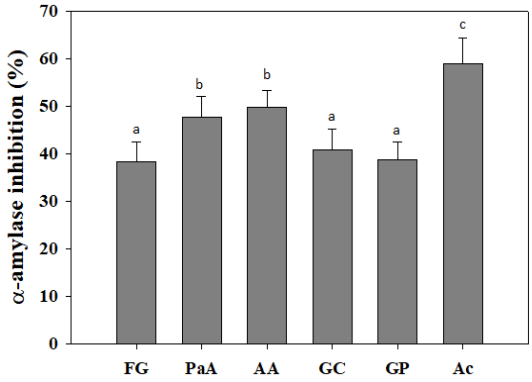

Figure 1. The alpha-amylase inhibitory activity of the extracts. Each determination was made in three independent experiments, and the data are shown as means ± SD. Different letters a–c indicate significant difference among groups ($p < 0.05$). *Ficus glomerata* (FG), *Pandanus amaryllifolia* (PaA), *Artocarpus altilis* (AA), *Gomphrena celosioides* (GC), *Gynostemma pentaphyllum* (GP), and Acarbose (Ac).

Glucose adsorption capacity of the extracts

In this assay, glucose adsorption capacity of FG, PaA, AA, GC and GP extracts at the concentration of 1% (w/v) were investigated *in vitro* (Figure 2). It was observed that GC extract exerted the highest glucose adsorption capacity at the value of 1.8 ± 0.16 mM glucose/g extract, followed by AA (1.7 ± 0.18), PaA (1.2 ± 0.15), GP (1.1 ± 0.11) and FG (1.0 ± 0.13) mM glucose/g extract.

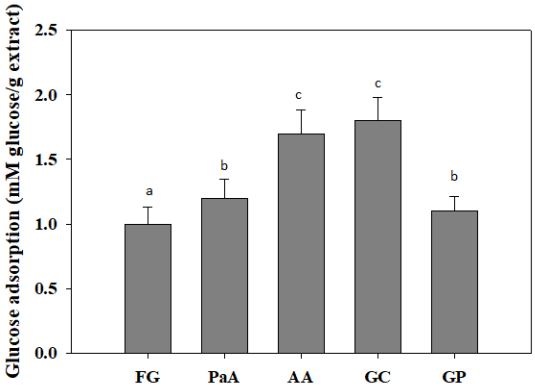

Figure 2. Glucose adsorption capacity of the extracts at glucose concentrations of 50 mM. Each determination was made in three independent experiments and the data are shown as means ± SD. Different letters a–c indicate significant difference among groups ($p < 0.05$). *Ficus glomerata* (FG), *Pandanus amaryllifolia* (PaA), *Artocarpus altilis* (AA), *Gomphrena celosioides* (GC) and *Gynostemma pentaphyllum* (GP).

The glucose uptake capacity of the extracts

In order to investigate whether the extracts are able to stimulate glucose uptake, hepatic LO-2 cells were pre-treated with different extracts before incubated with glucose solution. The percentage of glucose uptake was indicated by measuring the rest of glucose level in the cell culture supernatant. The results showed that PaA and AA extracts possessed the highest stimulation of glucose uptake as compared to the control group. The glucose uptake levels of PaA and AA were up to 143 ± 11.3% and 142 ± 9.1%, respectively, followed by GC - 128 ± 1.0%, FG - 115 ± 1.0% and GP - 115 ± 9.1%. Meanwhile, metformin-treated cells significantly increased glucose uptake up to 197 ± 11.3% at the concentration of 20 µg/ml.

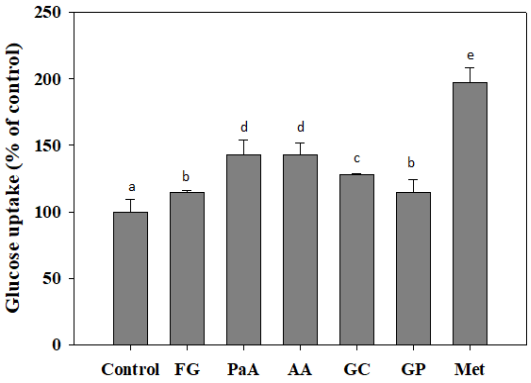

Figure 3. Glucose uptake capacity of the extracts on human hepatic LO-2 cells. Each determination was made in three independent experiments and the data are shown as means ± SD. Different letters a–e indicate significant difference among groups ($p < 0.05$). Control is the untreated cell group. *Ficus glomerata* (FG), *Pandanus amaryllifolia* (PaA), *Artocarpus altilis* (AA), *Gomphrena celosioides* (GC), and *Gynostemma pentaphyllum* (GP), and metformin (Met).

Free radical scavenging activity of the extracts

Antioxidant activity of the extracts was investigated by measuring DPPH and ABTS+ radical scavenging ability. In Figure 4A, high DPPH scavenging activity was exposed by AA (54.5 ± 2.5)% and FG (41.2 ± 2.2)%, followed by GP (37.5 ± 4.8)%, PaA (34.9 ± 3.5)%, and GC (30.6 ± 5.1)% at the concentration of 100 µg/ml. Likewise, these extracts also exhibited ABTS+ scavenging activity up to 52.4 ± 4.6% for FG, 51.1 ± 5.2% for AA, 36.6 ± 4.7% for GC, 36.3 ± 2.9% for PaA, and 31.8 ± 3.5% for GP (Figure 4B). Besides, vitamin C was indicated as a powerful scavenger of DPPH (81.8 ± 4.3%) and ABTS+ (98 ± 3.8%) radicals.

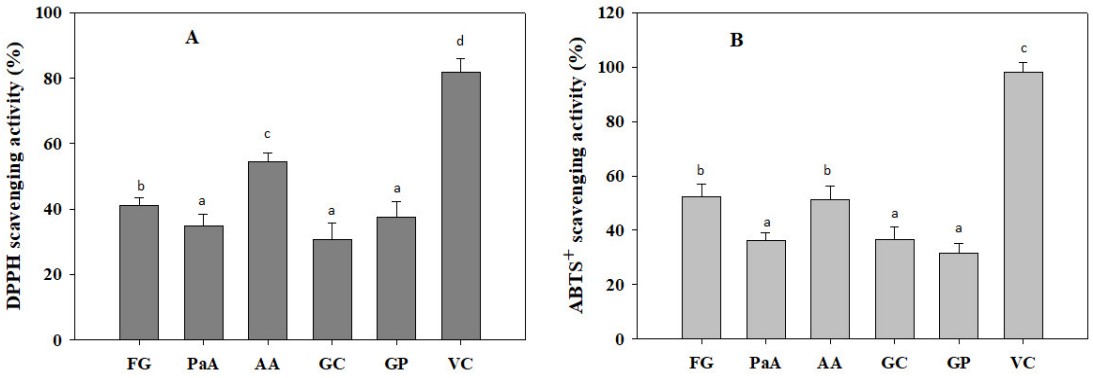

Figure 4. DPPH (A) and $ABTS^{+}$ (B) scavenging activity of the extracts.
Each determination was made in three independent experiments and the data are shown as means ± SD.
Different letters a–d indicate significant difference among groups ($p < 0.05$).
Ficus glomerata (FG), *Pandanus amaryllifolia* (PaA), *Artocarpus altilis* (AA),
Gomphrena celosioides (GC), and *Gynostemma pentaphyllum* (GP), and Vitamin C (VC).

Effect of the extracts on cell viability

The effect of FG, PaA, AA, GC, and GP extracts on cell viability of human hepatic LO-2 cells and human gastric BGC-823 cells was investigated *in vitro* (Figure 5). The cell viability was shown in a range of 86 – 99% for LO-2 cells and 83 – 95% for BGC-823 cells as compared with the blank group (Absence of extract). This indicates that FG, PaA, AA, GC, and GP extracts did not cause any cytotoxic effect on human hepatic cells and human gastric cells at the concentration of 100 µg/ml.

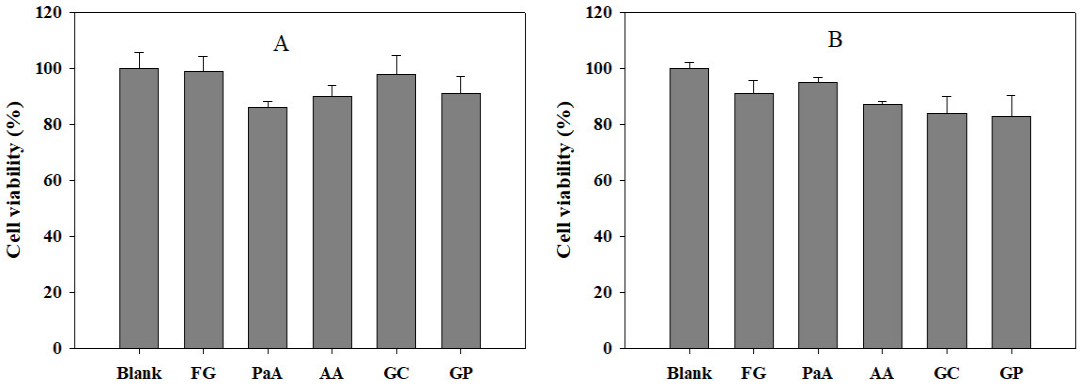

Figure 5. The effect of the extracts on cell viability of human hepatic LO-2 cells (A) and human gastric BGC-823 cells (B) using MTT assay. The cells were treated with the extracts for 24 h before adding MTT reagent. The results were expressed as compared with blank group.
Each determination was made in three independent experiments and the data are shown as means ± SD.

DISCUSSION

Alpha-amylase plays an important role in hydrolysis of carbohydrates to small fragments of sugars in intestinal mucosa. Normally, the excess level of sugar will be converted to energy sources such as glycogen. In diabetic patients, the excess activity of alpha-amylase enzyme results in hyperglycaemia due to insulin deficiency or insulin resistance (29). In this sense, the inhibition of alpha-amylase activity can reduce postprandial hyperglycemia and prevent the risk of diabetes development (30, 31). In this study, the extracts of AA, PaA, GC, GP and FG were found to possess inhibitory activity against alpha-amylase. Notably, the inhibitory activity of AA and PaA extracts on alpha-amylase was more effective than that of *Momordica charantia* (IC_{50} = 0.267 ± 0.024 mg/ml) (32). Currently, acarbose, miglito, and voglibose are used as common anti-diabetic drugs for down-regulation of carbohydrate digestion enzymes such as alpha-amylase, sucrose,

maltase, and alpha-glucosidase (33). It was evidenced that mice treated with acarbose could slow the breakdown of sucrose and starch (34, 35). Hence, the inhibitory activity of these extracts, especially AA and PaA, on alpha-amylase may contribute to the diminution of hyperglycaemia in type 2 diabetes patients.

Indeed, the hypoglycemic effect of medicinal plants not only associates to the inhibitory activity of carbohydrate digestion enzymes, but also relates to their adsorption capacity of glucose. Notably, various hypoglycemic medicinal plants such as ginseng, bitter melon, fenugreek, banaba, *Gymnema sylvestre* and *Coptis chinensis* have been known due to their glucose adsorption capacity (36). Herein, FG, PaA, AA, GC, and GP extracts were also evidenced as potential adsorptive agents of glucose. The glucose adsorption capacity of these extracts may be attributed by their phytoconstituents such as insoluble and soluble constituents and fibers (37). As the result, these extracts, especially GC extract, were suggested to be able to decrease transport across of glucose into intestinal lumen and reduce the postprandial hyperglycemia.

It was reported that insulin resistance or insulin deficiency causes decrease in glucose uptake and increase in endogenic hepatic glucose production in diabetic patients (38). Thus, bioactive agents possessing glucose uptake capacity play an important role in hypoglycemia via stimulating glucose uptake into skeletal muscle, adipose tissue and liver (39). Notably, FG, PaA, AA, GC and GP extracts were determined to stimulate glucose uptake into hepatic LO-2 cells significantly. The glucose uptake capacity of these extracts, especially PaA and AA, may be associated to the recruitment of glucose transporters from intracellular pool to plasma membrane of the cells, thus stimulating glucose uptake in its target tissues and attenuating the hyperglycemia in type 2 diabetes (40).

Free radicals cause oxidation of cell components and molecules such as lipids, proteins, and DNA (41). Notably, free radical activity has been previously implicated in the development of diabetic vascular complications in diabetes mellitus (42). Long term complication of diabetes mellitus is associated with various oxidative reactions, free radical generation and oxidative stress (43). Therefore, antioxidants play a central role in neutralization of free radicals and prevention of the pathogenesis as well as complications of diabetes mellitus (44). Interestingly, antioxidant activity of FG, PaA, AA, GC and GP extracts was found due to scavenging DPPH and $ABTS^+$ radicals. It was evidenced that the transgenic antioxidant enzyme expression or antioxidant compounds have the capacity to prevent the development of experimental diabetic retinopathy, nephropathy, neuropathy and cardiomyopathy (42). Zatalia et al. (44) recently listed the beneficial effects of antioxidant agents for the treatment of diabetes and its complications in animals and humans. These experimental and human studies have suggested nutritional values in the prevention of diabetic complications. Thus, the potential antioxidant property of these extracts, especially FG and AA, may contribute to delay the development of free radicals-related diabetes complications.

Obviously, medicinal plants have long been used for maintaining human health and continued to be a valuable source of pharmaceuticals up to now (45). Besides great significance in therapeutic treatments, they also possess cytotoxic potential due to producing various cytotoxic substances for defence purposes (46). Thus, an assessment of their cytotoxic potential is necessary to ensure relatively safe use of medicinal plants. In the present study, MTT assay has revealed that the ethanol extract of these medicinal plants did not cause any cytotoxicity on hepatic LO-2 cells and gastric BGC-823 cells at the tested concentrations (100 µg/ml). Moreover, the results indicate that BGC-283 cell viability was lower after AA, GC and GP treatment as compared to the blank. According Jamil et al. (47), AA extract reduced cell viability of HeLa cervical cancer cells up to 44% at 100 µg/ml. Similarly, GP extract lowered cell viability of A549 human lung epithelial cells up to 21% at 100 µg/ml (48). Therefore, a further study is needed due to cytotoxic effect of these extracts using an *in vivo* model to achieve the adequate knowledge regarding safe use of these medicinal plants.

CONCLUSIONS

Herbal medicines have traditionally been used for the prevention and treatment of various diseases up to now. The pharmacological researches using *in vitro* as well as *in vivo* models have evidenced numerous health-beneficial effects of herbal medicines. In this study, different medicinal plants including *F. glomerata, P. amaryllifolia, A. altilis*, *G. celosioides and G. pentaphyllum* have been analysed as a promising hypoglycemic agents due to inhibiting starch digestive enzyme, possessing glucose adsorption and glucose uptake capacity and scavenging free radicals. Especially, *A. altilis* was observed to be effective in all assays, indicating its promising therapeutic role in the management of hyperglycaemia in type 2 diabetes. However, further studies related to safety and efficacy of *A. altilis* also need to be evaluated for future development of health-beneficial products.

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ETHIC APPROVAL

Non applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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EXPRESSION OF CYTOKINES AND CYTOKINE RECEPTORS-GENES IN PATIENTS WITH DIFFERENT FORMS OF THYROID PATHOLOGY IN UKRAINIAN POPULATION

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ABSTRACT

Multiple susceptibility genes can be involved in the development of Hashimoto's thyroiditis. Some of these genes are implicated in other autoimmune diseases, while others are specific to thyroid autoimmune response. 153 patients with thyroid pathology were enrolled in the study (152 women and 1 man, the average age was 46,02±14,3). They were divided into 3 groups: 16 patients with postoperative hypothyroidism; 65 patients with hypothyroidism resulting from autoimmune thyroiditis, and 72 patients with both AIT and elevated serum an anti-thyroglobulin and anti-thyroid peroxidase antibodies. We used a pathway-specific real-time Polymerase chain reaction array to identify and verify cytokines and receptor pathway-associated gene expression in peripheral white blood cells in randomly selected 12 individuals from each group. In the patients with postoperative hypothyroidism and those with hypothyroidism resulting from autoimmune thyroiditis, the expression of Chemokine (C-X3-C motif) receptor 1, Chemokine (C-X-C motif) receptor 4, Interleukin 6, and Interleukin 6 receptor significantly decreased, while the expression of IL6ST and IL10RA increased. In contrast, mRNA levels of Chemokine (C-X3-C motif) receptor 1, Chemokine (C-X-C motif) receptor 4, Interleukin 6, and Interleukin 6 receptor increased in the autoimmune thyroiditis patients with elevated serum anti-thyroglobulin and anti-thyroid peroxidase antibodies, while the expression of Interleukin 6 signal transducer and Interleukin 10 receptor, alpha decreased in this group of patients. The patients with hypothyroidism resulting from autoimmune thyroiditis and patients with elevated serum anti-thyroglobulin and anti-thyroid peroxidase antibodies had significantly lowered expression of Interleukin 10, while the expression of Interleukin 1, beta and Interleukin 1 receptor, type I was elevated. autoimmune thyroiditis and hypothyroidism affect the mRNA-level expression of cytokines and cytokine receptor genes in a gene-specific manner, and these changes to gene expression can be among the triggers of autoimmune inflammation progression in the thyroid gland. Transcriptional activity of cytokines, inducer, and receptor genes in the peripheral white blood cells can be used as an important minimally invasive prognostic marker of the autoimmune thyroid disease severity.

Keywords: *Autoimmune thyroiditis, cytokines, hypothyroidism, mRNA, receptors.*

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INTRODUCTION

Hashimoto's thyroiditis (HT) is among most common autoimmune thyroid diseases (AITDs). It is a T cell-mediated organ-specific autoimmune disorder that results in thyroid damage and subsequent clinical hypothyroidism. The disorder is mediated by infiltrating and/or locally activated thyroglobulin (Tg)-specific T cells. Surveys indicate that HT is the leading cause of hypothyroidism in iodine-sufficient areas of the world (1).

A significant factor involved in the induction of autoimmune response is a defect in or deficiency of the immune regulation, particularly disequilibrium between the effector T cells (Teff) and regulatory T cells (Treg) which normally prevent the development of autoimmunity (2,3). Multiple susceptibility genes may be involved in the disease development, some of which are also implicated in other autoimmune diseases (4,5), while others are specific to thyroid autoimmunity (6,7). However, data about the expression of cytokines and cytokine receptor pathway-focused genes in patients with different forms of thyroid pathology from Europe are limited, and no such data from Ukraine has been internationally published.

Cytokines can influence the autoimmune process through a number of mechanisms, including the recruitment of inflammatory cells and the activation of molecules required to maintain an inflammatory response in the affected area. Possible direct exposure to cytokines on thyroid function by affecting TFC distribution and modulation of expression and function molecules involved in the synthesis of thyroid hormones (2). Earlier, when studying the type of cytokine response in ATD, it was revealed that in HT, the Th1 response predominates (2).

Cytokines, their receptors, and the signaling pathways they utilize are potentially attractive therapeutic targets in AITDs (8). For instance, T helper (Th1) cytokine is frequently prevalent in HT, as well as in experimental autoimmune thyroiditis as a result of the infiltration of T cells and macrophages into thyroid tissue (9). Furthermore, the thyroid follicular cells can themselves produce several types of cytokines (10).

The aim of the study was to detect changes in the expression of the genes involved in the production of cytokines and their receptors in patients with different forms of thyroid pathology.

METHODS

153 patients with thyroid pathology were enrolled in the study. They were divided into 3 groups: Group 1 included 16 patients with postoperative hypothyroidism; Group 2 included 65 patients with hypothyroidism resulting from autoimmune thyroiditis (AIT), and Group 3 included 72 patients with both AIT and elevated serum an anti-thyroglobulin (anti-Tg) and anti-thyroid peroxidase (anti-TPO) antibodies. The control group included 25 healthy individuals, which were recruited randomly, without matching for age or sex. Clinical characteristics of the subjects are shown in table 1.

Hypothyroidism was diagnosed following the recommendations of the American Association of Clinical Endocrinologists, 2012. The diagnosis of AIT was based on detected circulating antibodies to thyroid antigens (Anti-TPO and anti-TG) and reduced echogenicity on the thyroid sonogram in a patient with relevant clinical features (11).

Blood specimens were collected between 8 and 10 AM after an overnight fast. Free thyroxin (fT4, normal range 6.0-13.0 pmol/L for males and 7.0-13.5 pmol/L for females), thyroid-stimulating hormone (TSH, normal range 0.3-4.0 mIU/mL), anti-thyroid peroxidase (anti-TPO, normal range 0-30 IU/mL) and anti-thyroglobulin (anti-TG, normal range 0-65 IU/mL) antibodies levels were determined in every individual using STAT FAX303/Plus analyzer (Awareness Technology Inc, USA).

Patients under the age of 18 or those suffering from malignancy, inflammation associated with rheumatic diseases or acute/chronic infection, diabetes mellitus, cardiovascular or cerebrovascular diseases, chronic hepatic or renal diseases, as well as pregnant women and those taking any drugs that could interfere with thyroid function, were excluded from the study.

We used a pathway-specific PCR array (Neurotrophins and Receptors RT^2 Profiler PCR Array, QIAGEN, Germany) to identify and verify cytokines and receptor pathway-associated gene expression in randomly selected 12 individuals from each group using a real-time PCR following the procedure described below.

Experimental procedures. RNA isolation. Total RNA was isolated from white blood cells using NucleoZOL kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. NucleoZOL is intended for the isolation of total RNA (small and large RNA) in single or separate fractions. White blood cells were lysed and homogenized in NucleoZOL guanidinium thiocyanate and phenol based reagent.

cDNA synthesis. The RNA quality was determined and it was reverse transcribed. The concentration and quality of the isolated total RNA were determined on a NanoDrop spectrophotometer (Thermo Scientific™, USA). For the reverse transcription procedure with a cDNA conversion RT² First Strand Kit (QIAGEN, Germany, Cat. no. 330401), RNA samples with the following parameters were selected: ratio A260/A280 within the range of 1.8-2.2.

The RT^2 HT First Strand Kit procedure comprises 2 steps: elimination of genomic DNA contamination, and reverse transcription with an RT master mix, as well as random

hexamers and oligo-dT prime reverse transcription to capture more difficult-to-detect genes.

PCR Array. The cDNA was then used with RTI Profiler PCR Array (QIAGEN, Cat. no. PAHS-031Z) in combination with RTI SYBR® Green qPCR Mastermix (QIAGEN, Cat. no. 330504), following the complete RT^2 Profiler PCR Array procedure (www.qiagen.com). Samples were assigned to control and study groups. CT (cycle threshold) values were normalized based on the automatic selection from the full panel of reference genes.

Any CT value >35 was considered to be a negative call. The RT^2 Profiler PCR Array data analysis software calculates the fold change using the ΔΔCT method. The formula first calculates delta CT between the gene of interest (GOI) and an average of reference genes (HKG). In the second step, delta-delta CT is calculated as (delta CT (Test Group)-delta CT (Control Group)). Fold change is then calculated as 2^(-ΔΔCT). The data analysis report was exported from the QIAGEN web portal at GeneGlobe. The software allows defining the best reference genes for normalization.

A list of cytokines and receptor pathway-associated genes selected for this study is given in table 2.

Ethics

The ethical principles contained in the Declaration of Human Rights adopted in Helsinki, in 1975, and revised in 2008, were fully respected in our study. The enrolled subjects, participated in this study voluntarily, and completed and signed a written informed consent. The protocol of the study was approved by the local ethics committees of HSEEU "Bukovinian State Medical University", Chernivtsi Regional Endocrinology Center, and I. Horbachevsky Ternopil National Medical University.

Statistical analysis of PCR array data

The RT2 Profiler PCR Array Data Analysis software calculates p-values using a Student's t-test (two-tail distribution and equal variances between the two samples) based on the triplicate 2^(-ΔΔCT) values for each gene in the experimental group compared to the control group. Published results from the Microarray Quality Control (MAQC) confirm that such ranked lists of genes based on fold-change and associated p-value is sufficient to demonstrate reproducible results using the RT^2 Profiler PCR Arrays.

RESULTS

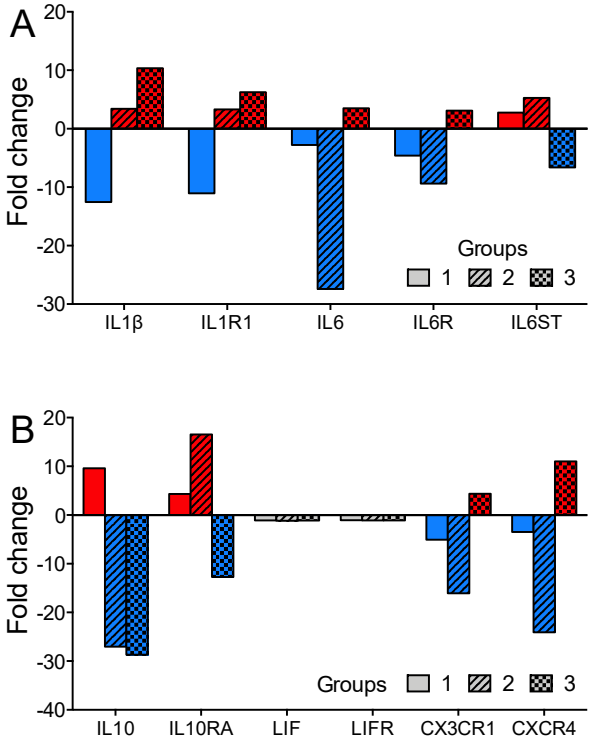
Using the Pathway-Focused PCR Array Profiling (Neurotrophins and Receptors RT2 Profiler PCR Array) we examined the Cytokines and receptor pathway-associated gene expression of patients with primary hypothyroidism as a result of AIT and postoperative hypothyroidism and patients with AIT with rising serum autoantibodies, such as anti-Tg and anti-TPO.

As it is shown in Table 1, there was a probable decrease in fT4 levels in the groups of patients with postoperative hypothyroidism and primary hypothyroidism as a result of AIT in 2.6 and 2.16 times, respectively, compared with the Control group. At the same time, TSH levels were significantly higher in these groups by 3.2 and 2.65 times, respectively. According to our studies, in patients with primary hypothyroidism as a result of AIT, the level of anti-TPO was 11.2 times higher, and the level of anti-TG in this group was 2.16 times higher than in the Control group. Patients in Group 3 had a 9.7-fold increase in anti-TPO and 2.4-fold anti-TG levels compared with the Control group.

The results from RT2 Profiler Neurotrophins & receptors pathway-focused genes expression analysis is given in table 3.

Genes expression analysis showed that in the study Group 1, which included the patients with postoperative hypothyroidism, the expression of IL10 and IL10RA increased 9.6 and 4.3-fold respectively (Figure 1B), while expression of the following genes decreased: CX3CR1 (5.04-fold), CXCR4 (3.5-fold) (Figure 1B), IL1β (12.5-fold), and IL1R1 (11.1-fold) (Figure 1A). Declines in IL6 (2.8-fold) and IL6R (4.6-fold) mRNA levels were also detected in Group 1 (Figure 1A).

Figure 1. Gene expression profiles for cytokines and receptors with systemic pro-inflammatory effects (A) and suppressors of pro-inflammatory signals and those with multidirectional effects on the inflammatory process (B). Fill pattern represents patients of different groups.



Red color shows upregulation, blue – downregulation and grey - no changes.

As it is shown in Table 3, in the patients with hypothyroidism resulting from AIT (Group 2), the expression of the following cytokines and receptor pathway genes increased: IL10RA (16.5-fold), IL1β (3.4-fold), IL1R1 (3.3-fold), and IL6ST (5.3-fold). The expressions of CX3CR1 (16.1-fold), CXCR4 (24.1-fold), IL10 (27.03-fold) (Figure 1B), IL6 (27.4-fold), and IL6R (9.4-fold) (Figure 1A) decreased.

In Group 3, which includes patients with AIT and elevated serum anti-Tg and anti-TPO auto-antibodies, IL10 and IL10RA were down-regulated, 28.6 and 12.7 times respectively (Figure1B). The mRNA levels of IL6 (3.5-fold), and IL6R (3.1fold) significantly increased (Figure1B). IL6ST mRNA levels in the Group 3 were reduced 6.6-fold, while the expression of IL1β (10.4-fold) and IL1R1 (6.2-fold) significantly increased (Table 3). Expression of LIF and LIFR was not different in all groups of patients (Figure 1B).

All the effects are summarized in figure 2. Notably, Group 1 patients solely have up-regulated genes for IL10 and down-regulated for IL1β and its receptor 1L1R1 (Figure 2A).

Figure 2A. Gene expression profiles for cytokines and receptors with systemic pro-inflammatory effects (IL1β, IL1R1, IL6, IL6R, IL6ST); suppressors of pro-inflammatory signals (IL10, IL10RA, LIF, LIFR) and those with multidirectional effects on the inflammatory process (CX3CR1 and CXCR4) in patients with postoperative hypothyroidism.

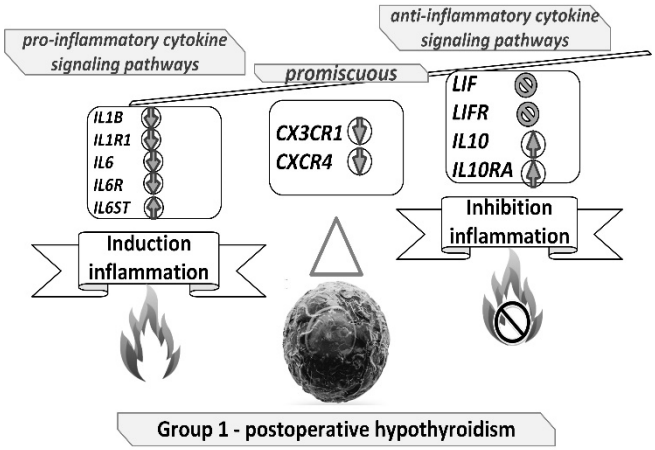


Figure 2B. Gene expression profiles for cytokines and receptors with systemic pro-inflammatory effects (IL1β, IL1R1, IL6, IL6R, IL6ST); suppressors of pro-inflammatory signals (IL10, IL10RA, LIF, LIFR) and those with multidirectional effects on the inflammatory process (CX3CR1 and CXCR4) in patients with hypothyroidism resulting from autoimmune thyroiditis

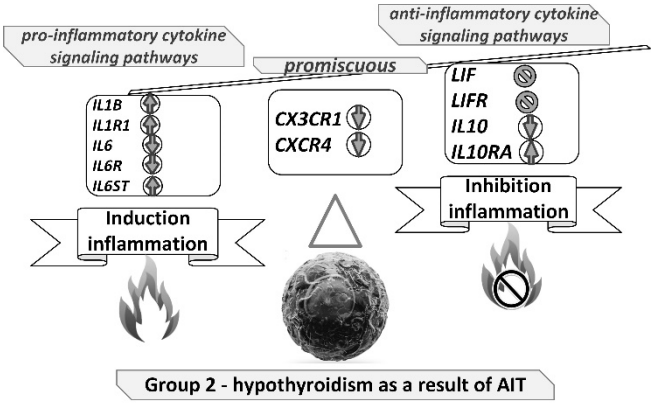
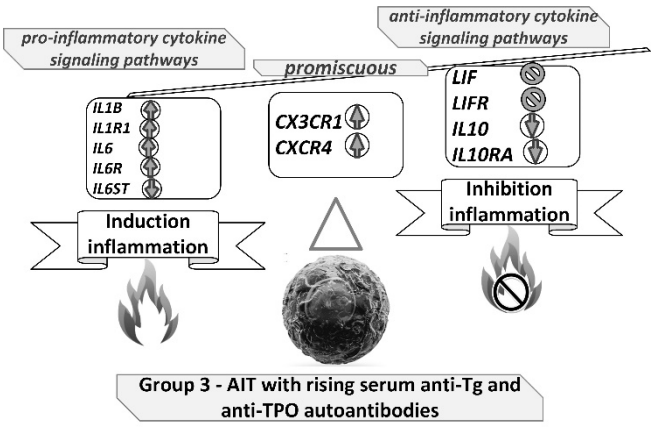


Figure 2C. Gene expression profiles for cytokines and receptors with systemic pro-inflammatory effects (IL1β, IL1R1, IL6, IL6R, IL6ST); suppressors of pro-inflammatory signals (IL10, IL10RA, LIF, LIFR) and those with
multidirectional effects on the inflammatory process (CX3CR1 and CXCR4) in patients with AIT and elevated serum anti-Tg and anti-TPO antibodies.



Patients of the Group 3, unlike the others, have up-regulated genes of IL6 and IL6R, while down-regulated IL6ST and IL10RA (Figure 2C). Interestingly, patients of Group 3 have some expression changes similar to those of Group 2 (Figure 2B), but also some unique to Group 3 (Figure 2C).

Table 1. Clinical characteristics of the subjects

	Referent values	Control group (n=25)	Patients with postoperative hypothyroidism (Group 1) (n=16)	Patients with hypothyroidism as a result of AIT (Group 2) (n=65)	Patients with AIT with rising serum anti-Tg and anti-TPO autoantibodies (Group 3) (n=72)
Age (years)		46.1 ± 14.6	47.3 ± 12.3 (p>0.05)	46.7 ± 15.49 (p>0.05)	45.0 ± 13.7 (p>0.05)
fT4 (pmol/L)	7.0-13.5	8.91 ± 0.97	3.44 ± 0.31 (p<0.001)	4.13 ± 0.52 (p<0.001)	8.51 ± 0.82 (p>0.05)
TSH (mIU/mL)	0.3-4.0	2.67 ± 0.52	8.61 ± 0.84 (p<0.001)	7.09 ± 0.50 (p<0.001)	2.38 ± 0.62 (p>0.05)
anti-TPO (IU/mL)	0-30	34.0 ± 3.70	36.1 ± 2.78 (p>0.05)	380 ± 73.4 (p<0.001)	330 ± 50.2 (p<0.001)
anti-TG (IU/mL)	0-65	15.3 ± 1.97	15.5 ± 1.90 (p>0.05)	33.0 ± 4.27 (p<0.001)	36.4 ± 7.70 (p<0.001)
Current dose of L-thyroxine (μg/day)		None	111 ± 5.25	88.5 ± 1.55	None

Data are expressed as mean ± standard deviation
The p values are calculated based on a Student's t-test in the control group and study groups

Table 2. Cytokines and receptor pathways-associated genes

UNIGENE	REFSEQ	SYMBOL	DESCRIPTION
Hs.78913	NM_001337	CX3CR1	Chemokine (C-X3-C motif) receptor 1
Hs.593413	NM_003467	CXCR4	Chemokine (C-X-C motif) receptor 4
Hs.193717	NM_000572	IL10	Interleukin 10
Hs.504035	NM_001558	IL10RA	Interleukin 10 receptor, alpha
Hs.126256	NM_000576	IL1β	Interleukin 1, beta
Hs.701982	NM_000877	IL1R1	Interleukin 1 receptor, type I
Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
Hs.135087	NM_000565	IL6R	Interleukin 6 receptor
Hs.532082	NM_002184	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
Hs.2250	NM_002309	LIF	Leukemia inhibitory factor (cholinergic differentiation factor)
Hs.133421	NM_002310	LIFR	Leukemia inhibitory factor receptor alpha

Table 3. Differential expression of mRNA cytokines and receptors pathway-focused genes in patients with different thyroid pathology

GENE SYMBOL	UP-DOWN REGULATION (COMPARING TO THE CONTROL GROUP)		
	Patients with postoperative hypothyroidism (Group 1)	Patients with hypothyroidism as a result of AIT (Group 2)	Patients with AIT and rising serum anti-Tg and anti-TPO autoantibodies (Group 3)
	Fold change	Fold change	Fold change
CX3CR1	-5.04 (p=0.003)	-16.1 (p=0.0009)	4.36 (p=0.09)
CXCR4	-3.5 (p=0.001)	-24.1 (p=0.001)	10.9 (p=0.04)
IL10	9.6 (p=0.01)	-27.03 (p=0.000001)	-28.6 (p=0.00004)
IL10RA	4.3 (p=0.04)	16.5 (p=0.04)	-12.7 (p=0.0003)
IL1β	-12.5 (p=0.001)	3.4 (p=0.006)	10.4 (p=0.04)
IL1R1	-11.1 (p=0.009)	3.3 (p=0.01)	6.2 (p=0.04)
IL6	-2.8 (p=0.01)	-27.4 (p=0.001)	3.5 (p=0.03)
IL6R	-4.6 (p=0.005)	-9.4 (p=0.0006)	3.1 (p=0.05)
IL6ST	2.7 (p=0.01)	5.3 (p=0.06)	-6.6 (p=0.003)
LIF	-1.1 (p=0.37)	-1.17 (p=0.15)	-1.14 (p=0.15)
LIFR	-1.06 (p=0.76)	-1.1 (p=0.54)	-1.08 (p=0.45)

The p values are calculated based on a Student's t-test of the replicate 2^(- Delta CT) values for each gene in the control group and study groups

DISCUSSION

The cytokines and receptor pathway genes involved in this study can be assigned into 3 functional groups: 1) those with predominantly systemic pro-inflammatory effects (inflammation inducers and regulators of its intensity): IL1β, IL1R1, IL6, IL6R, IL6ST; 2) suppressors of pro-inflammatory signals (inflammation repressors): IL10, IL10RA, LIF, LIFR; and 3) those exerting multidirectional effects on the inflammatory process, depending on the type of cell and tissue in which their expression is studied: CX3CR1 and CXCR4 (Figure 2A, B,C).

For a number of autoimmune and inflammatory diseases it was demonstrated that a key prognostic indicator of their course was the extent of changes to the transcriptional activity in the immune system cells (12, 13). An important task is to find new drugs that can reduce the risk of neurological complications in patients with thyroid disease (14). An important role in AITDs is played by chemokines; for instance, studies showed overexpression of CC and CXC chemokine in both HT and experimental autoimmune thyroiditis (9).

CX3CR1 is a seven-transmembrane G-protein coupled receptor. It has a sole ligand, CX3CL1, also known as fractalkine or neurotactin (15). In the immune system cells, CX3CR1 helps to recognize the CX3CL1 gradient (chemotaxis), which attracts the cells to the inflamed tissue and elicits the innate immune response (16,17). In this study, the expression of CX3CR1 was reduced in the patients with hypothyroidism resulting from AIT as well as with postoperative hypothyroidism. On the other hand, CX3CR1 was significantly up-regulated in the group of patients with elevated serum autoantibodies.

CXCL12 is constitutively expressed by various cells and tissues. Interacting with CXC chemokine receptor 4, CXCL12 can elicit several responses, such as migration of inflammatory cells across the endothelium and leukocyte mobilization (18,19). The principal source of CXCL12 in the thyroid are thyrocytes; this is in contrast to its receptor CXCR4, which is mainly expressed by T and B cells (18).

Similarly, thyroid epithelial cells produce cytokines CCL2, CXCL9, and CXCL10. Various factors can up-regulate production of these cytokines in the thyrocytes. For instance, iodine-induced thyrocyte necrosis stimulates production of IL1 and TNFα by the resident macrophages. This prompts synthesis of CXCL12 by the nearby thyrocytes (20). In mice with autoimmune thyroid diseases, the expression of both CXCL12 and CXCR4 is elevated. In a study of HT patients Armengol et al. showed up-regulated CXCL12 mRNA expression and elevated protein levels in the thyroid glands compared to non-autoimmune thyroid glands; the predominant source of CXCL12 in AIT was thyrocytes (20). In this study we found that expression of CXCR4 was reduced in the patients with hypothyroidism resulting from AIT as well as postoperative hypothyroidism. In contrast, CXCR4 was up-regulated in the group of patients with elevated serum autoantibodies. These results suggest that a high level of serum autoantibodies, such as anti-Tg and anti-TPO, can be linked to up-regulation of CX3CR1 and CXCR4.

Thyroid cells synthesize several cytokines, including IL-1, IL-6, IL-8, IL-12, IL-13, and IL-15 (21). The IL-1 family consists of IL-1α, IL-1β, and two receptors, IL-1 receptor 1 (IL1R1) and IL-1 receptor 2 (IL1R2); these receptors are found to be associated with thyroid carcinogenesis (22). IL-1β is found to be produced in the thyroid gland of HT patients by infiltrating monocytes and macrophages, activated endothelium, fibroblasts, and thyrocytes. In normal thyrocytes, it can induce Fas expression, resulting in massive thyrocyte apoptosis and tissue destruction (23). In this study, IL-1β was significantly down-regulated in the patients with postoperative hypothyroidism. In contrast, IL-1β was up-regulated in the patients with elevated serum autoantibodies anti-Tg and anti-TPO and the patients with hypothyroidism resulting from AIT. This corresponds to the findings of higher IL-1β mRNA expression in the peripheral blood mononuclear cells (PBMCs) of HT patients compared to Graves' disease (GD) patients, while the normal controls had the lowest level of IL1β expression (25). Similar trend was also detected in the thyroid tissues of the same groups of patients and normal controls. These results suggest IL-1β involvement in pathogenesis of AITDs and its utility as a biological marker to distinguish HT from any other AITDs (24). We found that expression of IL1R1 increased in the patients with elevated serum anti-Tg and anti-TPO antibodies and the patients with hypothyroidism resulting from AIT. In contrast, IL1R1 was down-regulated in the group of patients with postoperative hypothyroidism.

IL-10 is a Treg cytokine, crucial for maintaining and controlling inflammation. This cytokine is also shown to increase antibody production, in particular of anti-thyroid peroxidase antibodies (TPOAbs) that play an important role in the development of HT (25). Moreover, the complex interactions within the cytokine network can switch it from producing typical anti-inflammatory responses to pro-inflammatory ones. This complex cytokine interaction, resulting in the addition of IL-10 pathogenic effect to B-cell autoantibody production, could be mediating the IL-10 role in HT susceptibility (26). An IL10 polymorphism IL10−592A/C (27) and let-7e, a miRNA that regulates sIL10 expression (28), are both associated with HD severity. We found that patients with postoperative hypothyroidism had significantly lower expression of IL10 compared to the control group. On the other hand, high levels of serum anti-Tg and anti-TPO antibodies were associated with increased expression of IL10.

IL10RA (Interleukin-10 receptor alpha) is a subunit of IL-10 receptor heterotetramer which belongs to the family of IFNR-like receptors (28, 29). In humans, IL-10 and its receptors together contribute to controlling intestinal mucosal immune responses. Genome-wide association studies linked nucleotide sequence polymorphisms in the IL10 gene to the elevated risk of developing inflammatory bowel disease (IBD), as reported in (30). In this study, decrease in IL10RA mRNA was found in the patients with elevated serum autoantibodies anti-Tg and anti-TPO, while in the patients with postoperative hypothyroidism and with hypothyroidism resulting from AIT, the expression of IL10RA increased.

A major mediator of the host response to injury and infection, IL-6 is a key interleukin contributing to inflammation, inflammation-associated cancers, and autoimmune processes (31). Graves' disease, an autoimmune thyroid disorder, is characterized by elevated IL-6 levels (32, 33). IL-6 is involved in regulation of thyroid cells growth and differentiation, while its expression in these cells regulates infiltration of the lymphocytes (32). In this study, we found reduced IL-6 expression in the patients with hypothyroidism resulting from AIT as well as with postoperative hypothyroidism. In contrast, IL-6 was significantly up-regulated in the group of patients with elevated serum autoantibodies. These results suggest that the high level of serum autoantibodies, including anti-Tg and anti-TPO, can up-regulate IL-6. Previous studies support the correlation between increased IL-6 levels and Hashimoto's thyroiditis (33, 34). For instance, El-Shenawy et al. found high IL-6 levels in different groups of individuals with HT (33). IL-6 has been proposed as the immune system mediator in the pathogenesis of HT disease, and the association between the levels of IL-6 and inflammation severity in HT points out to a direction for further studies (33, 34).

An IL-6 receptor locus polymorphism (Asp358Ala) is correlated with changes in serum IL6R levels and to a lesser extent, with IL-6 levels. The presence of IL6R 358Ala allele increased the expression of soluble ILR isoform in functional studies, but reduced the levels of membrane-bound isoform in CD4 T cells and monocytes, reducing overall IL6R response (35). In our study, IL6R was down-regulated in the patients with hypothyroidism resulting from AIT and in patients with postoperative hypothyroidism. Conversely, in the group of patients with AIT and elevated serum autoantibodies, the expression of IL6R did not change. These

results suggest that hypothyroidism results in suppressed expression of IL6R.

IL-6 transducer (IL6ST or gp130), is the signal-transducing subunit of the IL-6 cytokine family receptors activated by the IL-6/IL6Rα complex. IL6R is composed of two different proteins: IL6Rα, or ligand-binding subunit, and IL6ST/gp130, or signal-transducing subunit. IL6ST/gp130 is expressed in almost all organs. IL6Rα can be released as a soluble receptor (sIL6Rα); binding IL-6 it then interacts with a gp130 located on the surface of any cell. This trans-signaling process is likely the chief mechanism of IL-6 signaling in humans, because the sIL6R/IL-6 complex can exert an agonist effect. The soluble receptor form appears to be essential in the regulation of IL-6 action (36). Several studies linked increased sIL6R levels to different autoimmune diseases as well as their severity (36, 37). In this study, IL6ST expression increased in the patients with hypothyroidism resulting from AIT as well as with postoperative hypothyroidism. In contrast, IL6ST was significantly down-regulated in the group of patients with elevated serum anti-Tg and anti-TPO antibodies. These results suggest that a high level of serum autoantibodies will down-regulate the expression of IL6ST.

These results indicate that even in the patients which currently do not present clinical manifestations (those with AIT and elevated serum anti-Tg and anti-TPO autoantibodies), there is a significant transcriptional induction of pro-inflammatory cytokine genes and their receptors IL1β, IL1R1, IL6, IL6R against the background of suppressed expression of cytokine inhibitors of inflammation IL10 and IL10RA.

Leukemia inhibitory factor (LIF) belongs is a member of the IL-6 family with neuroprotective and anti-inflammatory properties (38). Pro-inflammatory cytokines IL-6 and tumor necrosis factor stimulate production of LIF. The beneficial role of LIF was demonstrated on both histological and functional models of different cell types and maturity. LIF receptor (LIFR) is activated after an injury, binding LIF, and is a type 1 cytokine receptor located in the nuclei of neuronal cells (39).

In this study, we did not detect changes in the transcriptional activity of LIF and LIFR genes in patients with primary hypothyroidism and AIT. However, while there are only a few studies on the association of HT with LIF and LIFR, some indications of TSH involvement in the mediation of LIF signaling exist. For instance, TSH up-regulated LIF expression in monkey thyroid tissue (40). A study of the LIF signaling pathway in the culture of endometrium tissue showed that in stromal cells TSH elevated LIF expression. Furthermore, TSH promotes the mRNA expression of LIFR (41). In an animal study, high levels of TSH affected the LIF/STAT3 pathway, resulting in poor fetus implantation outcomes (42).

CONCLUSION

This study demonstrated that autoimmune thyroiditis and hypothyroidism can affect the mRNA-level expression of cytokines and cytokine receptor genes in a gene-specific manner and that these changes to genes expression can be among the triggers of autoimmune inflammation progression in the thyroid gland. Transcriptional activity of cytokines, inducer, and receptor genes in the peripheral white blood cells can be used as minimally invasive prognostic marker of autoimmune thyroid disease and its severity.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study. The protocol of the study was approved by the local ethics committees of HSEEU “Bukovinian State Medical University”, Chernivtsi Regional Endocrinology Center, and I. Horbachevsky Ternopil National Medical University.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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EXPERIMENTAL SUBSTANTIATION OF AUTOPLASMA APPLICATION AS A HAEMOSTATIC AGENT IN ENDOSCOPIC OPERATIONS IN THE DIGESTIVE TRACT

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ABSTRACT

In endosurgery of the digestive tract, 'cellular technologies' are gaining popularity, including the use of blood components or blood cells with a haemostatic purpose. In this regard, the objective of the study is evaluation of the effectiveness of bleeding control and safety of resection of the hollow organs of the digestive tract in the experimental trauma models of the abdominal organs in laboratory animals. The study was conducted in 20 mature male Chinchilla rabbits with the mean body mass of 2450 ± 210 g. All animals were divided into four experimental groups: the control group (without bleeding control); the group that received the infiltration of the wall of a hollow organ with saline; the group in which bleeding was controlled by argon plasma coagulation (APC), and the group wherein animals underwent haemostasis with autoplasma. In the control group and the physiological saline group, no statistical difference was observed in the time of bleeding control. Autoplasma, due to preventive local administration, is more effective than APC and has a high haemostatic potential. Autoplasma has been found to be highly effective and safe for bleeding control in the gastrointestinal tract in the experimental model, which has paved the way for new possibilities for operations of various scales, including endoscopic operations.

Keywords: *Bleeding, hemostasis, autoplasma.*

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INTRODUCTION

Currently, most medical technologies in abdominal surgery involve removal of pathologically altered tissues. Any surgical intervention is associated with complications, among which the most frequent and threatening are perforation of the hollow organ and bleeding. At present, physical methods of haemostasis are widely used in endosurgery, creating comfortable conditions for performing the operation and obtaining, in general, positive results (1). However, it should be noted that 'cellular technologies' are gaining popularity in endosurgery of the digestive tract, including the use of blood components or blood cells with a haemostatic purpose. There are reports that the use of these blood components allows optimizing local haemostasis with the maximum efficiency, and it improves conditions for surgical dissection, preventing perforation of the hollow organs (2-4). P.S. Randelli et al note that the use of auto plasma during the arthroscopic treatment of injuries to the rotatory cuff of the shoulder can statistically reduce the intensity of pain in all patients (5). G. Filardo et al obtained a good clinical result when using autoplasma to treat athletes suffering from tendinitis of their own patellar ligament (6). However, available literature data on the efficacy and safety of the use of blood autologous components at local applications are contradictory. In this regard, the **objective of the study is** to evaluate the effectiveness of bleeding control and safety of resection of the hollow organs of the digestive tract in the experimental trauma models of the abdominal organs in laboratory animals.

MATERIALS AND METHODS

All experimental work was performed at the premises of the Oncology Research Institute of Bashkir State Medical University (BMSU) following the national recommendations of the 'Guidelines for the Preclinical Study of New Pharmacological Substances,' good laboratory practice (GLP) and international ethical standards and rules (7). The study was approved by the ethics committee of Bashkir State Medical University (protocol No.10 of February 11, 2018).

The study was conducted using 20 mature male Chinchilla rabbits with the mean body mass of 2450 ± 210 g. Animals were quarantined for 7 days in a separate box of vivarium of BMSU. The animals had a full-time free access to drinking bowls, received a set of natural products (vegetables and grain) and a standard diet, presented in the form of extruded pelleted feed for the housed laboratory animals.

All animals were divided into four experimental groups (five animals each): I-the control group that did not use any methods of bleeding control, II-the group that received infiltration of the hollow organ wall with physiological saline, III-the group that underwent physical haemostasis by the electrosurgical unit and argon plasma coagulation (APC) apparatus and IV-the group of animals that underwent local biologically controlled haemostasis using autoplasma. To receive autoplasma, the whole blood collection was performed from the rabbit's ear in the amount of 2 ml before laparotomy. For a stabilizer, sodium citrate was used in the ratio of 1:9 with blood. The blood was centrifuged at 1500 rpm for 5 min. The ready-to-use autoplasma was injected into a zone of the prospective resection of mucosa of the rabbit's digestive tract. The group of intact animals was included in the experiment to compare the condition and behaviour of these animals with experimental animals.

Under general anaesthesia (Zoletil 7 mg/kg, Virbac Sante Animale, France), after preparation of the surgical field (shaving and aseptic treatment), laparotomy and mobilization of the stomach, duodenum and colon were performed. The main studies in the stomach were carried out in a zone of the body and its antrum where anatomically good and intense blood circulation was observed. Next, the bleeding was caused by a scalpel transversely crossing the intestine. The time to bleeding control was recorded by a stopwatch and evaluated visually.

The results of the study were processed using the statistical package Statistica 10.0 (StatSoft Inc, USA). The test for normality of distribution of the actual data was performed using the Shapiro-Wilk test. It was determined that the type of distribution of the obtained data differs from the normal one; therefore, in further analysis, we used nonparametric methods. The data are presented as medians (Me), 25 and 75 percentiles. The analysis of variance was performed using the Kruskal-Wallis test. The significance level p for statistical criteria was taken equal to 0.05.

RESULTS

The results of evaluating the effectiveness of different methods of bleeding control in the experiment are presented in Table 1.

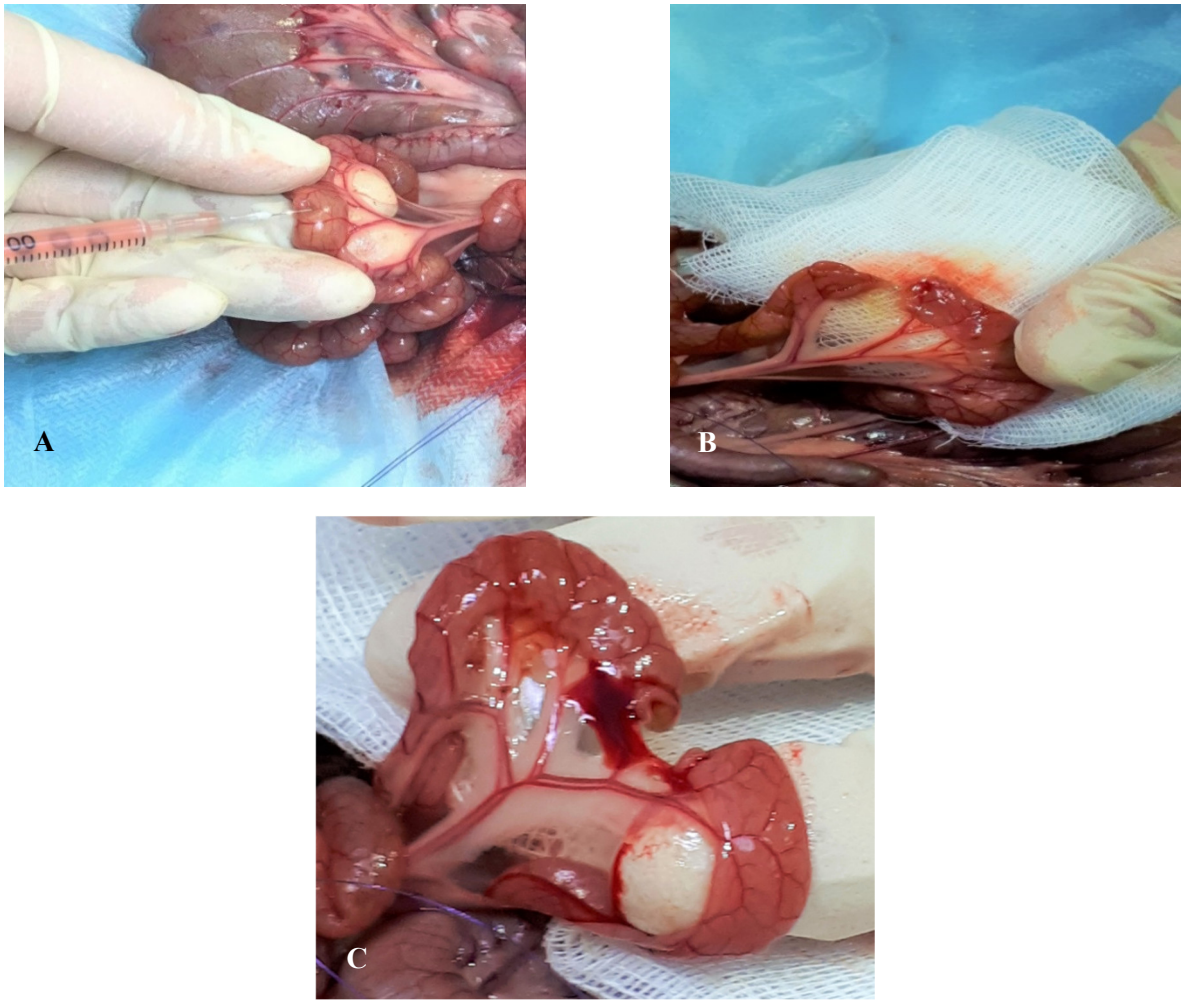
In the control group (I) and in the physiological saline group (II), there was no statistical difference in the time of bleeding control; however, in the group II, one more episode of bleeding was seen. The data presented in Table 1 demonstrate that autoplasma has a high haemostatic potential following the preventive local administration (group IV) (Figure 1).

Table 1. The main indicators of the results of bleeding control in the studied groups of laboratory animals, Me (0.25-0.75)

Group	I	II	III	IV
Time to bleeding control, s	147.2 (140.4-153.6)$^{\#, \dagger}$	130.1 (120.5-145.8)$^{\#, \dagger}$	89.6 (85.4-100.2)$^{*, \dagger}$	17.8 (15.4-20.7)$^{*, \#, \dagger}$
Recurrent bleeding, abs. (%)	2 (16.7) $^{\#, \dagger}$	1 (8.3)$^{*, \#, \dagger}$	0 (0.0)*	0 (0.0)*

Medians of the bleeding time for all injured organs (n = 12) are presented. The level of statistical significance differs between the animal groups: * $p < 0.05$-compared with the group I, # $p < 0.05$-compared with the group III, † $p < 0.05$-compared with the group IV.

Figure 1. Comparison of the intensity of bleeding when using autoplasma (A-administration of the drug, B-intestinal dissection) with the control (C).



At the same time, electrocoagulation is expectedly more effective than physiological saline, but haemostasis with APC is characterized by the rapid formation of a necrotic zone, which may have unfavourable consequences in the future.

DISCUSSION

Till date, physical methods (clips, acupressure, coagulation, etc.) and non-contact coagulation agents (APC and local haemostatic agents) have been used for bleeding control (8). All physical methods for bleeding control require adequate visualization of the source for accurate positioning of the endoscope to the source of bleeding, adequate pressure on the tissue and sufficient duration of exposure for complete coagulation. Simultaneously, prolonged coagulation increases the risk of deep tissue damage and perforation, whereas insufficient pressure or duration of exposure can aggravate bleeding (9). The use of clips can also be difficult when placed on bleeding vessels at the bottom of a large fibrous ulcerative defect due to deficiency of the surrounding tissues (10). The disadvantages of endoscopic suture devices are their lack of accessibility, the need for a two-channel endoscope, technical complexity and limited manoeuvrability, making it difficult to access certain areas of the stomach (11).

Non-contact haemostatic devices such as APC and local haemostatic agents do not require clear positioning of the bleeding source, which greatly simplifies their use (12). Complications from APC are rare and include gastric distension with gaseous argon, submucosal emphysema, pneumomediastinum, pneumoperitoneum and perforation (10). A number of authors attribute these complications to technical errors during the procedure-the probe's contact with the tissue, which causes the influx of argon gas into submucosa. This method is often ineffective-any fluid (for example, blood) between the probe tip and the bleeding tissue can cause formation of the coagulation film, which may interfere with the adequate surgical haemostasis (13).

The benefits of topical haemostatic agents include their ease of use and potential efficacy in bleeding control at various sites (14, 15). One of the most common and inexpensive measures are injections of diluted adrenaline. Adrenaline injections contribute to primary haemostasis, but this effect gets depleted, with the subsequent risk of rebleeding. This haemostasis can be used to monitor initially active bleeding and improve visualization; it should be combined with another method to reduce the risk of rebleeding. Complications of the injection therapy are usually associated with the effects of the drug injected (10). Among the most threatening complications are tissue necrosis, ulceration and perforation, as well as hypertension, and cardiac arrhythmia (13, 16). Today, in addition to the injection therapy, which should not be used as monotherapy, there are few convincing data that would definitely give preference to specific methods of haemostasis. However, there are sufficient numbers of experimental and fundamental studies offering various synthetic drugs as systemic or local injection drugs (17-19). While these agents for local and systemic haemostasis are at different stages of preclinical and clinical studies, the effective alternative is to use autologous coagulation factors to increase haemostatic potential. The results of our study have demonstrated high efficiency and safety of autoplasma in bleeding control in the gastrointestinal tract in the experimental animals.

From the point of view of prevention of complications associated with perforations, there are also approaches that have proven their effectiveness in a large number of patients (12). However, our endosurgical experience allows us to note that not all classical methods, including electrosurgical, allow achieving the stable haemostasis in the wound sites of the digestive tract, given their high vascularization (20). We have observed that the 'lifting effect' used for the convenience of dissection gets exhausted rapidly, and its protective effect disappears. This, in turn, increases the risk of iatrogeny and possible complications.

CONCLUSION

In conclusion, high efficiency of autoplasma has been established for the bleeding control in the gastrointestinal tract in the experiment, which paves the way for new possibilities for operations of various scales, including endoscopic operations. The potential application of this method of bleeding and perforation prevention in patients with underlying disorders of the haemostatic system aiming to increase locally the content of coagulation factors should be emphasised.

ETHICS APPROVAL

All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU) and approved by the Ethics Committee of Faculty of Medical Sciences, University of Kragujevac, Serbia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INFLUENCE OF "SNEZNIK-1/79" MINERAL WATER ON ANTHROPOMETRIC, FUNCTIONAL AND BIOCHEMICAL PARAMETERS OF PROFESSIONAL BASKETBALL PLAYERS: ROLE OF OXIDATIVE STRESS

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ABSTRACT

Adequate hydration represents the balance between the water intake and loss and has an unambiguous significance for public health and it is essential to sustain life. The changes in electrolyte balance which occur during and after training affect on athletes health and performance. Therefore, fluid replacement with adequate mineral composition is of utmost importance. The aim of the present study was to examine the influence of low mineral water from the well Sneznik-1/79 on anthropometric, functional, biochemical parameters and redox status of professional basketball players. In total, 17 male basketball players were included, during the pre-competitive mesocycle, and after the initial testing, they were randomly divided into two groups: group 1 - consumed the commercial drinking water for four weeks (n=7), and group 2 - consumed water from the well Sneznik-1/79 for four weeks (n=10). Determination of the anthropometric, functional, biochemical parameters and redox status was performed. Our results pointed out that consumption of mineral water from the well Sneznik is completely safe from the aspect of affecting various anthropometric, functional and biochemical parameters as well as systemic oxidative stress of professional athletes. In addition, existence of discretely better effects over commercial drinking water indicates that a long period of monitoring may certainly be of interest for further investigation.

Keywords: *Alkaline water, anthropometry, function, oxidative stress, Sneznik.*

INTRODUCTION

Adequate hydration represents the balance between the water intake and loss and has an unambiguous significance for public health and it is essential to sustain life. This balance has a very important role in general population, during the changed physiological conditions, disease, physical activities, in work environment (1).

During exercise, losing of the body water has a big impact on physiological and perceptual strain. This condition can impair endurance performance, especially in hot environments, leading to the increased exertional heat illness risk, whereby the fluid loss up to 4% of the body mass does not compromise performance (2). Consuming fluid during the exercise has a very important role in decreasing the perceived exertion and thirst sensation as well as reducing the cardiovascular and thermal stress and it mostly depends on the type of exercise (3).

Strategies for the fluid replacement during the exercise include a large spectrum of different thirst sensations, amount consumption and electrolyte compensation with a well-planned and structured intake (4). According to pH values, two different types of water can be used as supplementation and rehydration in athletes, acidic and alkaline water. Today, acidic rich water is suggested to be used in healthy people and some medical conditions (5, 6), while alkaline water is intended for general public. Recent study suggest that acidic water can be associated with maintained peak power output trained male cyclists (7).

On the other hand, metabolic acidosis that occurs during the high intensity training,represents metabolic alteration, emerging due to the lactate acid production in a working muscle. A part of the produced lactate can be realised in the blood leading to the acid-base disbalance and decreased pH blood values. In this state, cells are forced to rely on anaerobic ATP shifting that leads to proton releasing and the blood pH decreasing that can impair performance (8). Moreover, lactate produced during the exercise, generally remains in plasma while hydrogen ions are largely buffered in red blood cells due to the fact that red blood cells have a higher buffering capacity than the blood plasma (8). Although sodium bicarbonate has shown a positive effect on the strength and speed endurance, it has a lot of limitations such as gastrointestinal disorders including nausea, stomach pain, diarrhea, vomiting, metabolic alkalosis, even edema due to sodium overload (9). However, the newest data showed that treatment with acidic water can decrease blood lactate levels and enhance ventilatory efficiency in healthy males (10).

THE AIM OF THE PAPER

Having in mind all mentioned above it is assumed that the daily intake of low mineral content water Sneznik-1/79 could be important and useful for maintaining the physiological function of professional athletes in terms of influencing both sports performance and recovery from the training processes. Therefore, the aim of the present study was to examine the influence of low mineral water from the well Sneznik-1/79 on the anthropometric, functional, biochemical parameters and redox status of professional basketball players.

SUBJECTS AND METHODS

Study design

In accordance with the aim of our study, we included male basketball players (n = 17), 21 ± 3 years of age with years of professional basketball experience, members of the senior Basketball Club Borac , Cacak. The basketball players were put to the test during the pre-competitive mesocycle, from August 15th to September 15th, 2019.

After the initial testing, the participants were randomly divided into two groups: group 1 - consumed commercial drinking water for four weeks (n = 7), and group 2 - consumed water from the well Sneznik-1/79 for four weeks (n = 10).

During the aforementioned monitoring period, each participant received his own drinking bottle while the water intake was carefully monitored and recorded. Water was controlled immediately before, during and after the training or preparation matches. The total of 2 ± 0.5 liters/day was the average of consumed water during the indicated period.

Following at least a 3-hour fasting period, the testing began at the same time at 10:00 am. Blood was collected from the cubital vein in Vacutainer tubes (BD Vacutainer Blood Collection System) in a quiet, air-conditioned, temperature-controlled room (22-24°C). Sweat was collected from the back skin in 1.5 ml mini plastic tubes and stored in the freezer until the appropriate electrolytes were measured.

The study was conducted in accordance with the principles outlined in the Declaration of Helsinki and principles of Good Clinical Practice (GCP). The written informed consent was obtained from all participants, and the study protocol was approved by the Ethics Committee, prior to the onset of the study.

Anthropometric parameters

The following anthropometric parameters were examined: the body height, body weight, body composition indicators (bone mass, soft tissue mass, total fat mass, skeletal muscle mass (SMM), fat percentage, total body water, basal metabolism, body mass index (BMI), intracellular water (ICW), extracellular water (ECW)). The basic anthropometric indicators were determined during the standard sports-medical examination. The body composition parameters were measured by the bioelectrical impedance analysis (BIA)

(before and immediately after Ergospirometry). BIA uses the electrical properties of the human body to alternate the current flow and measures resistance values to estimate the body water content and composition.

Functional parameters

The functional parameters were determined during the cardiopulmonary exercise test (CPET) - the ergospirometric test on a treadmill (Trackmaster TMX428 Stress Treadmill), conducted at the beginning of the monitoring period (the initial, first measurement) and after four weeks of drinking commercial or Sneznik drinking water (the final, second measurement). This test provides an assessment of the integrative exercise responses involving the cardiovascular, pulmonary, neuropsychological, and skeletal muscle systems, which are not adequately reflected through the measurement of the individual organ system functions. The following functional parameters were examined: the absolute and relative maximum oxygen consumption (VO_{2max}), respiratory exchange ratio (RER), heart rate (HRmax), respiratory reserve, anaerobic threshold, aerobic efficiency).

Evaluation of Systemic Redox State

The redox status was evaluated spectrophotometrically by measuring the levels of prooxidative parameters, hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), nitrites (NO_2^-) and index of lipid peroxidation (TBARS) in plasma. Activities of the corresponding antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were measured in erythrocytes in the same manner.

Determination of prooxidative parameters

The degree of lipid peroxidation in plasma was estimated by measuring of thiobarbituric acid reactive substances (TBARS) using 0.4 ml 1% of thiobarbituric acid (TBA) in 0.05 NaOH mixed with 0.8 ml of plasma, incubated at 100 °C for 15 min and measured at 530 nm. Distilled water was used as a blank probe. The TBA extract was obtained by combining 0.8ml plasma and 0.4ml TCA (trichloroacetic acid). Thereafter, the samples were put on ice for 10 min, and centrifuged for 15 min at 6000 rpm (11). Nitric oxide (NO) decomposes rapidly to form the stable metabolite nitrite/nitrate products. The method for detection of the plasma nitrite levels is based on the *Griess* reaction. Nitrites were determined as an index of NO production with the *Griess* reagent (forms purple diazocomplex) (12). 0.1 ml 3 N PCA (perchloric acid), 0.4 ml 20mM EDTA (ethylenediaminetetraacetic acid), and 0.2 ml plasma were put on ice for 15 min, then centrifuged for 15 min at 6000 rpm. After pouring off the supernatant, 220 µl K_2CO_3 were added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe. The level of O_2^- was measured using Nitro Blue Tetrazolium (NBT) reaction in TRIS-buffer with plasma and read at 550 nm. Distilled water was used as a blank probe (13). The determination of H_2O_2 concentration is based on oxidation of phenol red using hydrogen peroxide, in the reaction catalyzed by the enzyme peroxidase from horse radish (POD) (14). 200 µl sample with 800 µl PRS (phenol red solution) and 10 µl POD were combined (1:20) and measured at 610 nm.

Determination of antioxidative enzymes activity

Isolated RBCs were washed three times with 3 volumes ice-cold 0.9mmol/l NaCl and hemolysates containing about 50g Hb/l, prepared according to McCord and Fridovich (15) were used for the determination of catalase (CAT) activity. The determination of CAT activity was done according to Beutler (16). Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove hemoglobin. Then 50 µl CAT buffer, 100 µl sample, and 1ml 10mM H_2O_2 were added to the samples. The detection was performed at 360nm. Distilled water was used as a blank probe. The determination of SOD activity is based on epinephrine method of Misra and Fridovich (17). 100µl lysate and 1 ml carbonate buffer were mixed, and then epinephrine in a volume of 100 µl was added. The detection was performed at470 nm. This method belongs to the 'negative' type group of methods, since it monitors the decrease of autoxidation speed in alkaline medium, which is dependent of $O_2^{\cdot-}$. The level of GSH concentration was determined based on GSH oxidation with 5.5-dithiobis-6.2-nitrobenzoic acid, using Beutler method (18). The measurement of absorbance is carried out at a wavelength of the maximum absorption of 420 nm.

Laboratory analyses

The laboratory analyses of leukocytes (WBC), erythrocytes (RBC), platelets (PLT), lymphocytes (Lymph), granulocytes (Gran) (Beckman Coulter LH780 analyzer), hemoglobin (HBG, Cyanmethemoglobin method, Beckman Coulter LH780 analyzer), hematocrit (HCT, MCV(fl) x number of erythrocytes (10^{12})), mean corpuscular volume (MCV, Beckman Coulter LH780), mean corpuscular hemoglobin (MCH, hemoglobin (g/L)/erythrocyte count (10^{12})); mean corpuscular hemoglobin concentration (MCHC, hemoglobin (g/L)/hematocrit value); mean platelet volume (MPV, Beckman Coulter LH780); plateletcrit (PCT, Beckman Coulter LH780); Na, K, Cl (Ion-selective electrodes ISE method, biochemical analyser Beckman Coulter AU680); Ca (Colorimetric method - Arsenazo III, biochemical analyser Beckman Coulter AU680); P (Colorimetric method - modification of Daly and Ertingshausen method, biochemical analyser Beckman Coulter AU680) were performed.

Points of interest

All mentioned anthropometric, functional and oxidative stress parameters were determined at the beginning of the monitoring period (the initial, first measurement) and after four weeks of consumption of Sneznik drinking water (the final, second measurement) and compared between the groups which consumed different types of water as well as

within the groups. In addition, electrolytes from blood and sweat as well as the haematological parameters, due to their dynamics during the initial and final measurements, were determined at three time points of interest: 1. at rest (before the ergospirometric test); 2. during the maximum effort (immediately after completion of the ergospirometric test); 3. in the 15th minute of recovery and compared between the groups that consumed different types of water as well as within the groups.

Statistics

IBM SPSS Statistics 20.0 Desktop for Windows was used for the statistical analysis. Distribution of the data was checked by Shapiro-Wilk test. Where distribution between the groups was normal, statistical comparisons were performed using the one-way analysis of variance (ANOVA) tests with a Tukey's post hoc test for multiple comparisons. Kruskal-Wallis was used for comparison between the groups where distribution of the data was different than normal. The values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Anthropometric parameters

Body height and age

The studied groups did not differ statistically by the age and height (Table 1). There was also no difference in the body height between the first and second measurements, i.e. after the follow-up period. These data are important to ensure adequate homogeneity of the study sample, which will allow for the subsequent relevance of other results.

Body composition

The results of the body composition parameters are shown in Table 2. It can be observed that there was no statistically significant difference in the body weight of basketball players at the beginning and at the end of the study, both between the groups and within groups. In addition, the subjects did not differ in terms of the amount of total body water in both body compartments (extracellular and intracellular). The proportion of muscle mass in both groups was no significantly higher after four weeks, while at the same time the percentage of body fat decreased. The body mass index and basal metabolism were almost identical at the first and second measurements in both groups with no differences between the groups. These results indicate that there were no changes in the body composition between the groups after four weeks of the follow-up period, as it was expected. Improvements that exist discreetly in both groups (the fat and protein content) support a good training process carried out during the preparatory period.

Ergospirometry

The results of the ergospirometry parameters are shown in Table 3 and Figure 1. Based on the findings obtained, it can be observed that the absolute and relative maximum oxygen consumption was higher after the follow-up period in both basketball groups with a discreetly higher increase in the group that consumed Sneznik water. The results related to the maximum heart rate and anaerobic threshold showed no significant differences within as well as between the groups.

On the other hand, it is interesting that the athletes who consumed Sneznik water after four weeks of monitoring, had almost an unchanged respiratory reserve, unlike the group of athletes who consumed commercial drinking water and that had a decrease in this parameter during the same period. In addition, a more pronounced increase in the aerobic efficiency was observed in the group that used Sneznik water. Although there was no statistical significance between the groups, the unambiguous difference would probably be more noticeable after a longer follow-up period.

Biochemical parameters

Oxidative stress parameters

The results of the redox status are shown in Figure 2 and 3. It can be observed that the values of NO2-, in both groups, were not significantly higher after the follow-up period. On the other hand, the values of O2- were insignificantly higher in the group that consumed commercial water, after four weeks, while in the group of athletes who consumed Sneznik water, decreasing of this parameter was recorded (without the statistical significance) (Figure 2). Furthermore, H2O2 was significantly decreased after the four-week consumption of both, commercial and Sneznik water, while TBARS was significantly increased (Figure 2).

The activity ofantioxidative enzymes was insignificantly higher in both groups of participants after the follow-up period, which again goes in a favour of the physiological adaptation to the training process. There were no statistical differences in the achieved dynamics between the groups (Figure 3).

Electrolytes from blood and sweat

Table 4. represents the effects of the four-week Sneznik water consumption on the electrolyte values at different points of interest in blood and sweat. The obtained results showed that there were no statistically significant differences in ionograms from the blood samples at all measurement times, both within and between the groups. Interestingly, the concentration of sodium, potassium and chlorine in sweat of both groups was higher at the end of the study with minor deviations in the group that consumed Sneznik drinking water. All these differences were statistically insignificant (Table 4).

Laboratory analysis

The effects of the four-week water consumption from the well Sneznik on the values of blood parameters of interest are shown in Table 5. The results of total leukocytes as well as the absolute and relative leukocyte formulas show that there was no statistically significant difference between and within the groups. The parameters of erythrocytes (the total erythrocyte count, hematocrit and hematological indexes) did not also change during the follow-up period within the groups, with no differences between them. The same trend was observed in the parameters related to platelets (the total number as well as distribution and volume of platelets). On the other hand, it is interesting that the hemoglobin values in both groups were higher at the end of the study with no statistical significance (Table 5).

Table 1. Characteristics of study participants.

	Age (years)	Height (cm)	
		1st measurement	2nd measurement
Commercial water	20.14 ± 4.60	195.17 ± 9.15	195.50 ± 8.95
Sneznik water	22.00 ± 3.06	195.78 ± 7.87	196.33 ± 7.68

Results are expressed as mean ± standard error.

Table 1. Characteristics of study participants.

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Commercial water	20.14 ± 4.60	195.17 ± 9.15	195.50 ± 8.95
Sneznik water	22.00 ± 3.06	195.78 ± 7.87	196.33 ± 7.68

Results are expressed as mean ± standard error.

Table 2. Effects of four-week water consumption from the well Sneznik on body composition.

	Commercial water		Sneznik water					
	1st measurement		2nd measurement		1st measurement		2nd measurement	
	before	after	before	after	before	after	before	after
ICW	36.40 ± 4.88	36.33 ± 4.91	37.85 ± 5.40	38.98 ± 5.30	37.70 ± 3.46	37.52 ± 3.44	39.04 ± 3.36	38.73 ± 3.40
ECW	21.87 ± 2.91	21.86 ± 3.00	22.35 ± 3.21	23.06 ± 3.14	22.41 ± 2.38	22.20 ± 2.45	22.97 ± 2.22	22.77 ± 2.22
Proteins	15.73 ± 2.14	15.73 ± 2.14	16.35 ± 2.33	16.84 ± 2.30	16.31 ± 1.48	16.21 ± 1.49	16.88 ± 1.45	16.74 ± 1.47
Minerals	5.29 ± 0.66	5.25 ± 0.69	5.54 ± 0.75	5.62 ± 0.74	5.64 ± 0.65	5.50 ± 0.66	5.80 ± 0.66	5.74 ± 0.60
Bone mass	4.34 ± 0.54	4.31 ± 0.56	4.52 ± 0.61	4.59 ± 0.60	4.66 ± 0.55	4.51 ± 0.58	4.80 ± 0.58	4.73 ± 0.51
Total fat mass	6.81 ± 3.13	6.44 ± 3.16	5.30 ± 2.04	4.82 ± 1.81	8.31 ± 3.16	7.85 ± 3.27	6.43 ± 2.90	6.60 ± 2.74
Total body water	58.27 ± 7.79	58.19 ± 7.91	60.20 ± 8.58	62.04 ± 8.43	60.11 ± 5.80	59.72 ± 5.86	62.01 ± 5.56	61.50 ± 5.61
Soft tissue mass	74.91 ± 9.90	74.84 ± 10.16	77.57 ± 11.0	79.92 ± 10.9	77.38 ± 7.36	76.91 ± 7.46	79.90 ± 7.13	79.24 ± 7.19
Fat free mass	79.33 ± 10.57	79.14 ± 10.71	82.12 ± 11.7	84.52 ± 11.5	82.03 ± 7.91	81.43 ± 8.02	84.69 ± 7.69	83.98 ± 7.69

	Commercial water 1st measurement	Sneznik water 2nd measurement	1st measurement	2nd measurement				
	before	after	before	after	before	after	before	after
Weight	86.14 ± 10.22	85.59 ± 10.34	87.42 ± 11.1	89.34 ± 10.7	90.34 ± 10.68	89.28±1 1.05	91.12 ± 10.31	90.58±10. 12
SMM	45.49 ± 6.36	45.41 ± 6.38	47.33 ± 7.05	48.80 ± 6.92	47.16 ± 4.49	46.94 ± 4.52	48.91 ± 4.38	48.48 ± 4.44
BMI (kg/m^2)	22.39 ± 1.75	22.23 ± 1.74	22.82 ± 1.72	23.00 ± 1.73	23.55 ± 1.68	23.12 ± 1.89	23.60 ± 1.58	23.46 ± 1.54
Fat percentage (%)	8.00 ± 3.62	7.43 ± 3.89	6.20 ± 2.55	5.56 ± 2.41	8.96 ± 2.52	8.54 ± 2.59	6.83 ± 2.50	7.08 ± 2.30
Basal metabolism	2082.71±228.3	2079.14 ± 231	2143 ± 251	2194.6 ± 247	2141.8 ± 171	2128.40 ± 173	2195.89 ± 161	2183.3 ± 166

Results are expressed as mean ± standard error. Before - before ergospirometric test; After - after ergospirometric test. ICW - intracellular water; ECW - extracellular water; SMM - skeletal muscle mass; BMI - body mass index

Table 3. Effects of four-week water consumption from the well Sneznik on ergospirometric parameters.

	Commercial water		Sneznik water	
	1st measurement	2nd measurement	1st measurement	2nd measurement
VO_2 (ml/min)	4348.86 ± 435.15	4723.00 ± 528.56	4282.70 ± 497.69	4552.44 ± 453.66
RER	1.15 ± 0.08	1.08 ± 0.03	1.18 ± 0.15	1.09 ± 0.04
HR max	184.00 ± 5.83	184.80 ± 5.31	181.60 ± 5.56	184.00 ± 8.44
Vt BTPS (L)	130.03 ± 28.53	141.94 ± 28.53	126.56 ± 19.55	135.04 ± 20.77
Respiratory reserve	39.14 ± 18.85	35.60 ± 16.59	41.60 ± 9.56	41.67 ± 8.03
RR (number/min)	46.14 ± 12.76	46.80 ± 13.18	45.40 ± 7.76	43.56 ± 12.14
ANP	1.00 ± 0.00	1.00 ± 0.00	0.99 ± 0.01	1.00 ± 0.00

Results are expressed as mean ± standard error. Before - before ergospirometric test; After - after ergospirometric test. VO_2 - (absolute and relative maximum oxygen consumption); RER - respiratory exchange ratio; HR - heart rate; Vt BTPS - tidal volume at body temperature and pressure saturated; RR - respiratory rate; ANP - anaerobic threshold

Table 4. Effects of four-week water consumption from the well Sneznik on electrolyte values in blood and sweat at different points of interest.

	Commercial water		Sneznik water	
	1st measurement	2nd measurement	1st measurement	2nd measurement
Ca (Peace)	2.38 ± 0.19	2.29 ± 0.04	2.39 ± 0.18	2.28 ± 0.13
Ca (Max)	2.43 ± 0.15	2.43 ± 0.07	2.47 ± 0.20	2.45 ± 0.10
Ca (Rest)	2.36 ± 0.16	2.30 ± 0.04	2.33 ± 0.19	2.33 ± 0.13
Ca (Sweat)	5.06 ± 2.92	3.34 ± 1.09	4.40 ± 2.36	3.26 ± 1.40

	Commercial water		Sneznik water	
	1st measurement	2nd measurement	1st measurement	2nd measurement
Na (Peace)	139.08 ± 1.93	137.64 ± 2.17	139.20 ± 2.19	136.99 ± 2.19
Na (Max.)	139.52 ± 2.06	139.12 ± 1.84	139.06 ± 2.34	138.99 ± 2.04
Na (Rest)	139.65 ± 2.59	138.30 ± 1.00	138.63 ± 2.40	138.29 ± 1.79
Na (Sweat)	111.95 ± 17.17	138.44 ± 34.94	100.06 ± 28.38	115.01 ± 25.99
K (Peace)	4.64 ± 0.59	4.17 ± 0.34	4.71 ± 0.51	4.32 ± 0.21
K (Max)	4.50 ± 0.57	4.81 ± 0.46	4.76 ± 0.39	4.69 ± 0.41
K (Rest)	4.31 ± 0.54	4.46 ± 0.37	4.53 ± 0.34	4.39 ± 0.33
K (Sweat)	18.64 ± 9.48	21.46 ± 2.42	20.31 ± 6.06	25.06 ± 3.61
Cl (Peace)	99.03 ± 5.11	97.43 ± 1.00	98.37 ± 3.89	97.71 ± 4.02
Cl (Max)	101.92 ± 3.21	98.83 ± 3.82	102.03 ± 4.50	101.47 ± 3.24
Cl (Rest)	100.68 ± 3.63	98.14 ± 3.26	99.95 ± 3.83	100.06 ± 3.84
Cl (Sweat)	102.71 ± 42.50	130.69 ± 53.55	75.48 ± 30.34	96.25 ± 32.85
P (Peace)	1.14 ± 0.08	1.23 ± 0.16	1.19 ± 0.09	1.19 ± 0.11
P (Max)	1.40 ± 0.10	1.66 ± 0.10	1.39 ± 0.16	1.50 ± 0.06
P (Rest)	1.23 ± 0.06	1.28 ± 0.10	1.21 ± 0.06	1.26 ± 0.03
P (Sweat)	0.60 ± 0.37	0.47 ± 0.05	0.48 ± 0.10	0.44 ± 0.02

Results are expressed as mean ± standard error. Peace - before ergospirometric test;
Max - immediately after ergospirometric test; Rest - 15 minutes after ergospirometric test.

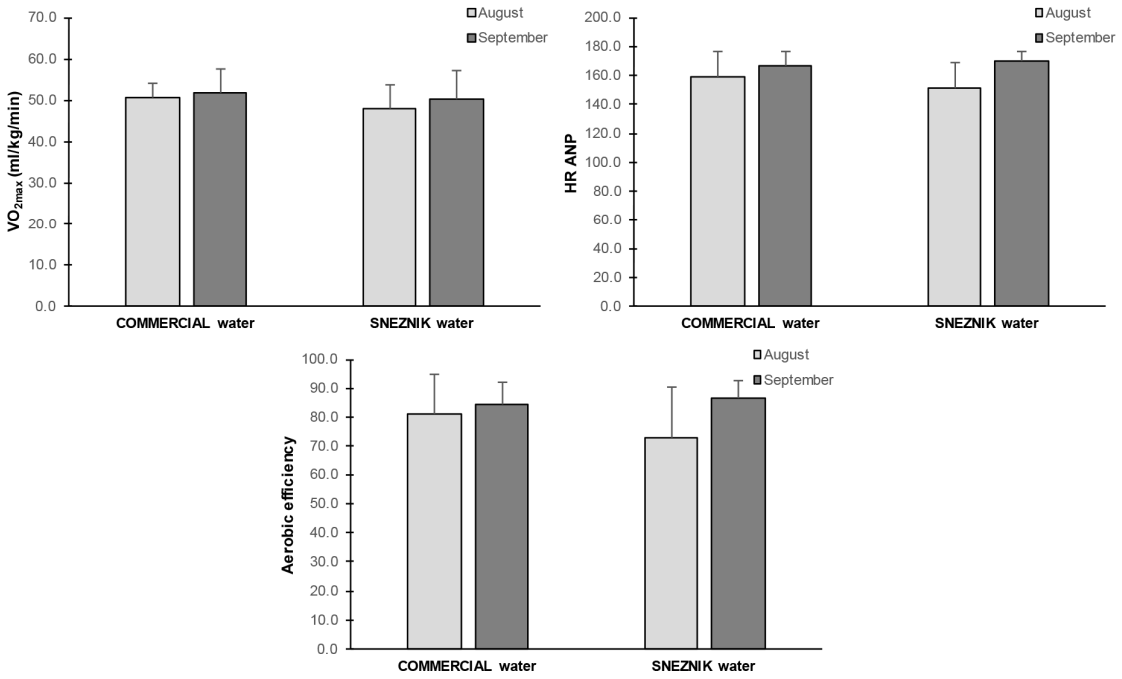
Table 5. Effects of four-week water consumption from the well Sneznik on values of parameters of interest in blood picture

	Commercial water		Sneznik water	
	1st measurement	2nd measurement	1st measurement	2nd measurement
WBC - Peace (10^9/l)	5.06 ± 1.60	5.22 ± 1.40	5.32 ± 1.17	5.17 ± 1.19
WBC - Max (10^9/l)	7.87 ± 2.28	8.92 ± 2.05	8.17 ± 1.52	8.96 ± 1.58
WBC - Rest (10^9/l)	4.97 ± 1.55	5.40 ± 1.68	4.95 ± 1.11	5.12 ± 0.96
Lymph - Peace (10^9/l)	1.66 ± 0.40	1.88 ± 1.88	1.75 ± 0.44	1.80 ± 0.46
Lymph - Max (10^9/l)	3.30 ± 0.82	3.90 ± 1.67	3.35 ± 0.68	4.03 ± 0.90
Lymph - Rest (10^9/l)	1.60 ± 0.54	2.02 ± 0.61	1.50 ± 0.34	1.43 ± 0.44
Gran - Peace (10^9/l)	3.00 ± 1.20	2.92 ± 0.92	3.20 ± 0.78	2.97 ± 0.79
Gran - Max (10^9/l)	3.77 ± 1.37	3.88 ± 1.43	4.08 ± 0.94	4.09 ± 1.06
Gran - Rest (10^9/l)	2.94 ± 1.07	2.90 ± 1.05	3.11 ± 0.77	3.06 ± 0.77
Lymph - Peace %	34.04 ± 8.06	36.55 ± 6.35	32.90 ± 4.73	34.82 ± 5.94
Lymph - Max %	42.83 ± 7.37	46.60 ± 6.95	41.44 ± 4.98	45.07 ± 7.51
Lymph - Rest %	32.91 ± 8.21	37.78 ± 5.13	30.18 ± 2.92	33.12 ± 7.82
Gran - Peace %	57.80 ± 7.94	54.72 ± 7.06	59.61 ± 4.82	56.80 ± 6.14

	Commercial water		Sneznik water	
	1st measurement	2nd measurement	1st measurement	2nd measurement
Gran - Max %	47.36 ± 6.78	44.54 ± 10.15	49.57 ± 5.15	45.42 ± 8.42
Gran - Rest %	58.07 ± 7.97	52.50 ± 5.81	62.33 ± 3.05	59.06 ± 7.24
HGB - Peace (g/l)	138.43 ± 8.50	146.33 ± 9.97	145.20 ± 9.80	148.44 ± 6.54
HGB - Max (g/l)	145.86 ± 9.53	156.80 ± 10.87	154.30 ± 8.87	160.44 ± 6.97
HGB - Rest (g/l)	136.71 ± 6.92	146.00 ± 9.22	146.00 ± 9.10	148.89 ± 7.24
RBC - Peace (10^{12}/l)	4.51 ± 0.19	4.61 ± 0.23	4.69 ± 0.33	4.65 ± 0.25
RBC - Max (10^{12}/l)	4.73 ± 0.26	4.99 ± 0.30	4.94 ± 0.32	5.01 ± 0.32
RBC - Rest (10^{12}/l)	4.43 ± 0.15	4.66 ± 0.20	4.64 ± 0.35	4.73 ± 0.27
HCT - Peace %	41.79 ± 2.34	42.60 ± 2.75	43.79 ± 3.04	43.66 ± 1.78
HCT - Max %	44.16 ± 1.72	46.32 ± 3.18	46.38 ± 3.03	46.54 ± 2.30
HCT - Rest %	40.96 ± 1.61	42.78 ± 2.18	43.29 ± 3.21	43.84 ± 2.16
MCV - Peace (fL)	92.66 ± 2.98	92.52 ± 3.14	93.52 ± 2.04	93.98 ± 2.26
MCV - Max (fL)	93.47 ± 3.18	92.92 ± 3.15	94.06 ± 2.12	94.58 ± 1.97
MCV - Rest (fL)	92.50 ± 2.26	91.96 ± 2.88	93.48 ± 2.12	93.68 ± 2.20
MCH - Peace (pg)	30.60 ± 1.11	31.68 ± 1.14	30.92 ± 0.75	31.87 ± 0.82
MCH - Max (pg)	30.63 ± 1.29	31.36 ± 1.02	31.23 ± 0.66	31.76 ± 0.85
MCH - Rest (pg)	30.77 ± 1.23	31.30 ± 1.26	31.23 ± 0.87	31.73 ± 0.60
MCHC - Peace (g/L)	330.57 ± 5.41	343.00 ± 1.67	331.20 ± 7.00	340.56 ± 6.04
MCHC - Max (g/L)	329.71 ± 5.35	338.00 ± 2.55	332.40 ± 4.48	337.33 ± 3.35
MCHC - Rest (g/L)	332.86 ± 6.99	340.60 ± 4.39	334.60 ± 5.27	339.00 ± 4.18
PLT - Peace (10^{9}/l)	173.71 ± 36.89	182.67 ± 27.38	191.88 ± 73.93	223.67 ± 39.84
PLT - Max (10^{9}/l)	204.86 ± 37.87	220.60 ± 31.91	217.44 ± 85.11	264.89 ± 36.89
PLT - Rest (10^{9}/l)	173.71 ± 47.95	188.40 ± 23.41	191.89 ± 75.55	226.33 ± 40.51
MPV - Peace (fl)	9.04 ± 0.98	9.35 ± 0.44	8.98 ± 0.55	9.19 ± 0.51
MPV - Max (fl)	9.21 ± 0.99	9.56 ± 0.52	9.06 ± 0.61	9.33 ± 0.55
MPV - Rest (fl)	9.01 ± 0.76	9.46 ± 0.40	8.87 ± 0.50	9.12 ± 0.48

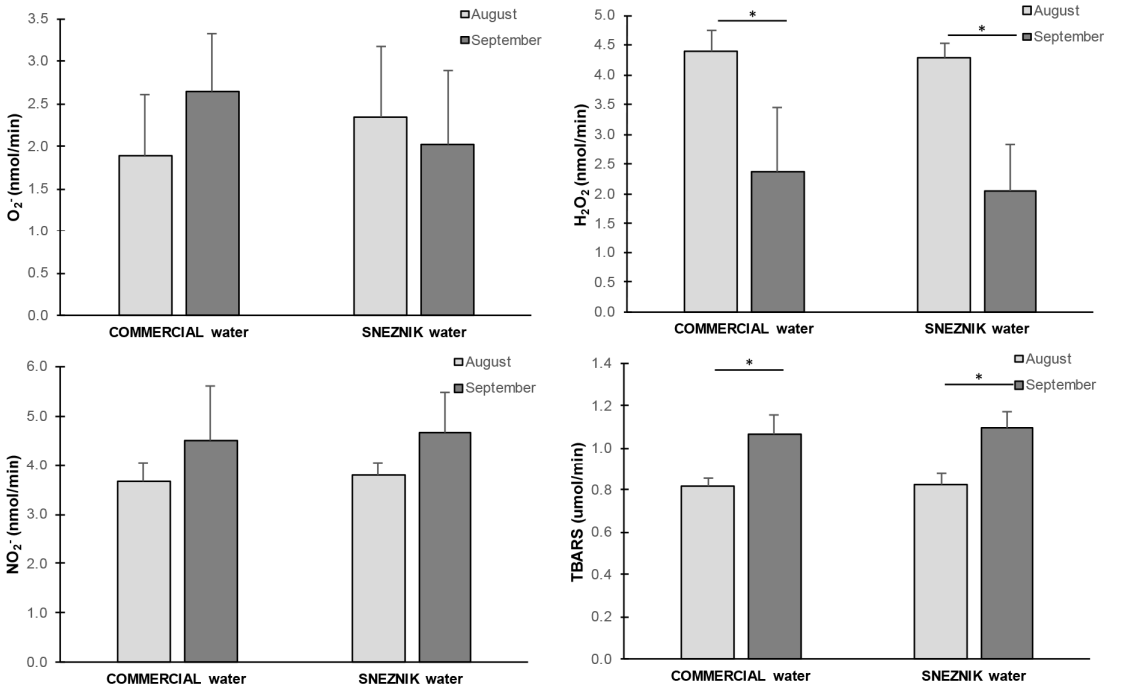
Results are expressed as mean ± standard error. Peace - before ergospirometric test; Max - immediately after ergospirometric test; Rest - 15 minutes after ergospirometric test. WBC - leukocytes, RBC - erythrocytes, PLT - platelets, Lymph - lymphocytes, Gran - granulocytes, HBG -hemoglobin, HCT - hematocrit (MCV(fl) x number of erythrocytes (10^{12})), MCV - mean corpuscular volume, MCH - mean corpuscular, MCHC - mean corpuscular hemoglobin concentration; MPV - mean platelet volume, PCT -plateletcrit

Figure 1. Effects of four-week water consumption
from the well Sneznik on selected ergospirometric parameters.



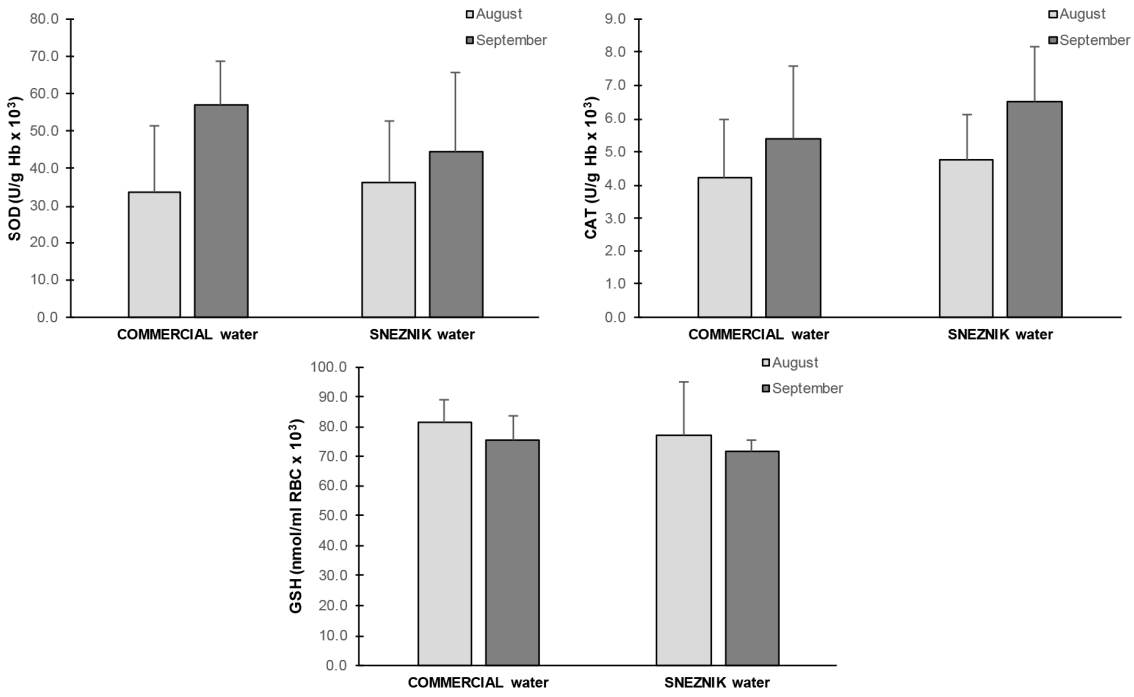
Results are expressed as mean ± standard error.
VO_{2max} - absolute and relative maximum oxygen consumption,
HR ANP- heart rate at anaerobic threshold.

Figure 2. Effects of four-week water consumption
from the well Sneznik on prooxidative parameters.



Results are expressed as mean ± standard error. O_2^- - superoxide anion radical;
H_2O_2 - hydrogen peroxide; NO_2^- - nitrites; TBARS - index of lipid peroxidation. * $p < 0.05$.

Figure 3. Effects of four-week water consumption
from the well Sneznik on parameters of antioxidant protection.



Results are expressed as mean ± standard error.
SOD -superoxide dismutase; CAT - catalase; GSH - reduced glutathione.

DISCUSSION

Present study aimed to estimate the potential benefits of alkaline water consumption from the Sneznik well and its potentially positive effects on the body composition, anthropometric and laboratory values as well as oxidative stress in professional sports. In order to examine these effects, we compared the results before and after consumption of commercial and Sneznik water, where we found out that Sneznik water is completely safe from the aspect of affecting various anthropometric, functional and biochemical parameters as well as systemic oxidative stress of professional basketball players.

Evaluation of the body composition is required in order to monitor the training outcomes, nutrition and general health. The body composition among athletes varies across different types of sport. This is very important in order to develop specific dietary regimes as well as adequate and optimal trainings. The fat mass and fat-free mass are mostly used to monitor the nutrition needs and energy consumption. Moreover, an increase in the body fat can impair the physical performances (19). Furthermore, the total body bioimpedance can predict the body composition compartments due to the fact that the body's fluid is equally distributed and body segmental lengths are proportional to segmental, which is very important for the appropriate hydration (20).

The results of the previous study which indicate that there is no statistical difference in the basic anthropometric characteristics, body mass, bioimpedence, including total body water and its active transport (TBW - total body water / ICW - intracellular water / ECW - extracellular water) in athletes who consumed alkaline high or low as well as commercial water, correlate with our results (21, 22). We haven't noticed any significance between and within the followed groups in terms of the body composition.

According to our results, from the aspect of the water Sneznik influence on the body composition, we might say that consumption of water from the mentioned well did not disturb the parameters of the body composition and with an efficient and healthy training process, it can be associated with the trend of positive effects on the body composition, which is supposed to be more obvious during longer basketball follow-up time (more than four weeks).

The exchange of ions, CO_2, and water between the intracellular and extracellular compartments helps to restore the acid-base balance following the intensive exercise. During the exercise, well trained athletes can decrease muscle pH from 7.0at rest to the values as low as 6.4-6.5 during the exercise (23). There are some data indicating that supplementation with acidic water cane have an alkalizing effect in young

physically active men. (24). In correlation with these results, the ergospirometric test showed that consumption of water from the well Sneznik was associated with a slightly better functional capacity of the tested athletes, and it did not disturb the parameters of the ergospirometry. In addition, the positive trend achieved after consuming Sneznik water would be statistically confirmed over a longer monitoring period, indicating that further research would certainly be of interest.

It is believed that water rich in hydrogen ions may improve neutralization of ROS by stimulation of various antioxidant proteins, whereby hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals (25). Moreover, hydrogen-rich water can be mainly beneficial in different metabolic disorders (26). On the other hand, alkaline water, with high pH values, mainly has an important role in increasing the lactate utilization through the effect on the acid-base balance, following anaerobic exercise (21). Furthermore, the fluid replacement following dehydration reduces oxidative stress during the recovery (27). We wanted to examine whether weakly mineralized and weakly acid might have an antioxidative potential.

Our results showed significantly lower values of H_2O_2, after four weeks of different water consumptions which can point out that water from Sneznik potentially has an antioxidative role and it doesn't disturb the redox status in athletes. On the other hand, in trained individuals, the antioxidant system is more efficient due to the adaptation to exercise (21) which supports our unchanged values of antioxidative enzymes.

Interestingly, the level of TBARS was significantly increased after fourweeks in both groups, which goes in favor of an ongoing training process and micro-injuries that occur physiologically due to the intense physical activity. The previous studies indicate that prooxidative markers can be recorded in a higher concentration in athletes during the supramaximal exercise (21).

Based on these results, it can be observed that consumption of water from the well Sneznik was not associated with negative changes in the redox balance of the athletes and thus greater oxidative damage. In addition, as in the previous cases, it can be assumed that discrete positive changes would be more noticeable after a long-time exposure to this water.

Regarding the effect of water on the blood parameters, the previous study showed a significant difference in the whole blood viscosity when assessing high-pH, electrolyte water versus acceptable standard purified water (22). In our study, initiation of the training process did not affect changes of the most important blood parameters i.e., it was not associated with any hematological disorders. From the aspect of the water Sneznik influence on the examined blood parameters and electrolytes, we might say that consumption of water from the mentioned well is completely safe. This can indicate that low mineral and weakly acid water might maintain the adequate hydration. Also, although statistically unconfirmed, a smaller deviation in the concentration of the most important sweat ions between the first and second measurements compared to the group that used commercial drinking water speaks in favor of potentially beneficial effects whose validation requires further research. A longer follow-up time is suggested for confirmation and deeper analysis of these results.

CONCLUSION

In summary, our results pointed out that consumption of low mineral water from the well Sneznik is completely safe from the aspect of affecting various anthropometric, functional and biochemical parameters as well as systemic oxidative stress of professional basketball players.

In addition, existence of discretely better effects over commercial drinking water indicates that a long period of monitoring may certainly be of interest for further investigation.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethicalstandards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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FOLLOWING THE PRINCIPLES OF ERGONOMICS AND MUSCULOSKELETAL DISORDERS IN ULTRASONOGRAPHERS

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ABSTRACT

It has been noticed that doctors who regularly perform ultrasound examinations have different clinical manifestations of musculoskeletal disorders. The aim of this research is to examine the level of knowledge of ultrasonographers regarding preventative measures and health consequences of the forced body positions during work. The total of 75 doctors of both genders, aged 31-50 years, who use ultrasound diagnostics participated in the research. The research was conducted throughout 2016, as a cross-sectional study, with the aid of a structured questionaire. The total of 60% subjects either rarely use the break during working hours or do not have a break at all. The participants most commonly use the movable monitor and the ability to wrap the cable around their arm (73,3%). This percentage is similar in all age groups. The respondents most often stated that they walk daily (41,3%), with no differences among age groups. 25% of the respondents negate any spine related pains, while over a half said that they experience pain in one segment of the spinal column (cervical, thoracic or lumbar), regardless of the age group. 52% said that they serch for information on their own accord, while 36% stated that they are not at all informed. Only in less than 10% of cases is information provided by the authorities in the institution where the participants are employed, and during regular health examinations, information is given by the designated doctor in 2,7% of cases.The respondents implement the recommendations regarding safe work and other preventative measures to a very small extent, regardless of gender and age. Most of the respondents experience pain in the spine and joints, which can be related to the work in ultrasound diagnostics. Additionally, doctors are not well-informed regarding the preventative measures.

Keywords: *Ergonomics, ultrasonography, musculoskeletal disorder.*

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INTRODUCTION

Due to its non-invasiveness, in many areas of medicine, such as cardiology, gynecology or urology, the ultrasound examination is a part of the protocol for diagnosis, as well as for long-term follow-up of a patient's health condition and monitoring of the effects of the therapy.

It has been noticed that a large number of doctors, of various specialties, who regularly perform ultrasound examinations during their practice have different clinical manifestations of musculoskeletal disorders. Work-related musculoskeletal disorders can be defined as injuries which occurred or got worse due to the activities at work. The share of these disorders rises up to 60% out of all work-related diseases. Various terminology related to these disorders can be found in the literature and some of them are: injury due to repeated straining, cumulative trauma, injury caused by repeated movement (1-3). Among the population of doctors who deal with ultrasound diagnostics, this disorder was first identified in 1993 (4). The prevalence of musculoskeletal symptoms connected to the work of ultrasonographers, in large scale researches, goes up to 80% (5) and 90% (6). The typical areas of symptoms reported in previous surveys are the neck and lower back (5-10). These musculoskeletal disorders have different intensities and can manifest as discomfort or intensive pain.

Many following studies showed that ultrasonographers are at a higher risk of numerous musculoskeletal disorders, including wrist disorders (carpal tunnel syndrome, carpal instability, tendinitis), elbow disorders (bursitis, epicondylitis), pain in the shoulders, neck and spine (11,12).

When it comes to the pains in the spinal column, considering the risk factors, some are connected to the work environment and work process, while some are not. Individual risk factors include the previous injury history and spinal column diseases, anthropometric characteristics, gender and age. The highest risk period for men is around the age of 40, and for women between the age of 50 and 60.

Studies have also shown that the relative risk of the occurrence of pain in the lumbar area of the spine is greater in men taller that 180 cm and women taller than 170 cm, compared to 10cm shorter persons. People with higher body mass index, of both genders, are also at a higher risk, as well as those with decreased lumbar lordosis. Nowadays, it is believed that individual risk factors define the basis of the likelihood for the occurrence and the development of musculoskeletal disorders, but that the illness will develop depending on the other factors, most important of which is work environment, i.e. irregular position of the body during work (13). Also, overworking, prolonged straining of certain muscle groups, work operations which require strong muscle contractions, long forced body positions, repeated movements and prolonged static load - the transducer is held pressed onto the surface of a patient's body, are also relevant (13).

The symptoms of work related musculoskeletal disorders can be intermittent or temporary, they can last during the entire work day or they can occur at night. If the first symptoms are disregarded, they can lead to serious, chronic, incapacitating symptoms and disorders such as the loss of sensibility or muscle weakness (13).

In cases of the work related disorders in ultrasonographers, the most common diagnosis is tendon and/or tendon sheath inflammation, i.e. tendinitis or tenosynovitis. The most common cause of tendinitis is repeated straining, i.e. performing the same type of examination without sufficient time for rest between two examinations. Ultrasonographers specialized only for one type of examination, such as gynecologists or cardiologists, are at a particular risk. The conclusion of a doctoral dissertation published in Sweden in 2018, which included almost all female doctors which perform heart ultrasonography, is the recommendation that visual ergonomics and optimal adaptability of the equipment should be improved, i.e. that the equipment is designed in a way which allows for variations of work positions, since none of the applied techniques was optimal (14).

In order for the muscles to relax, sufficient recovery time should be allocated after prolonged work in a forced body position. A short recess is recommended after every three examinations, during which it is advised to stand up, walk around the room and stretch. Such preventative measures should become a habit, which will ensure health protection despite occupational risk.

The aim of this research is to examine the level of knowledge of ultrasonographers regarding preventative measures and the level of application of these measures, as well as health consequences of the forced body positions during work.

METHODS

In total, 75 respondents of both genders and different age groups who practice ultrasound diagnostics took part in the research. They were divided into three groups in a ten-year range from 31 to over 50 years of age. The research was conducted in of seven institutions in total - primary, secondary and tertiary health care institutions, in Belgrade and Obrenovac. All the participants submitted a written informed consent for the participation in the research.

The data was collected through a structured questionnaire which had been adapted according to the questionnaires used in similar researches (15,16,17). The questionnaire was firstly validated and it consisted of five groups of questions referring to: demographic data, workload, preventative measures implementation during work, health consequences and awareness of the topics relevant to this research. With the aim of quantification, the obained data regarding the implementation of preventative measures during work, health

consequences and awareness was translated from categories to numerical values by evaluating the responses according to the key.

The data was presented using the methods of descriptive statistics: arithmetic mean, standard deviation and proportion frequency. Normality of distribution was examined using the Kolmogorov-Smirnov test. The significance of the differences among the groups was examined using the Kruskal-Wallis test, i.e. the χ^2 test for the qualities in terms of categories. Finally, for *post hoc* analysis, Mann-Whitney test was used. The relation among the examined qualities was tested using Spearman's rank correlation coefficient (ρ). The results were processed in the statistical programmes Excel for Windows and Origin. Statistical significance was accepted at $p<0.05$.

The research was designed as a *cross-sectional study* and was conducted during 2016.

RESULTS

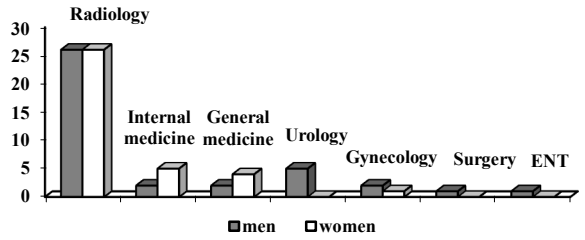
1. Demographic data

At the moment of the research, the average number of years of service of the respondents was 19, and on average, they had 11 years of experience in ultrasound diagnostics. On average, they perform ultrasound examinations 3.5 days a week, 4.7 hours a day and they see 16 patients a day.

The total of 75 respondents participated in the study, 39 (52%) of which were men and 36 (48%) were women. All three age groups consisted of a similar number of respondents ($p>0.05$), with similar gender distribution ($p>0.05$).

The majority of the respondents, 52 of them (69.3%), were radiologists, men and women equally. However, out of seven internists and six general physicians, the majority were women, while the specialists in different surgical branches (urology, general surgery and otorhinolaryngology) were all men and there were seven of them.

Image 1. The number of respondents of both genders in different specialties



2. Workload

On average, the respondents had 19,30±9,14 years of service (from 9.43±3.37 years in the youngest to 28.80±4,51 in the oldest age group). The number of years of work in the field of ultrasound diagnostics follows the total number of years of service and increases with the age (from 4.05±2.22 to 17.70±6.13). However, the number of days per week when the respondents are engaged in ultrasound examinations, as well as the number of hours per day during which they perform the examinations, do not differ among age groups ($p>0.05$). When the engagement in the ultrasound diagnostics is expressed on a weekly basis, a slightly lower number of examinations per week can be noticed, as well as a lower number of hours per week, in the group of the participants of over 50 years of age compared to the two younger groups, though without statistical significance ($p>0.05$). These results are presented in Table 1.

3. The implementation of preventative measures during work

Out of all the respondents, the total of 60% either rarely have a break during working hours or do not have a break at all, and the percentage is similar in all age groups ($p>0.05$). A very small number of participants have a break after each examination (6.7%), the smallest number of these doctors is in the group above the age of 50 (only 3.6%), which is statistically significantly less compared to both younger age groups ($p<0.05$). In respect to this parameter, men and women do not differ, regardless of the age group ($p>0.05$). The results are shown in Table 2.

Table 1. Workload characteristics of the respondents in different age groups

Yrs	Years of service	Ultra-sound (years of service)	Ultra-sound (days per week)	Ultra-sound (hours per day)	No. of patients per day	Ultra-sound (hours per week)	Ultra-sound (patients per week)
31-40	9.43 ±3.37	4.05 ±2.22	3.48 ±1.57	5.10 ±1.81	18.33 ±9.18	17.67 9.97	67.14 ±52.03
41-50	17.00 ±5.20	10.80 ±5.80	3.90 ±1.50	4.60 ±2.30	16.90 ±11.10	17.60 ±11.70	66.10 ±57.00
Over 50	28.80 ±4.51	17.70 ±6.13	3.32 ±1.79	4.43 ±2.07	14.54 ±5.01	12.43 ±7.38	47.32 ±30.11
Total	19.30 ±9.14	11.00 ±7.50	3.57 ±1.65	4.69 ±2.09	16.41 ±8.71	15.67 ±9.97	59.38 ±47.42

Table 2. The use of a break during working hours, in respect to gender and age groups

Yrs	Break	Total	Men	Women
31-40	Rarely/never has a break	12 (57.1%)	8 (61.5%)	4 (50%)
	As they choose	5 (23.9%)	4 (30.8%)	1 (12.5%)
	Predetermined	2 (9.5%)	0 (0%)	2 (25%)
	After each examination	2 (9.5%)	1 (7.7%)	1 (12.5%)
41-50	Rarely/never has a break	17 (65.4%)	10 (76.9%)	7 (53.8%)
	As they choose	4 (15.4%)	1 (7.7%)	3 (23.1%)
	Predetermined	3 (11.5%)	2 (15.4%)	1 (7.7%)
	After each examination	2 (7.7%)	0 (0%)	2 (15.4%)
Over 50	Rarely/never has a break	16 (57.1%)	6 (46.1%)	10 (66.7%)
	As they choose	3 (10.7%)	2 (15.4%)	1 (6.7%)
	Predetermined	8 (28.6%)	4 (30.8%)	4 (26.6%)
	After each examination	**1 (3.6%)***	1 (7.7%)	0 (0%)
Total	Rarely/never has a break	45 (60.0%)	24 (61.6%)	21 (58.3%)
	As they choose	12 (16.0%)	7 (17.9%)	5 (13.9%)
	Predetermined	13 (17.3%)	6 (15.4%)	7 (19.5%)
	After each examination	5 (6.7%)	2 (5.1%)	3 (8.3%)

* the group of respondents over 50 years of age compared to both younger groups $p<0.05$;

In accordance with the previously stated, not only do respondents rarely have a break during working hours, but also the break between examinations. If they do use it, it is usually no longer than five minutes. Age groups do not differ in this respect, nor do men and women within an age group, except in case of the group of respondents over 50 years of age, where all 15 women (100%) reported that they do not have a break at all between two examinations, which is statistically significantly different from the women in other age groups, as well as from the men in the same age group ($p<0.05$) (Table 3).

Table 3. The duration of a break between examinations in respect to gender in different age groups

Yrs	Duration of a break	Total	Men	Women
31-40	Non-existent	14 (66.7%)	9 (69.2%)	5 (625%)
	Up to 5 minutes	6 (28.6%)	4 (30.8%)	2 (25.0%)
	Over 5 minutes	1 (4.7%)	0 (0%)	1 (12.5%)

Yrs	Duration of a break	Total	Men	Women
41-50	Non-existent	17 (65.4%)	10 (76.9%)	7 (53.8%)
	Up to 5 minutes	8 (30.8%)	3 (23.1%)	5 (38.5%)
	Over 5 minutes	1 (3.8%)	0 (0%)	1 (7.7%)
Over 50	Non-existent	24 (85.7%)	9 (69.2%)	**15 (100.0%)***
	Up to 5 minutes	4 (14.3%)	4 (30.8%)	0 (0%)
	Over 5 minutes	0 (0%)	0 (0%)	0 (0%)
Total	Non-existent	55 (73.3%)	28 (71.8%)	27 (75.0%)
	Up to 5 minutes	18 (24.0%)	11 (28.2%)	7 (19.4%)
	Over 5 minutes	2 (2.7)	0 (0%)	2 (5.6%)

* $p<0.05$; women over 50 compared to women in younger groups;
* $p<0.05$; women over 50 compared to men over 50.

As a convenience during work, the respondents mostly use the movable monitor and the possibility to wrap the cable around their arm. This combination is used by 73.3% of all the respondents, and this percentage is similar in all age groups ($p>0.05$). Five respondents (6.7%), besides these two conveniences, also use a cuff, while only one respondent claimed that they did not use any of the conveniences, not even the movable monitor (Table 4). None of the respondents in the entire study stated that they used a cushion or a rolled-up towel as a support for the arm with which they hold the transducer.

Table 4. The use of conveniences during work in different age groups

Yrs	Use of conveniences	Frequency
31-40	None	1 (4.8%)
	Only the movable monitor	3 (14.2%)
	The movable monitor and wrapped cable	16 (76.2%)
	The movable monitor, wrapped cable and a cuff	1 (4.8%)
41-50	None	0 (0%)
	Only the movable monitor	8 (30.8%)
	The movable monitor and wrapped cable	17 (63.4%)
	The movable monitor, wrapped cable and a cuff	1 (3.8%)
Over 50	None	0 (0%)
	Only the movable monitor	3 (10.7%)
	The movable monitor and wrapped cable	22 (78.6%)
	The movable monitor, wrapped cable and a cuff	3 (10.7%)
Total	None	1 (1.33%)
	Only the movable monitor	14 (18.7%)
	The movable monitor and wrapped cable	55 (73.3%)
	The movable monitor, wrapped cable and a cuff	5 (6.7%)

When describing their physical activity, the participants mostly stated that they go for a walk every day (41.3% of all the respondents), and there is no difference among age groups ($p>0.05$). A similar percentage said that they were not at all physically active, except in in the 41-50 age group, where the percentage of the physically inactive is 26.9%. This is statistically significantly less compared to the other two groups (42.8% in the group of the respondents younger than 40, and 46.4% in the group of the respondents older than 50), $p<0.05$. Fewer than 25% of the respondents do an organized physical activity, and this percentage decreases with age, but with no statistical significance. Only one participant practiced yoga, as a way of stretching and relaxation of the locomotor system. The results are presented in Table 5.

Table 5. The type and amount of physical activity in different age groups

Yrs	Physical activity	Presence	Duration (hours/week)
31-40	Not physically active	9 (42.8%)	2.4±2.3
	Organized sports activity	5 (23.8%)	
	Yoga, Pilates	1 (4.8%)	
	Walk	6 (26.6%)	

Yrs	Physical activity	Presence	Duration (hours/week)
41-50	Not physically active	**7 (26.9%)***	2.6±1.7
	Organized sports activity	5 (19.2%)	
	Yoga, Pilates	0 (0%)	
	Walk	14 (53.9%)	
Over 50	Not physically active	13(46.4%)	2.0±1.9
	Organized sports activity	4 (14.3%)	
	Yoga, Pilates	0 (0%)	
	Walk	11 (39.3%)	
Total	Not physically active	29 (38.7%)	2.3±1.6
	Organized sports activity	14 (18.7%)	
	Yoga, Pilates	1 (1.3%)	
	Walk	31 (41.3%)	

* $p<0.05$; 41-50 age group compared to the other two age groups

4. Health issues analysis

About a quarter of the respondents negated any spinal column related pain, while over a half stated that they have pains in one segment of the spinal column (cervical, thoracic or lumbar). This percentage is similar in all age groups ($p>0.05$). Similar distribution can be noticed when the frequency of pain in arm joints is examined (shoulder, elbow and wrist), but there is statistically significantly higher percentage of persons with pain in two joints in 41-50 age group compared to the other two age groups ($p<0.05$). The results are presented in Tables 6 and 7.

Table 6. The presence of spine related pain in different age groups

Yrs	Pain in the spine (cervical, thoracic, lumbar)	Frequency
31-40	Does not experience pain in the spine	5 (23.8%)
	Experiences pain in one segment	13 (61.9%)
	Experiences pain in two segments	1 (4.8%)
	Experiences pain in all three segments	2 (9.5%)
41-50	Does not experience pain in the spine	6 (23.1%)
	Experiences pain in one segment	16 (61.5%)
	Experiences pain in two segments	4 (15.4%)
	Experiences pain in all three segments	0 (0%)
Over 50	Does not experience pain in the spine	8 (28.6%)
	Experiences pain in one segment	14 (50.0%)
	Experiences pain in two segments	5 (17.8%)
	Experiences pain in all three segments	1 (3.6%)
Total	Does not experience pain in the spine	19 (25.3%)
	Experiences pain in one segment	43 (57.3%)
	Experiences pain in two segments	10 (35.7%)
	Experiences pain in all three segments	3 (10.7%)

Table 7. The presence of pain in joints in different age groups

Yrs	Pain in joints (wrist, elbow, shoulder)	Frequency
31-40	Does not experience pain in the joints	5 (23.8%)
	Experiences pain in one joint	16 (76.2%)
	Experiences pain in two joints	0 (0%)
	Experiences pain in all three joints	0 (4.8%)
41-50	Does not experience pain in the joints	7 (26.9%)
	Experiences pain in one joint	12 (46.1%)
	Experiences pain in two joints	**6 (20.1%)***
	Experiences pain in all three joints	1 (3.9%)
Over 50	Does not experience pain in the joints	5 (17.9%)

Yrs	Pain in joints (wrist, elbow, shoulder)	Frequency
	Experiences pain in one joint	20 (71.4%)
	Experiences pain in two joints	3 (10.7%)
	Experiences pain in all three joints	0 (0%)
Total	Does not experience pain in the joints	17 (22.7%)
	Experiences pain in one joint	48 (64.0%)
	Experiences pain in two joints	9 (12.0%)
	Experiences pain in all three joints	1 (1.3%)

When health issues are ranked, relatively low scores are obtained in all three age groups (5.64 to 5.71 points out of the maximum 30). Age groups did not show statistically significant differences (p>0.05). Although there is a trend of higher scores in the female subgroup compared to men in all age groups, the differences are not statistically significant. (Table 8).

Table 8. Average score values of health issues in different age groups

Yrs	Total	Men	Women
31-40	5.71±4.30	5.23±3.61	6.5±5.42
41-50	5.69±3.99	4.92±3.88	5.66±3.85
Over 50	5.64±3.36	4.77±3.11	6.40±3.48
Total	5.68±3.78	4.90±3.45	6.44±4.06

Even though health issue scores are low, there is still statistically significant correlation between that parameter and workload of the respondents, observed through the number of patients examined weekly, in the 41-50 age group. In this group, health issue score also significantly correlates with the number of hours per week during which the respondents are engaged in ultrasound diagnostics (Images 2 and 3). Health issue score also significantly correlates with the number of performed ultrasound examinations per day, not only in this, but in the younger age group as well (Image 4).

Image 2. Correlation between the number of patients per week and the health issue score in different age groups

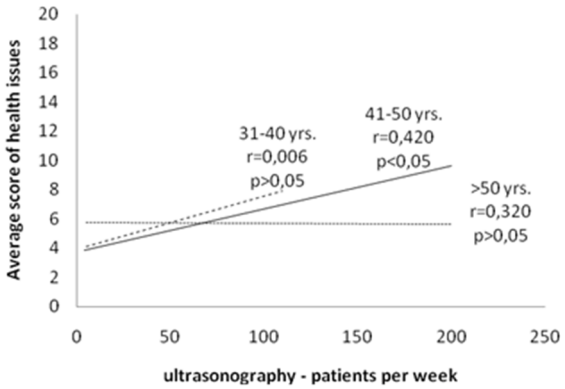


Image 3. Correlation between the number of hours at ultrasound diagnostic jobs per day and the health issues score in different age groups

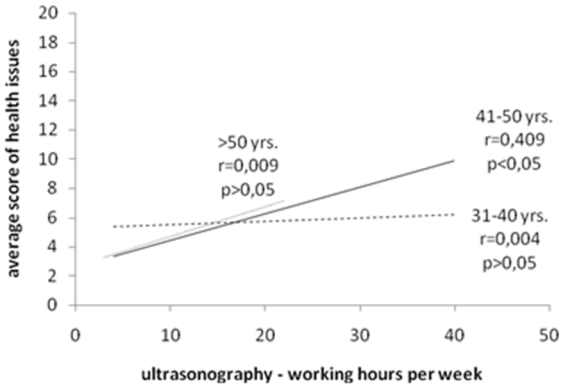
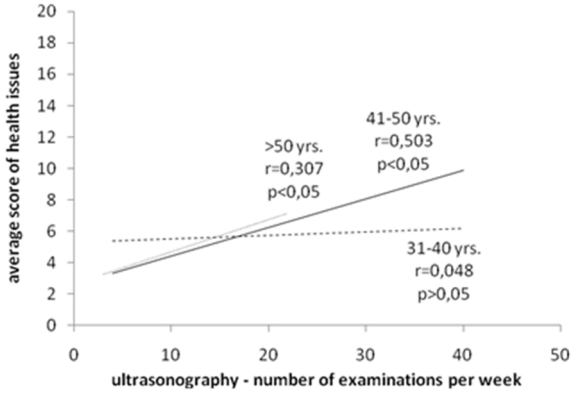


Image 4. Correlation between the number of examinations per week and the health issues score in different age groups



With the aim to determine whether the application of preventative measures influences the mitigation of health issues,

we examined the correlation between the preventative measures implementation score and the health issues score. The obtained correlation coefficients did not point to statistically significant correlation of these two indicators in any of the age groups.

5. Awareness of the respondents

The part of the questionnaire which refers to the employees' awareness of their body position during work and the application of other preventative measures gave the following results:

When it comes to the way in which the respondents get informed about these topics, over a half (52%) stated that information search was self-initiated, while 36% said they were not at all informed. Only in less than 10% of cases was the information provided by the authorities in the institution in which they are employed, and during regular health examinations, information was given by the designated doctors in 2,7% of cases. To the question who is responsible for providing the information regarding these topics in the institutions in which they are employed, almost half of the participants (48%) responded that they think that it is none of the employees, while additional 28% do not know who is responsible. 10,7% of the respondents knows that occupational health service is responsible, 9,3% said that it is persons responsible for health and safety at work, and 4% said that it is the direct management. 40% of the respondents said that the communication with regard to such topics in their institutions is informal, while 26,7% thinks that there is no any communication. Only 20% of the respondents communicate about these topics in meetings, expert seminars and within continuous medical education, while the remaining 13,3% use the Internet as the only communication tool. Half of the respondents think that not enough attention is given to these topics in the institutions where they work, nor are they satisfied with the amount and usability of the obtained information. However, the number of respondents who assessed their knowledge on this topic as sufficient is significantly higher than expected - it is 38,7%. 37,3% of the respondents are partially satisfied with their knowledge, while 24% are not satisfied.

The fact that the awareness of these topics is very low is supported by the average awareness scores. Out of the maximum 34 points, average scores are 12,00±6,87 in the 31-40 age group, 12,15±8,23 in the 41-50 age group and in the over 50 age group the score is the lowest (8,21±6,58), which is statistically significantly less compared to both younger groups ($p<0.05$) (Table 9).

The majority of the respondents (72%) believe that it would be best if the information regarding this issue were presented through training and practical work and through printed materials (61.3%). They think that they would benefit from mandatory meetings and seminars to a lesser extent (46.7%), and the least from consulting the Internet sites (22.7%).

Table 9. Average score values for the awareness in different age groups

Yrs	Total	Significance of the difference
31-40	12.00±6.87	*n.s.*
41-50	12.15±8.23	*n.s.*
Over 50	8.21±6.58	***p*<0.05** compared to the younger groups
Total	10.64±7.42	***p*<0.05**

Discussion

In modern world, there is an increasing need for highly specialized experts in various fields of work. Ultrasound diagnostics is one such field which is more and more present in medical practice. Beside the increased need for experts of such profile, there is also the need for the upgrade of the work environment in terms of the improvement of the ergonomic characteristics of the work place.

Some of the conducted studies showed that more than 80% of ultrasonographers in all specialties, occasionally or constantly feel pain while performing ultrasound examinations, which, on average, as a consequence resulted in 23 hours (2.96 days) of inability to do ultrasound examinations annually (4.18). Average time from the start of regular ultrasound examinations of patients to the occurrence of first musculoskeletal disorders is around five years (19). In order to better understand musculoskeletal disorders in ultrasonographers, various studies examined possible causes of the disorders, such as the number of ultrasound examinations per day and per week, the duration of a break between two examinations, etc. On the other hand, more and more attention is paid to the development of the preventative measures and procedures, which are introduced into the work process through the rules of good practice, and with the aim to limit the occurrence of detrimental positions so as to prevent the development of musculoskeletal disorders (20).

Since 1920s, numerous researches have been conducted which have established the incidence of the diseases of 84% among this population, although there are researches which show much higher incidence, rising up to 90% (21). Such increase if the incidence can be explained in several ways. Firstly, there has been an increase in the life expectancy of ultrasonographers. When the era of ultrasound diagnostics began 50 years ago, only 8.3% of ultrasonographers were older than 50. By 2008, this percentage had grown to 30%. Furthermore, workload has also increased. In the first research of this kind, conducted in 1992, on average, doctors performed about 1500 diagnostic procedures of this type a year, whereas in 2008, it was 2700 procedures, which was an increase of 55.7% (1). In our study, the percentage of doctors over the age of 50 who practice ultrasonography was 37.3% and they have a bit lower workload, i.e. they see 14.54 patients a day, compared to the two younger age groups. The number of examinations on a daily level has also increased

to approximately 10, which is the consequence of the widened range of indications for ultrasound examinations and the advancement of the method which is being increasingly used. The increase in the incidence of the musculoskeletal disorders among the population of ultrasonographers can also be partially explained by drawing attention to this problem. Namely, these diseases have become more and more associated with workplace risks, which was not the case initially: doctors suffered from pains outside workplace as well, sometimes at night, which can indicate that the problem is connected to other factors, rather than professional exposure. Workload of a person who performs ultrasound examinations is higher due to the increase in workload in general. This fact points to the reduction of time allocated for the muscle recovery from the forced position. Research has shown that a muscle can work all day without fatigue if the contraction force does not exceed 10% of the maximum, since blood flow through the muscle in this case enables unhindered elimination of the acidic metabolic products, with simultaneously good supply of oxygen and energy. However, when the frequency and the duration of the load exceed the capabilities of muscles and tendons to adapt, inflammation occurs, which is then followed by degeneration, microtraumas, and finally, scarring (22).

In our research, men and women were equally present, both in the total number of the respondents and in each age group. In the majority of the available literature, researches predominantly involved women (2,3,21,23), which makes our results somewhat more interesting.

In the segment of the questionnaire dealing with workload, the obtained results point to the fact that the respondents over the age of 50 are a bit less burdened, observed through the number of examinations per week, as well as the number of hours performing these jobs per week. In one of the first studies which examined the prevalence of these problems among the population of ultrasonographers and the correlation of their physical symptoms with workload and work habits, a questionnaire was also used (2). The total of 149 questionnaires were analyzed, mostly (80%) filled in by women. The author proved a positive correlation between the severity of the health issues on one hand, and the number of years of service in ultrasound diagnostic jobs, the number of hours and the number of examinations per week on the other. Our results are in accordance with this study, since we also confirmed a direct correlation between the number of examinations per week and the severity of health issues, though only in the 41-50 age group. Nevertheless, in all age groups, except the youngest one, we proved a statistically significant correlation between the number of examinations per day and the severity of health issues. The same authors state that, in their study, they identified 66% of ultrasonographers with health issues connected with work habits (2). In our study, the percentage of the respondents with health issues is somewhat bigger, around 70% in all age groups, regardless of whether the issues are related to the spine or joints. Other studies, however, report an even larger percentage of persons with health issues related to musculoskeletal system and it is between 80% and 90% (5,6,7,12,24,25). A survey study which examined pain in the spinal column in doctors who practice ultrasonography, aged around 28, showed that 84% of the respondents suffered from pain. Our results in the 31-40 age group are quite similar, showing that approximately 23% do not suffer from pain in the spinal column (25). A research conducted with the sample of 26 female sonographers, who had filled in a standardized questionnaire, also showed a significant influence of workload on the frequency of musculoskeletal symptoms. They were present in 96% of cases, despite the respondents' young age (37 years of age on average) and lower number of years of service, both total (9.2 years) and on ultrasonography jobs (6,4 years), compared to our respondents (3).

The data in the literature shows that the risk of these disorders rapidly increases after five years of work in ultrasound diagnostics (26), and our respondents have far more years of service in these jobs (11 years on average, for the whole group). By further analyzing workload, it is seen that the number of ultrasound examinations per week is 60, which is lower than the bottom limit (100 examinations per week) for the development of health issues (26).

Ultrasonographers have various preventative measures at their disposal, some of them are technical-technological, requiring additional equipment, while others refer to programmed physical activity and better work organization (27,28,29,30). It has been shown that doctors spend 68% of examination time in a position which implies shoulder abduction greater than 30º, 63% of time with the external shoulder rotation greater that 30º, and 37% of time with their neck leaning forward, to the side or twisted at the angle greater than 20º. Other than that, with the aid of electromyographic testing of the shoulder muscles, the authors have confirmed a higher risk due to the neck and shoulder muscles straining. In a study from 2016, the authors concluded that ultrasonographers consider their work stimulating but also physically exhausting. They are aware of the ergonomic positions during work, though the comfort of the patient and obtaining good ultrasonographic images often took precedence over the work position (31).

Physical activity is of the utmost importance for the preservation of the overall well-being, especially for the health of the locomotor system. Our respondents do not have a habit of keeping fit by doing an organized physical activity, at least three times a week in the duration of one hour - running, playing football, basketball, tennis, swimming, or doing any other predominantly aerobic sport. Instead, they stated that they go walking (41.3%) or that they are not physically active at all (38.7%).

Health issue scores also do not differ among different ages, but the trend of greater scores can be noticed in case of women compared to men. Although without statistical significance, this trend is in accordance with the results of other researchers (2,3,21,23), with female ultrasonographers more often reporting issues of this kind than their male colleagues.

OSHA (Occupational Safty and Health Administration) guides for safe work state that information and education are also very important for the prevention of work related musculoskeletal disorders, along with the implementation of ergonomic positioning in the work of ultrasonographers (1,27,28). It is said that it is necessary to motivate doctors to learn what is needed for easier and safer work in ultrasound diagnostics, so that it leads to the increase in their efficacy and effectiveness (1,27). Our results, sadly, point to a very low level of awareness among the examined population. Over a half of the respondents, in all age groups, stated that they it was up to them to find information, which is in accordance with the results obtained in other studies, where ultrasonographers also mostly found information on these topics on their own (23). The same authors said that their respondents reported that their superiors in health care institutions did not pay attention to the implementation of the preventative measures and that they did not follow the advancements in the development of ergonomically better equipment. Almost half of our respondents think that no one in the institution where they work is responsible for giving information on these topics, or they do not know who is responsible. The level of awareness is especially low in the over 50 age group, which can be explained by greater use of the Internet in case of the younger groups and obtaining information in this way.

Research points to the fact that insufficient physical activity and too little attention paid to the work conditions are what most ultrasonographers in different studies have in common. The results of these studies are available in the literature (33,34). Unfortunately, our results confirm the ones found in the literature.

CONCLUSION

Over 70% of the participants experience pain in the spine and joints which can be connected to ultrasound diagnostics work, regardless of age. These issues are slightly more frequent in women than in men. Health issues are in a positive correlation with the number of examinations per day and the number of working hours per day, as well as with the number of patients per week, but they do not correlate with the application of preventative measures during work.

The respondents implement the recommendations concerning safety at work and other preventative measures to a very small degree: they rarely have a break during work, they usually examine patients without stopping, they do not use conveniences during work enough and they are not physically active, regardless of age and gender.

Especially important conclusion is the nonexistence of good communication and very low implementation of preventative measures during work, which makes room for preventative-medical action among the population of ultrasonographers. Preventative-medical work should encompass becoming acquainted with the correct body positions during examinations, with the possibilities of the use of the conveniences which help to unburden the muscles and joints, as well as the height adjustment of the chair, desk and examination bed. It would be especially beneficial to organize interactive seminars, where information regarding the usefulness of preventative measures during work would be introduced through training and practical work. Printed materials, especially in the form of posters which would serve as reminders during work, would also have a purpose. However, the most important activity should be directed to motivating ultrasonographers to change their habits and accept recommendations.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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EVALUATION OF PHYSIOLOGICAL INTRACRANIAL CALCIFICATIONS IN CHILDREN USING COMPUTED TOMOGRAPHY

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ABSTRACT

Physiological intracranial calcifications have an increasing prevalence with the age and can be found in both children and in adults. These calcifications are basically asymptomatic and their presence can only be noticed through neuro-imaging. The aim of the paper was to evaluate physiological intracranial calcifications in children using computed tomography, in our conditions. The study was designed as a retrospective, observational, non-randomized clinical study. It was conducted at the Department of Radiology, Clinical Center Kragujevac, Serbia. The study included all the patients scanned by CT from 1st October, 2008 . to 30th September, 2018.. The criteria for the inclusion were: the patients aged up to 18 years who underwent a non-contrast computed tomography in the observed period, with diagnosed intracranial calcifications that do not have pathological etiology. Our study included 420 patients. Out of them, 213 (50.7%) were boys and 207 (49.3%) were girls . The mean age was 12.47. We divided the patients into two age categories: the first one included the patients aged 1 to 10 years and the other one included the patients aged 11 to 18 years. Our study has demonstrated that physiological intracranial calcifications are the most frequent in habenula (28.1%), followed by the pineal gland (22.6%) and choroid plexus (18.8%). There is a small number of studies with the subject of physiological intracranial calcification distribution, especially in children. It is important to know in which locations we can expect physiological intracranial calcifications, as well as the age in which they become detectable by imaging, in order not to mix them with hemorrhages, pathological tumor or metabolic mineralization.

Keywords: *Physiological intracranial calcification, computed tomography, children, pineal gland, habenula, choroid plexus.*

INTRODUCTION

Intracranial calcifications (IC) may be pathological and physiological (1, 2). Pathological calcifications, depending on the etiology, are classified as: infectious, endocrine and metabolic, vascular, neoplastic and congenital (3). There are also IC in which there is no evidence of a disease, they have no pathological cause and are classified as physiological intracranial calcifications (PIC) (2, 4). PIC arise due to the formation of deposits of calcium and iron in the blood vessels of various brain structures (5). These calcifications are basically asymptomatic and their presence can only be noticed through neuro-imaging. Computed tomography (CT) is still the gold standard and the most sensitive modality in IC diagnostics (6).

Physiological intracranial calcifications have an increasing prevalence with the age and can be found in both children and in adults. PIC can be found at the following locations: the pineal gland, choroid plexus, basal ganglia, falx, tentorium cerebelli, lens, petroclinoid ligament, dura mater, sagittal sinus and habenula (1, 7). Calcifications in the epiphysis (also referred as the pineal gland) appear from the fetal age, they are seen in two-thirds of the adults and their incidence increases over the years. Pineal calcifications that are greater than 1 cm in diameter or occur in children under 9 years of age may indicate a neoplasm (8, 2). Habenular calcification occurs in 15% of the elderly population and PIC in the basal ganglia have the incidence of 0.3% to 1.5%. Other locations have a total frequency of about 10% in the elderly (3). Before, it was thought that pineal calcification in children under 6 years of age is always pathological, but this does not apply in the modern era of CT. A study dealing with PIC prevalence in children showed that epithalamus (epiphysis and /or habenula) calcification occurred in 13% (63/500), and pineal in 5%, horrible in 12% (9). A significant association between the patients with choroid and pineal calcifications in children ($p = 0.003$) was reported (9).

There is a small number of studies with the subject of PIC distribution, especially in children. It is important to know in which locations we can expect physiological intracranial calcifications, as well as the age in which they become detectable by imaging, in order not to mix them with hemorrhages, pathological tumor or metabolic mineralization (10,11,12, 13,14).

The aim of this study is to evaluate physiological intracranial calcifications in children using computed tomography, in our conditions.

MATERIAL AND METHODS

The study was designed as a retrospective, observational, non-randomized clinical study. It was conducted at the Department of Radiology, Clinical Center Kragujevac, Serbia, and encompassed all the patients scanned by CT from 1st October, 2008 . to 30th September, 2018.

The criteria for the inclusion were: the patients aged up to 18 years who underwent a noncontrast computer tomography in the observed period, with diagnosed intracranial calcifications that do not have pathological etiology. The follow-up CT scans were not taken into account.

The presence of intracranial calcification on the computed tomography scans is defined as hyperatenuated matter in comparison to the gray matter. The diagnosis of IC was made by two radiologists with the experience in CT reading for 20 years.

The exclusion criteria were: pathological etiology, CT scans with artifacts, the use of contrast media, the patients who underwent surgery, presence of bleeding or neoplasms on CT which made the visualization of IC more difficult.

Imaging for all the patients was done on 64-slice MDCT (Aquilion 64, Toshiba, Japan). CT scans were done in the axial plane, with multiplanar reconstructions and 3D reconstructions. The analysis of the scans and MDCT data was done on the work station Vitrea 2 Workstation ver.4.1.14.0 (Vital-Images), while using commercially available software (Imaging Software ver.4.1.14.0, Vital-Images, Canon Group, USA).

The data were collected in Microsoft Office Excel and transferred to the SPSS (SPSS 21.0; IBM, Armonk, New York) for the analysis. The statistical analysis of the data was accomplished primarily with descriptive statistics. The correlations were made by using the Pearson product-moment correlation coefficient where p value less than 0.05 was considered significant.

RESULTS

Our study included 420 patients. Out of them, 213 (50.7%) were boys and 207 (49.3%) were girls . The mean age was 12.47. We divided the patients into two age categories: the first one included the patients aged 1 to 10 years and the other one included the patients aged 11 to 18 years. The prevalence of physiological intracranial calcifications on the explored locations, as well as their distribution by the gender and age are shown in Table 1.

Our results have shown that there is a significant correlation between pineal and habenular PIC ($p=0.003$), pineal and sagital sinus PIC ($p=0.009$) and tentorium cerebelli and petroclinoid PIC ($p=0.000$).

It has also been shown that there is a positive corretlation between the patients' age and presence of PIC ($p=0.001$). Intracranial calcifications were more frequent in older children, who were between 11 and 18 years old. Out of nine explored locations on computed tomography, two (basal ganglia and dura mater) showed no presence of PIC. Also, four examined

locations didnt have calcifications in children who were 10 years old and younger (Table 1).

Distribution of physiological intracranial calcifications in children depending on the age group are shown in Figure1, Figure 2 and Figure 3.

Figure 1. Chart shows the distribution of PIC in explored locations depending on age

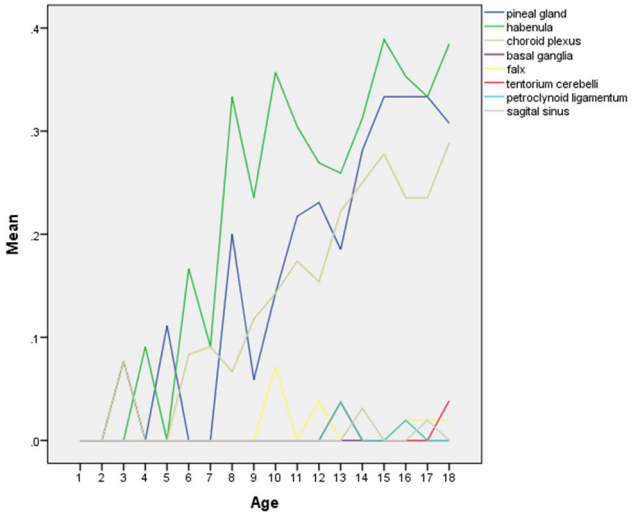

Figure 2. Distribution of pineal gland PIC by two age groups

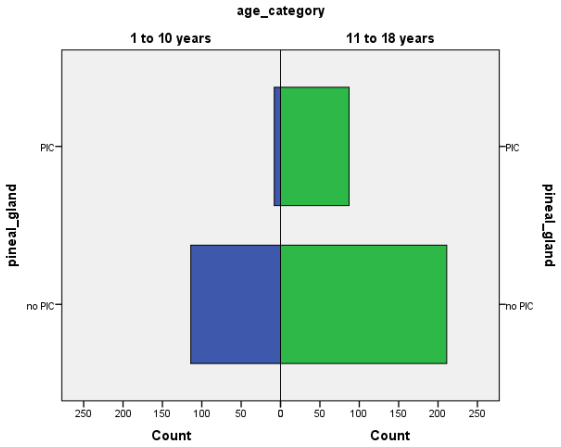

Figure 3. Distribution of habenular PIC by two age groups

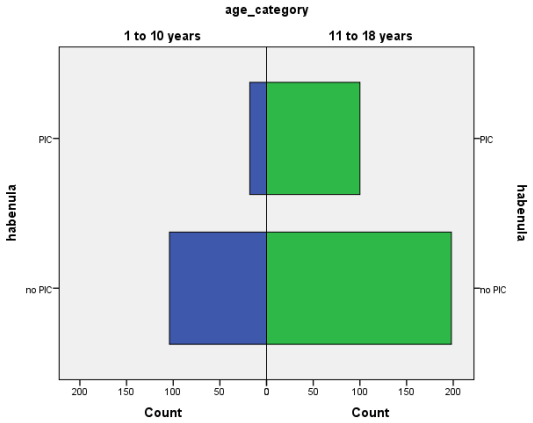

Table 1. Prevalence and distribution of the physiological intracranial calcifications on the explored locations by gender

PIC	Prevalence n, %	Gender n, %	Age from 1 to 10 n, %	Age from 11 to 18 n,%
Pineal gland	95 (22.6%)	m: 52 (54.7%) f: 43 (45.3%)	8 (1.9%)	87 (20.71%)
Habenula	118 (28.1%)	m: 66 (55.9%) f: 52 (44.1%)	18 (4.28%)	100 (23.81%)
Choroid plexus	79 (18.8%)	m: 33 (41.8%) f: 46 (58.2%)	8 (1.9%)	71 (16.9%)
Basal ganglia	0	m: 0 f: 0	0	0
Falx	6 (1.4%)	m: 2 (33.3%) f: 4 (66.7%)	1 (0.24%)	5 (1.19%)
Tentorium cerebelli	3 (0.7%)	m: 0 f: 3 (100%)	0	3 (0.71%)

PIC	Prevalence n, %	Gender n, %	Age from 1 to 10 n, %	Age from 11 to 18 n,%
Petroclynoid ligamentum	2 (0.5%)	m: 1 (50%) f: 1 (50%)	0	2 (0.48%)
Sagital sinus	2 (0.5%)	m: 2 (100%) f: 0	0	2 (0.48%)
Dura matter	0	m: 0 f: 0	0	0

Typical presentation of physiological intracranial calcifications in children are presented in Picture 1 and Picture 2.

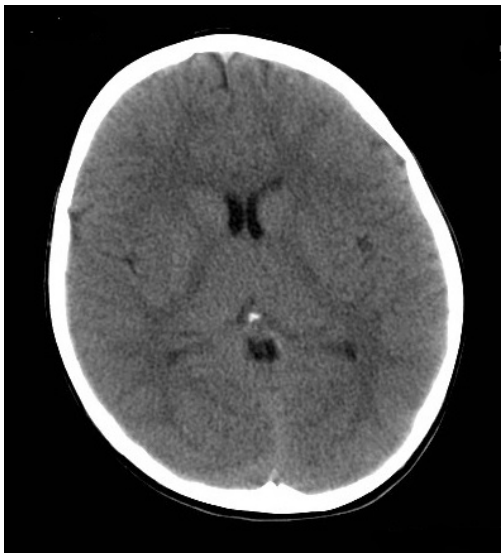

Figure 1. Habenular IC in 7 year old girl

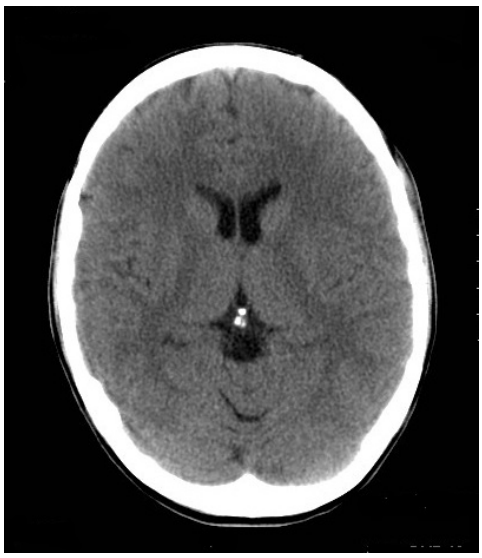

Figure 2. Pineal and habenular IC in 14 year old boy

DISCUSSION

Our study has demonstrated that physiological intracranial calcifications are most frequent in habenula (28.1%), followed by the pineal gland (22.6%) and choroid plexus (18.8%). Because of that, these three locations would be thoroughly discussed.

The previous study presented that PIC in habenula had the prevalence of 10% (the total number of patients was 500) and none younger than 2 years (9,15). Our prevalence was higher and our youngest patient with habenular PIC was 4 years old. Our study also showed a positive correlation between habenular and pineal PIC, which is understandable, due to their location and closeness (16,17).

Unlike habenula, there are more published data regarding pineal PIC in children. The prevalence of the patients younger than 10 years, was 8%, where the minimum age was 3 (15). The other one presented the prevalence of 5% and the minimum age was also 3 (9). In an older study, 3% of pineal PIC were found in babies before their first birthday (18). The prevalence of pineal PIC in our study was higher (22.6%), but our youngest patient was also 3 years old. The difference in the prevalence can be described by the age limit in the studies and different CT scanner. Some of the studies explored the patients from the birth, while others used the first birthday as the lower age limit. Few studies only explored the patients younger than 10 or 11 years. Also, the upper age limit varied from 15 to 20 years. Due to that, the incidence and other data varied from study to study. In addition to this, the technological advance of computed tomography allowed better resolution and more slices which enabled better diagnostic possibilities.

Choroid plexus PIC were the third most common PIC in our study (18.8%). The previous published data vary regarding this location (19,20,21,22). More recent studies reported the prevalence of 16% and 12% (9,19). Older studies had the prevalence of 0.5% in the first decade and 2% in the patients younger than 8 years (20,21). Our youngest patient was 3 years old. The prevalence in the first decade was 1.9% which is similar to the results published in 1980 and 1986. We haven't found a positive correlation between choroid plexus and pineal PIC which was reported previously (9,15).

CONCLUSION

There are very scarce data on physiological intracranial calcifications, seen by CT in children (23). However, these findings should be given more thought, especially due to the fact that its prevalence increases with the age. It would be important to follow up children with physiological intracranial calcifications and see if there is a long term correlation with other conditions and calcifications (24).

Also, other imaging modalities, like magnetic resonance imaging should be used (25-27).

This would require a large multicentric study and these results would give us better perspective for the clinical significance of physiological intracranial calcifications, which is why we recommend further investigations with this topic.

ETHICS APPROVAL

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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METHANOLIC EXTRACT OF TEUCRIUM POLIUM EXERTS IMMUNOMODULATORY PROPERTIES IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

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ABSTRACT

Teucrium polium has been used in traditional medicine around the world for centuries in treatment of various conditions and diseases. Many studies have confirmed pharmacological effects of its extracts, although the immunomodulatory effect has not been investigated. Therefore, the aim of our study was to examine the immunomodulatory effect of methanolic extract of T. polium (TPE) on peripheral blood mononuclear cells (PBMCs) derived from healthy donors and patients with hepatitis C virus HCV infection. We analyzed the effect of the extract on PBMCs viability using the MTT test. The cell death type was determined using Annexin V-FITC/7-AAD staining. Immunophenotyping using anti-CD8 FITC, anti-CD4 PE, anti-CD3 ECD, anti-CD20 PC5, anti-CD14 FITC and anti-CD25 PC7 was performed by flow cytometry. Results of the MTT test indicate that TPE stimulates proliferation of healthy PBMCs, while the HCV PBMCs viability was slightly reduced. The percentage of apoptotic HCV PBMCs was higher after TPE treatment compared to the control. The proportion of CD25-expressing cells was higher among the untreated HCV PBMCs than in the untreated healthy PBMCs. TPE treatment significantly and gradually increased CD25 expression in healthy PBMCs, whereas CD25 expression on HCV PBMCs increased only at the highest TPE concentration. The upregulation of double-positive CD3+CD25+, CD20+CD25+ and CD14+CD25+ cells was significant in TPE treated healthy PBMCs, while only the highest concentration was effective on HCV PBMCs. In summary, TPE exerts a strong immunomodulatory effect on healthy PBMCs and, only at the highest concentration, on HCV PBMNCs.

Keywords: *T. polium, immunomodulation, PBMC, HCV.*

INTRODUCTION

Since ancient times, plants have been used as an alternative source for treatment of different diseases. Inter alia, some plants were used in therapy of some diseases due to their immunomodulatory effect (1). Evaluation of the immunomodulatory activity of plant extract is an interesting and constantly growing area of research.

Excessive or suppressed immune response leads to autoreactivity, inflammation or increased susceptibility to pathogens. Therefore, immunomodulatory drugs are used in the treatment of many diseases with immunopathological background. Modulation of the immune system represents any change in the immune response regarding induction, expression, amplification, or inhibition of any part or stage of the immune response (2). Immunopharmacology, as a developing branch, aims to manipulate the immune system by modifying endogenous immune responses and many researchers are focused on ethnomedicinal plants and their products as a source of immunomodulatory substances (2, 3). The immune-response-modulation activity of plant extracts and their active compounds has become an acceptable therapeutic measure. Determination of immunomodulatory properties and the mechanism of action of herbal medicine provides affordable, easy access and low side effect agents as a complementary part of therapeutic protocols (4). Secondary metabolites of plants, such as sterols, alkaloids, glycoproteins, polysaccharides and flavonoids are responsible for immunomodulatory effects of plants (3).

Teucrium polium L. (Labiatae) is a traditional medicinal plant widely distributed in the Mediterranean countries. It has been traditionally employed for various types of pathophysiological conditions as antidiabetic, anti-inflammatory, antiulcer, hypotensive, antispasmodic, anorexic, wound-healing and antipyretic agent. Remedial effects are prescribed to tannins, terpenoids, saponins, sterols and flavonoids content. Numerous *in vitro* and *in vivo* studies confirmed the pharmacological effects of *T. polium* (5). Oral administration of the crude extract during 6 weeks in diabetic rats significantly reduced blood glucose concentration in streptozotocin-induced diabetes in rats (6). Antioxidant testing showed that the aqueous extract of *T. polium* inhibited β-carotene oxidation, AAPH-induced plasma oxidation, Fe^{2+}-induced lipid peroxidation in rat liver homogenates, scavenged free oxygen species, bound iron and increased intracellular GSH levels in HepG2 cells (7). Ethanolic and methanolic extracts exhibited a marked antimicrobial effect on both Gram-negative and Gram-positive bacteria (8). *In vitro* antitumor study underlined the methanolic extract as an effective and safe chemosensitizer in tumor therapy. Namely, the ethanolic extract in combination with vincristine, vinblastine or doxorubicin produced a strong inhibitory effect on tumor cell lines, whereas non-transformed fibroblasts were negligibly affected (9). In a study focused on the anti-inflammatory effect, the ethanolic extract significantly inhibited cotton-pellet granuloma and carrageenan- induced inflammation (10).

To the best of our knowledge, there is no study investigating the immunomodulatory effect of *T. polium* extract. Therefore, this research is focused on examination of the immunomodulatory potential of *T. polium* methanolic extract on peripheral blood mononuclear cells derived from healthy blood donors and patients with chronic hepatitis C infection (HCV).

MATERIALS AND METHODS

Subjects

Five patients with chronic HCV infection were recruited in the Clinical Center of Kragujevac, Serbia. An inclusion criterion was the presence of liver cirrhosis. The liver biopsies were performed and patients were graded and staged according to Knodell et al. (11). The patients coinfected with other hepatotropic viruses or with any possible causes of liver injury (alcohol, autoimmune diseases) were excluded from the study. None of the patients had been previously treated with immunomodulatory agents. Five healthy controls were selected from the hospital staff of similar age and with no history of liver diseases.

Isolation of peripheral blood mononuclear cells

Peripheral blood of healthy volunteers and HCV patients was collected in heparin-coated tubes and mononuclear leukocytes were isolated by density gradient centrifugation (Histopaque 1077, Sigma, Germany). Peripheral blood mononuclear cells (PBMNC) were then washed three times and finally suspended in the supplemented culture medium RPMI 1640 (10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin, all from Sigma, Germany). The cell number and viability were determined using Acridine orange/Ethidium bromide staining (all from Sigma, Germany).

Plant material and preparation of extract

Arial parts of *Teucrium polium* were collected from the natural population in the territory of Kragujevac – central Serbia, during June 2015. The voucher specimen was confirmed and deposited at the Herbarium of the Faculty of Science, the University of Kragujevac. The sampled material was dried at ambient temperature in a dark place. The air-dried material was milled in a grinder. The prepared plant material (10 g) was transferred into a dark-colored flask, filled with 200 ml of methanol and stored at room temperature. After 24 h, the infusion was filtered using Whatman No. 1 filter paper and residue was re-extracted with an equal volume of solvent. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 °C. The obtained extract was kept in a sterile sample tube and stored at + 4°C until the analysis. The stock solution was prepared as 100mg/ml DMSO and kept at +4°C. The content in *T. polium* methanol extract was defined by Stankovic et al. (12).

Cell viability assay

The effect of *Teucrium polium* extract (TPE) on peripheral blood mononuclear cells was determined by MTT assay that is widely used for assessing cell proliferation, cell viability, and/or cytotoxicity (13, 14). Isolated PBMNCs were grown in 96-well plates at a starting density of $0{,}2x10^6$ cells/well in presence of TPE (5, 10, 50, 100 and 500 µg/ml) or in medium alone (control). The cells were cultured for 24h at 37°C in 5% $C0_2$. The cultured cells viability was determined by assaying the reduction of MTT to formazan. In short, after the incubation, media was removed and MTT (0.5mg/1ml of PBS) was added to each well. The cells were then incubated at 37 °C for 4h, and DMSO (150µl/well) was added to dissolve the formazan crystals. Absorbance was measured at 550 nm with a multiplate reader (Zenith 3100, Anthos Labtec Instruments GmbH, Austria). The results were presented as relative to the control value (untreated cells).

Flow cytometric analysis

Freshly isolated PBMNCs were incubated with TPE (5µg/ml, 50µg/ml and 500µg/ml) or with media alone (control) for 24 h at 37 °C in an atmosphere of 5% CO_2 and absolute humidity. Then, the cells were harvested, washed in PBS and used for determination of apoptosis/necrosis and immunophenotyping.

Determination of apoptosis/necrosis

For apoptosis/necrosis detection, an Annexin V-FITC/7-AAD Kit was used according to the manufacturer's instructions (Beckman Coulter, USA). In short, PBMNCs ($2x10^5$ cells) were suspended in 100µL of ice-cold binding buffer. The cells were stained with 10µl of Annexin V-FITC and 20µl of 7-AAD and incubated for 15 minutes at +4ºC in the dark. Then, 400µl of binding buffer was added to each tube and the samples were immediately analyzed by flow cytometer Cytomics FC500 (Beckman Coulter). The data were analyzed using Flowing Software (http://www.flowingsoftware.com/).

Immunophenotyping

PBMNCs ($2x10^5$ cells) were suspended in 100µl of PBS and stained with anti-CD8 FITC, anti-CD4 PE, anti-CD3 ECD, anti-CD20 PC5, anti-CD14 FITC and anti-CD25 PC7 (Beckman Coulter) and isotype controls (Beckman Coulter) for 20 minutes in the dark. Then, 400µl of PBS was added to each tube and the samples were acquired on a Cytomics FC500. The data were analyzed with Flowing Software.

Statistics

Statistical analysis was performed in SPSS program (version 19.0, SPSS Inc., Chicago, IL). Normality of the data distribution was determined by Kolmogorov-Smirnov test. Comparing the data between the patient and control group was performed using Mann-Whitney *U* test. P-values <0.05 and <0.001 were considered statistically significant. The data are presented as column charts.

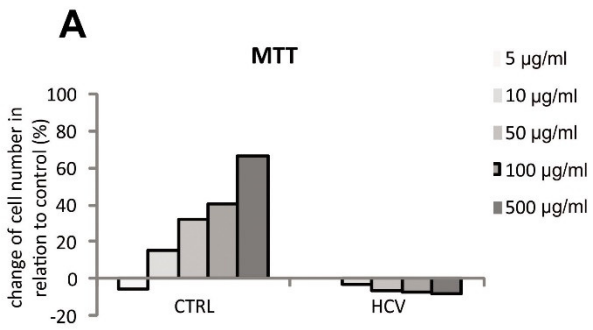
RESULTS

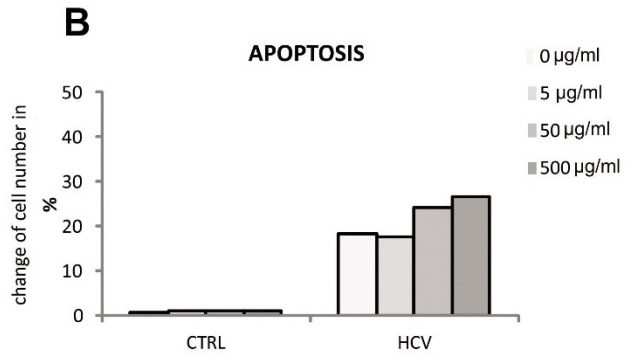
T. polium extract exerts different effects on viability of healthy and HCV patients PBMCs

Firstly, the effect of TPE on viability of isolated PBMCs was investigated by the MTT assay and flow cytometry. The MTT test showed not only that TPE is not cytotoxic to PBMCs from healthy individuals, but also it stimulates their proliferation, and this effect was dose-dependent: 10µg/ml induced 15,29%, 50µg/ml 32,04%, 100µg/ml 40,44% and 500µg/ml 66,79% increase in a cell number in relation to control, untreated cells.

Since we established that the TPE treatment stimulates proliferation of healthy PBMNCs, further, we included the TPE testing on PBMCs derived from HVC patients. Contrarily to healthy donors PBMCs, the MTT test showed that 24h-treatment with TPE slightly decreased viability of PBMCs from HCV patients (Figure 1A.). Flow cytometry using Annexin V/7-AAD staining demonstrated a high incidence of apoptosis in untreated HCV cells (18,2%) and an increase in percentage of apoptotic cells after the treatment with 50 and 500µg/ml TPE (24,19% and 26,63%, respectively). There was less than 1% apoptosis in healthy PBMCs in any concentration used (Figure 1B.). In both healthy and HCV PBMCs, the percent of necrotic cells was negligible.

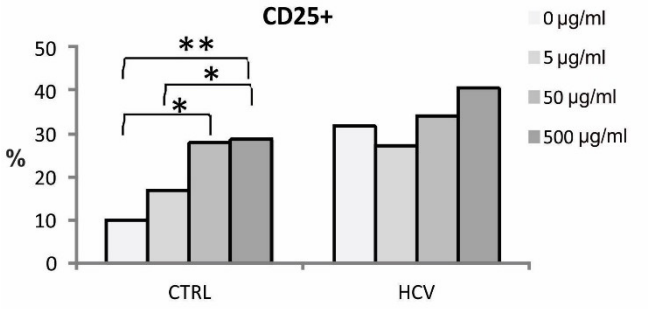
Figure 1. The effect of TPE on healthy (CTRL) and HCV PBMNCs after 24h-treatment determined by the MTT assay (A) and flow cytometry (Annexin V/7-AAD) analysis (B).





The results are median from five independent experiments.

Figure 2. Expression of CD25 in untreated and TPE treated PBMNCs of healthy individuals (CTRL) and HCV patients (HCV).



The results are median from five independent experiments. *p<0.05; **p<0.001

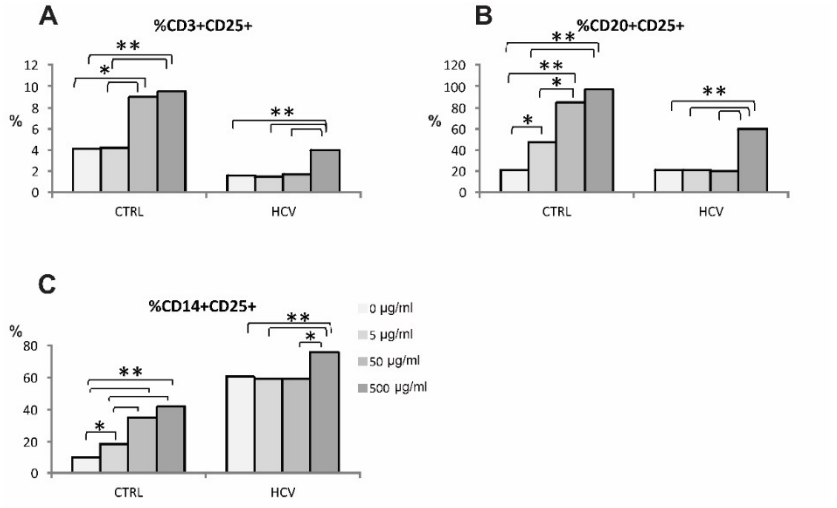
TPE-treated healthy and HCV patients PBMNCs have different immunophenotype characteristics

Further, we analyzed the expression of CD25 as an activation marker on peripheral blood mononuclear cells. In healthy untreated PBMCs, about 10% of population expressed CD25 and after the treatment with TPE, the number of activated cells significantly increased: 5µg/ml – 16,75%, 50µg/ml – 27,84%, p<0,05 and 500µg/ml – 28,56% (p<0,001). In the population of untreated PBMNCs isolated from HCV patients, there was a higher percent of CD25+ cells (31,76%) than in untreated CTRL PBMNCs. The TPE treatment resulted in a slight increase of CD25 expression (50µg/ml – 33,96 and 500µg/ml – 40,63%), but without statistical significance (Figure 2.).

In order to demonstrate better the effect of TPE on CD25 expression, we calculated the percentage ratios of CD25+ cells in PBMNCs populations. As shown in Figure 3, the TPE treatment in healthy controls engendered a gradual increase of CD25 expression in CD3+, CD20+ and CD14+ cells, while in HCV PBMNCs only the highest tested concentration affected CD25 expression.

In healthy controls, the treatment with 50 and 500µg/ml TPE resulted in more than a double increase in the percent of CD3+CD25+ cells, from 4,09% in untreated cells to 9,04% and 9,49%, respectively, while in HCV patients only the highest TPE concentration had the same effect (control: 1,63%; 500µg/ml: 4,02%). The percent of CTRL CD25+ B lymphocytes gradually increased from 20,87% in untreated cells to 97,65% in cells treated with 500µg/ml TPE. In HCV B lymphocytes, only the highest concentration of TPE influenced CD25 expression (60,11% CD25+ cells in comparison to 21,30% in untreated cells) (Figure 3B.). Similarly, in CD14+ subpopulation, the percent of CD25+ cells gradually rose from 9,30% to 42,04% in healthy controls, whereas in HCV patients, the expression of CD25 increased only after the treatment with 500µg/ml TPE (Figure 3C.).

Figure 3. The percentage ratio of CD25+ cells in CTRL and HCV CD3+ lymphocytes (A), CD20+ lymphocytes (B) and CD14+ cells (C).



The results are median from five independent experiments. *p<0.05; **p<0.001

DISCUSSION

The immune system is a highly regulated host defense mechanism playing the main role in maintaining homeostasis and disease-free state of the body. Modulation of the immune response, in terms of either stimulation or suppression, takes part in mitigating many diseases. Therefore, immunomodulators are used to manage a variety of disease conditions. This study examined the immunomodulatory activity of the methanolic extract of T. polium by assessing its effect on viability, apoptosis induction, immunophenotypic characteristics and activation status of PBMNCs obtained from healthy donors and HCV patients.

Chronic hepatitis C infection represents one of the leading cause of morbidity and mortality globally. Recent studies have recorded a significant increase of HCV-infected people in the last decade, with prevalence of HCV infection worldwide estimated at 2.5% (15). The WHO strategy for viral hepatitis, which includes the administration of antiviral drugs, vaccinations and various prevention programs, provides an assessment of the elimination of viral hepatitis worldwide by 2030. (16). Acute HCV infections are usually asymptomatic or with mild symptoms and in about 70-80% of cases, the infection goes into the chronic phase (17). The eradication of viruses in the acute phase results from the mechanisms of cell-specific immunity, in particular HCV-specific cytotoxic CD8 + T-lymphocytes and antiviral cytokines. The inability of the immune system to eradicate the virus results in persistent infection, which leads to chronic HCV infection. During the chronic infection, liver fibrosis develops in most patients, translating into cirrhosis in 15-25% of patients over a period of 10 to 40 years from the infection (18).

HVC persistence is repercussion of the viral ability to evade the immune surveillance by viral mutation, inhibition of innate immune cells by HCV proteins or by alteration of both arms, innate and adaptive, of the immune response which results in a gradually deterioration of the immune response. A strong and persistent cellular immune response is necessary for elimination of HCV virus. However, the adaptive immune response is often delayed in HCV infection, allowing HCV virus to spread before the host establishes effective T and B cellular response (19). Chronic antigen load and changes within targeted epitopes, resulting from a high viral error prone replication, trigger an excessive and prolonged activation of T cells, but they fail to hold on with viral epitope changes and elimination of viral infected cells (20). Waning of T cell response is depicted with an increased susceptibility to apoptosis (21, 22), disruption of differentiation, proliferation and effector functions (23). All of these are characteristics of the terminally exhausted immune response, particularly viral specific CD8+ T cells, that is mediated by the expression of numerous inhibitory molecules (23). Exhausted T cells highly express two or more inhibitory receptors such as, in the first place PD-1, CTLA-4, then lymphocyte activation gen 3 protein (LAG3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), 2B4 (known as CD244), but also T-box transcription factor and reduce expression of the IL-7 receptor α chain (CD127) (23, 24). In addition, directed T cell response collapses, resulting in a global immune dysfunction.

Based on the MTT test results and flow cytometry analysis, T. poluim methanolic extract exerted its non-cytotoxic and proliferative properties on healthy PBMNCs. As opposed to healthy PBMCs, untreated HCV PBMCs are prone to spontaneous apoptosis, whereby the TPE treatment further augmented apoptotsis in a dose dependent manner. Since spontaneous apoptosis and impaired proliferation are one of the main features of exhausted immune response in the chronically infected patients, HCV PBMCs under pressure of a proliferation stimulus to which they cannot respond spontaneously undergo apoptosis (21).

Many plants and plants-derived compounds have been shown to exert a proliferative effect on PBMCs (25-27). Primarily, we have to mention phytohemglutinin and concavalin A as well-known mitogens in common proliferation models (27). Several previous studies showed that some flavonoid and phenolic compounds increase a proliferative response of PBMCs from healthy individuals (25, 26), despite their reputation as immunosuppressants. Namely, flavonoid compounds like cyanidanol, derivates of (+)-3-methoxy-5,7,3',4'-tetrahydroxyflavan, (+)-3-palmitoyl-5,7,3',4'- tetrahydroxyflavan (25) and baicalin and baicalein derived from Plantago genus (26) stimulated proliferation of human PBMCs at lower concentration. Jose J et al. reported a similar effect of flavonoid from Phyllanthus niruri on un-stimulated human PBMCs (28). Further, the stimulatory effect of cyanidanol on in vitro lymphocyte responses in healthy individuals and in patients with chronic active hepatitis B was recorded (29, 30). Phenolic compounds like p-coumaric acid and vanillic acid have a high proliferative capacity with the stimulation index equal to 4.59 for PBMCs (26). The extracts of Teucrium genus plants have a high content of phenolic and flavonoid compounds which are carriers of various biological activities. Stankovic et al. have determined flavonoid and phenolic content of the examined extract, showing that the methanolic extract of T. polium leaves had a higher flavonoid concentration compared to the methanolic extract of Green tea as a reference substance and the highest phenolic content compared to the extract from other solvent (12). Flavonoids are known for their antioxidant properties, due to the ability to reduce free radical formation and to scavenge free radicals. The interaction of flavonoids with cell membrane lipids has been demonstrated. Also, some flavonoids inhibited catabolism of cyclic GMP leading to a high level of this nucleotide (31). Nevertheless, the mechanism of proliferative effect on PBMCs of some flavonoids has not been elucidated.

In the final step, we performed imunophenotyping of TPE-treated PBMCs in order to examine changes in the activation status and ratio of activated PBMCs population of both healthy individuals and HCV patient derived PBMCs.

Notable higher percentage of activated (CD25+) PBMCs in healthy controls is in line with previously mentioned prolonged and excessive activation of immune cells due to HCV agility (20). It should be noted that an increase of CD25 expression upon the treatment with TPE was more pronounced in all population of healthy PBMCs than in HCV PBMCs at the highest TPE concentration. HCV PBMCs inability to respond to weak stimuli, but only to stronger ones, such as the highest TPE concentration, is due to the outworn and exhausted immune response (23, 24). At the same time, the percent of CD14+CD25+ was multiple times higher in untreated HCV PBMCs than in healthy PBMCs, as a result of excessive stimulation present in chronic HCV infection (20).

CONCLUSION

In summary, the methanolic extract of *T. polium* exerts a strong immunomodulatory effect on healthy PBMCs. It stimulates the proliferation and activation of healthy T lymphocytes, B lymphocytes and monocytes, while the stimulation of HCV PBMNCs activation was present, albeit to a lesser extent, only at the highest concentration. Future research should be directed towards determining the type of immunomodulatory effect and possible complementary administration of TPE in the treatment of a patient with chronic HCV infection.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the local Ethics Committee (01-6427/5), and written informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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None.

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SUPEROXIDE DISMUTASE 2 VAL16ALA POLYMORPHISM IS ASSOCIATED WITH AMIODARONE-ASSOCIATED LIVER INJURY

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ABSTRACT

Association of SOD2 V16A single-nucleotide polymorphism (rs4880) with drug hepatotoxicity were reported but relationships with amiodarone prescriptions remained unexplored. Research was an exploratory, controlled prospective clinical trial. Patients hospitalized and treated in Clinical Center in Kragujevac, Serbia (in year 2017) were divided into experimental (using amiodarone, having liver injury, n=29, 19 males, the mean age 66.8±10.4 years), control A (neither amiodarone use nor hepatotoxicity, n=29, 19, 66.1±10.3) and control B group (using amiodarone, not having hepatotoxicity, n=29, 19, 66.8±9.8). From blood samples, among other routine biochemistry, genotyping for SOD2 polymorphism Val16Ala was conducted using real-time PCR method with TaqMan® Genotyping Master Mix and TaqMan® DME Genotyping Assay for rs4880. Patients taking amiodarone and having liver injury were mostly carriers of Val/Val (TT) genotype (13 of 24 patients, 54.2%) while Val/Ala (TC) and Ala/Ala (CC) genotypes prevailed in control group A (19 of 40, 47.5%) and control group B (9 of 23, 39.1%), respectively (2=10.409, p=0.034). Frequency of Val (T) and Ala (C) alleles were 0.51 and 0.49, respectively in the whole study sample (Hardy Weinberg equilibrium, 2=0.56, p=0.454). Carriers of TT genotype had significantly higher ALT (437.0±1158.0 vs 81.9131.5 U/L), total bilirubin (28.320.5 vs 15.313.0 mol/L) and total bile acid concentrations (10.910.2 vs 6.45.3 mol/L) compared to carriers of TC genotype (U=2.331, p=0.020, U=3.204, p=0.001 and U=2.172, p=0.030, respectively). Higher incidence of 47T allele of SOD2 was inpatients with amiodarone-associated liver injury as compared to patients on amiodarone not experiencing hepatotoxic effects.

Keywords: *Superoxide dismutase; polymorphism, single nucleotide; amiodarone, chemical and drug induced liver injury.*

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INTRODUCTION

Nowadays, amiodarone represents one of the most important antiarrhythmic drugs. Yet, its prescriptions are rather restricted due to numerous adverse reactions (1): it accumulates in tissues and cells, damaging phospholipid membranes, lysozymes and mitochondria (2-4). It is well known that amiodarone inhibits phospholipases (enzymes targeting the membrane phospholipids), but the effect on other signaling molecules is less understood. It has been reported that amiodarone could also increase the synthesis of hydrogen peroxide, leading to a state of oxidative stress (5).

Several studies have demonstrated association between the toxic effects of amiodarone and the activity of superoxide dismutase (SOD), an important enzyme for defense against oxidative stress, such as the induction of pulmonary fibrosis (6) or the direct cytotoxicity (7).On the other hand, amiodarone had variable influence on SOD activity within the patients' erythrocytes, depending on the existence and the type of adverse reactions (8). Differences in the observed effects of amiodarone could be based on different tissue and/or cellular distribution of individual forms of this enzyme. Superoxide dismutases (SODs) are involved in the reaction which converts superoxide into oxygen and hydrogen peroxide, and they also control the concentrations of several other reactive molecules (9). There are tree known isozymes of SOD in mammals: SOD1 (cytoplasmic form), SOD2 (mitochondrial form) and SOD3 (extracellular form) (10).

The role of genetic polymorphism of variety of proteins associated with drug-induced liver injury (DILI) has recently been emphasized (11) and several studies reported significant association of *SOD2* V16A single-nucleotide polymorphism (rs4880) with hepatotoxic effects of drugs (12,13). However, in the studies evidencing causal relation between SOD2 genetic polymorphism and DILI, patients did not use amiodarone (12-14). As the knowledge about importance of the genetic polymorphism of SOD enzymes on the organtoxicity of amiodarone, including liver damage, is lacking, the aim of our study was to investigate the association between amiodarone use, acute hepatotoxic events and *SOD2* polymorphism rs4880 in patients hospitalized due to cardiovascular disease.

PATIENTS AND METHODS

The research was designed as an exploratory, controlled, three-arm prospective study, with cross-sectional approach, which included 87 patients hospitalized and treated in Clinical Center "Kragujevac" in Kragujevac, Serbia, during the year of 2017. Experimental group consisted of patients who were treated with amiodarone and experienced the symptoms and/or signs of liver injury. There were two groups: the first (A) included patients not treated with amiodarone and not having hepatotoxicity, while the second (B) consisted of patients treated with amiodarone and not experiencing any symptoms or signs of hepatotoxicity during the entire follow-up.

The inclusion criteria for the experimental group were: age between 17 and 75 years, amiodarone use and the presence of 3-fold elevated liver enzymes and/or 2-fold elevated bilirubin level (in comparison with the levels on admission day) and/or signs and symptoms of manifested liver injury (abdominal pain in upper right abdominal quadrant, nausea, vomiting, jaundice, hepatomegaly, disturbances in hemostasis) during the hospital stay. Patients in control groups (1:1 design) were gender- and age-matched (within 5-year interval) with the patients in experimental group. The exclusion criteria were: previous and/or existing liver illness (including hepatitis of various origins, primary biliary cirrhosis, gallstones, cholangitis, fatty liver), alcohol abuse, thrombocytopenia, hemochromatosis, Wilson's disease, porphyria, as well as any other disease disabling patient's participation in the study.

The study data were collected prospectively (retrieved from patient's hospital records and/or acquired during physical examinations) and included hematological parameters and serum biochemistry parameters that were routinely measured during the hospital stay. Two additional blood samples were also provided from each patient: one for detecting the level of ornithine carbamoyltransferase (OTC) and total bile acids (TBA) (hepatic injury biomarkers not used in routine practice, but exploited for research purposes), and the other for detecting the presence of *SOD2* polymorphism Val16Ala (47T>C, rs4880). The first blood sample (10 mL) was centrifuged and serum was stored at -20°C until OTC and TBA measurement, which was performed using the enzyme-linked immunosorbent assay (ELISA) and spectrophotometric analysis on standard automated biochemical analyzer, according to the previous studies (15,16).

From the second additional blood sample DNA was extracted, and the genotyping for *SOD2* polymorphism Val16Ala was conducted using previously described methods (12,17). In short, genomic DNA was prepared using PureLink Genomic DNA kit (Invitrogen™, Thermo Fisher Scientific). *SOD2* genotyping was performed using real-time PCR method (SacaceSa96 PCR System, Sacace, Italy), with TaqMan® Genotyping Master Mix and TaqMan® DME Genotyping Assay for rs4880, C___8709053_10 (Applied Biosystems, Waltham, MA). The reaction included initial DNA denaturation for 10 minutes at 95°C, followed by 40 cycles of 15 seconds long denaturation at 95°Cand 1 minute long annealing at 65°C and was completed by a final extension step at 4 °C for 10 min.

The additional patients' clinical variables were: the total score on CIOMS/RUCAM scale (Council for International Organizations of Medical Sciences/ Roussel Uclaf Causality Assessment Method), a survey for evaluation of xenobiotic-induced liver damage (18); the drug exposure expressed as the number of defining daily doses (DDD) per 100 patient's days (PD) of hospitalization according to the international ATC (Anatomical Therapeutic Chemical) classification system (http://www.whocc.no/atc_ddd_index); and the total

score of Charlson Comorbidity Index (CCI) used for assessment of comorbidities (19).

The sample size calculation was based on data on *SOD2* rs4880 frequencies from 11 previously published studies (www.pharmgkb.org) and using appropriate software (20). Based on the imputed differences between frequencies of 13% and 47%, allocation ratio 1:1:1, study power 0.8, probability of alpha error 0.05 and two-sided analysis at least 27 subjects were required for each of three study groups, increasing the total study sample to 87 participants. The study data analysis included descriptive methods and hypothesis testing, with appropriate statistics according to the type and data distributions (Student's t-test or Man-Whitney U test, Chi-squared test, Fisher's exact test, Kruskal-Wallis test, one-way ANOVA - analysis of variance). To test for Hardy Weinberg equilibrium as well as to compare differences in *SOD2* allele frequencies and genotype distribution between patients with and without hepatotoxicity, chi-square test was used. The level of probability significance for differences was 5% (0.05) or less.

RESULTS

The patients' demographic and clinical characteristics between the study groups are presented in form of Table 1.

The patients having amiodarone-associated liver injury (experimental group) had been exposed to greater disease burden (both cardiovascular and non-cardiovascular) then the subjects in two other control groups, as measured by CCI. The median length of hospital stay was 2 days (range 2-7) for patients in all study groups (^{2}=0.0, p=1.0; Kruskal-Wallis test). The mean CIOMS RUCAM score in patients of experimental group was 8.48 points (standard deviation 1.20 points, minimal and maximal value 6 and 10 points, respectively) and 13 subjects had obvious symptoms and signs of hepatic disease on physical examination. Overall mortality rate was 3.4% and all 3 fatal outcomes were in patients of experimental group.

On the other hand, common hematological and serum biochemistry parameters, except those indicating hepatic damage, were fairly comparable between study groups. Statistically significant differences were found for C-reactive protein, urea and creatinine concentrations, but their magnitudes were mild-to-moderate and probably had no profound clinical importance (Table 2). The significant differences among the groups in terms of hepatocellular and cholestatic liver injury parameters (OTC and TBA, respectively) additionally confirmed the presence ofhepatic disease in the patients of experimental group comparing to the control ones.

Table 1. Demographic and clinical characteristics of study patients

Variable	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics*
Gender (male)	19	19	19	^{2}=0.0, p=1.0
Age (years)	66.8±10.4	66.1±10.3	66.8±9.8	F=0.040, p=0.961
Obesity	2 (6.9)	2 (6.9)	1 (3.4)	p=1.0
Fatty liver	14 (48.3)	0 (0)	2 (6.9)	^{2}=26.345, p<0.001
Hepatomegaly	18 (62.1)	1 (3.4)	4 (13.8)	^{2}=29.197, p<0.001
Heart failure	19 (65.5)	8 (27.6)	12 (41.4)	^{2}=8.644, p=0.013
Hypertension	13 (44.8)	17 (58.6)	15 (51.7)	^{2}=6.302, p=0.576
Coronary heart disease	25 (86.2)	5 (17.9)	20 (69.0)	^{2}=1.105, p<0.001
Arrhythmia	15 (51.7)	22 (75.9)	13 (44.8)	^{2}=29.452, p=0.043
Diabetes mellitus	6 (20.7)	8 (27.6)	7 (24.1)	^{2}=0.3766, p=0.828
Alcohol intake**	6 (20.7)	1 (3.4)	1 (3.4)	p=0.045
Smoking habit	5 (17.2)	4 (13.8)	1 (3.4)	p=0.326
CCI score (points)	6.8±0.6	3.6±1.3	3.9±1.1	^{2}=59.840, p<0.001

number represent the mean ± standard deviation (continuous variables), and number (percent) of patients (frequencies), as appropriate; p-probability for difference between the study groups; CCI- Charlson Comorbidity Index; *Chi-squared test, Fisher's exact test or Kruskal-Wallis test, depending on the data type and distribution; **-occasionally, not satisfying exclusion criteria (regular alcohol use was exclusion criterion, see methods)

Table 2. Laboratory parameters in patients of study groups

Variable	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics
ALT (U/L)	607.8±1047.6	29.3±17.8	23.5±11.6	^{2}=58.007, p<0.001
AST (U/L)	359.7±627.5	36.9±37.5	21.6±6.6	^{2}=34.677, p<0.001
GGT (U/L)	60.0±52.7	51.2±41.7	31.2±29.9	^{2}=6.826, p=0.033
Bilirubin total (mol/L)	35.1±19.4	12.9±6.5	11.7±4.5	F=34.375, p<0.001
ALP (U/L)	75.9±33.4	89.0±67.1	52.2±19.0	^{2}=9.345, p=0.009
LDH (U/L)	470.6±256.8	320.0±138.8	472.9±156.0	^{2}=9.291, p=0.010
CPK (U/L)	574.5±687.5	222.5±313.9	92.2±67.8	^{2}=7.863, p=0.020
OTC (ng/mL)	272.2±32.7	n.d.	241.4±28.8	t=3.800, p<0.001
TBA (mol/L)	11.9±10.2	6.5±3.7	5.7±4.2	^{2}=9.274, p=0.010
Amylase (U/L)	97.0±93.4	74.2±60.2	95.0±51.0	^{2}=2.843, p=0.241
Troponin (ng/mL)	1.2±1.8	1.5±3.7	0.1±0.1	^{2}=4.687, p=0.096
NT-proBNP (pg/mL)	8332.3±6488.2	1361.9±2323.3	2685.7±2142.7	^{2}=11.313, p=0.003
Proteins (g/L)	62.3±7.8	62.8±6.5	63.3±5.2	F=0.101, p=0.904
Albumin (g/L)	35.8±4.6	38.8±4.8	39.3±4.8	^{2}=9.337, p=0.009
Fibrinogen (g/L)	3.2±1.6	3.4±1.5	3.8±1.3	^{2}=4.032, p=0.122
C-reactive protein (mg/L)	36.9±42.9	17.7±20.2	16.8±27.8	^{2}=7.577, p=0.023
INR	1.7±0.8	1.3±0.3	2.0±1.6	^{2}=3.511, p=0.060
Glucose (mmol/L)	6.1±1.9	5.9±2.4	6.5±2.2	F=0.455, p=0.636
Cholesterol (mmol/L)	4.1±1.5	5.0±1.4	5.0±1.4	F=4.786, p=0.011
Triglycerides (mmol/L)	1.5±0.9	1.8±0.6	2.1±1.7	^{2}=0.936, p=0.626
Urea (mmol/L)	11.9±5.6	11.8±17.9	7.1±3.0	^{2}=13.595, p=0.001
Creatinine (mmol/L)	117.7±43.7	105.9±38.0	101.9±41.6	^{2}=2.679, p=0.009
Leukocytes (10^9/L)	11.2±5.3	9.7±2.9	8.8±3.0	F=2.150, p=0.124
Platelets (10^9/L)	215±86	228±80	202±46	F=0.735, p=0.483

numbers represent the mean ± standard deviation; p-probability for difference between study groups; ALT - alanine aminotransferase; AST - aspartate transaminase; GGT - gamma-glutamyl transferase; ALP - alkaline phosphatase; LDH - lactate dehydrogenase; CPK - creatine phosphokinase; OTC - ornithine carbamoyltransferase; TBA - total bile acids; NT-proBNP – N-terminal pro B-type natriuretic peptide; INR - international normalized ratio of prothrombin time; n.d. - not done; *Kruskal-Wallis test, one-way ANOVA (analysis of variance) or Student's t-test, depending on the data type and distribution

According to DDD analysis, no significant difference was observed in amiodarone use between experimental and control group B (195.5130.8 vs. 212.9171.8 DDD per 100 PD, U=415.0, p=0.932, Man-Whitney U test). Four patients in total used oral formulation of amiodarone, with no significant difference between the two groups in terms of amiodarone formulation (oral vs. parenteral; ^{2}=0.0, p=1.0).

Significant differences in the frequency of drug prescription (other than amiodarone) between the three study groups have been found for proton pump inhibitors (used by 21 patients in experimental group, 21 patients in control group A and 11 patients in control group B, ^{2}=9.656, p=0.008) and high-ceiling diuretics (22 vs. 11 vs. 13, ^{2}=9.503, p=0.009). The prescription of other drugs did not differ significantly between the study groups (p>0.05), including: acetylsalicylic acid, selective beta blockers, angiotensin converting enzyme (ACE) inhibitors, atorvastatin, enoxaparin sodium, organic nitrates, clopidogrel, xanthines, trimetazidine, H_2 receptor antagonists, spironolactone, benzodiazepines, metforminand dihydropyridines, which were prescribed to 55, 50, 49, 43, 40, 37, 36, 23, 22, 17, 15, 14, 10 and 9 patients in total, respectively.

Study patients had also taken some other drugs with possible hepatotoxicity but their uses was sporadic, precluding analysis of individual drug influence. Taking into account these drugs as a class, there were significant differences in their prescription between study groups (^{2}=10.963; p=0.004).

The distribution of *SOD2* genotypes was statistically different between study groups (Table 3). The patients taking amiodarone and having liver injury were mostly carriers of Val/Val (TT) genotype, while Val/Ala (TC) genotype prevailed in control group of patients without hepatic disease who did not use amiodarone, and Ala/Ala (CC) genotype was most frequent in patients using amiodarone experiencing no hepatoxicity. The frequency of Val (T) and Ala (C) alleles were 0.51 and 0.49, respectively in the whole study sample, with no deviation from Hardy Weinberg equilibrium (^{2}=0.56, p=0.454).

In addition, we found statistically significant differences in serum ALT and bilirubin concentrations among different SOD2 genotype subgroups (Table 4).

Table 3. *SOD2* genotype distribution in patients according to the study groups

SOD2 genotype Val16Ala (47T>C)	Experimental group n=29; n (%)	Control group A n=29; n (%)	Control group B n=29; n (%)	Statistics*
Val/Val (TT)	13 (44.8)	4 (13.8)	7 (24.1)	^{2}=10.409, p=0.034
Val/Ala (TC)	8 (27.6)	19 (65.5)	13 (44.8)	
Ala/Ala (CC)	8 (27.6)	6 (20.7)	9 (31.0)	
Total	29 (100)	29 (100)	29 (100)	

*Chi-square test

Table 4. The hepatic parameter values of patients according to *SOD2* genotypes

Variable	Genotype TT (n=24)	Genotype TC (n=40)	Genotype CC (n=23)	Statistics*
ALT (U/L)	437.01158.0	81.9131.5	234.4419.1	^{2}=6.698, p=0.035
AST (U/L)	224.2653.8	81.0180.2	152.5283.7	^{2}=2.592, 0.274
Bilirubin total (mol/L)	28.320.5	15.313.0	19.312.8	^{2}=10.635, p=0.005
OCT** (ng/mL)	250.535.1	257.834.6	263.033.6	F=0.623, p=0.540
TBA (mol/L)	10.910.2	6.45.3	7.75.4	^{2}=4.658, p=0.097

*Kruskal-Wallis test or one-way ANOVA (analysis of variance), depending on the data distribution;
**not measured in control group A (without both amiodarone and liver injury), data from 20, 21 and 17 patients in respective genotype subgroups

The carriers of TT genotype had significantly higher ALT, total bilirubin and total bile acid concentrations compared to carriers of TC genotype (U=2.331, p=0.020, U=3.204, p=0.001 and U=2.172, p=0.030, respectively; Man-Whitney U test), but not compared to carriers of CC genotype (p>0.05; Man-Whitney U test) (Figures 1 and 2).

Figure 1. The ALT values (U/L) in patients with different rs4880 SOD2 polymorphism (CC, TC and TT subgroups with 23, 40 and 24 patients, respectively); for the sake of clarity outliers are removed; asterix - p=0.020 comparing to carriers of TC genotype

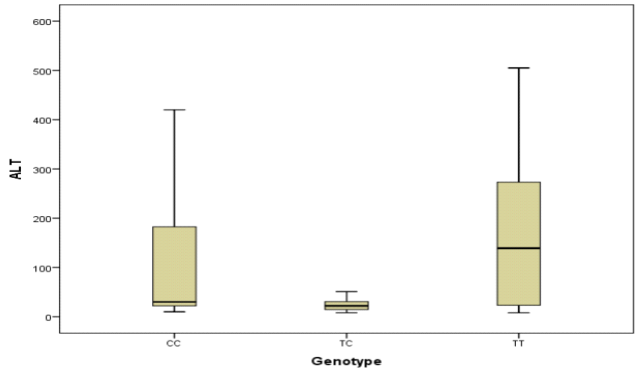
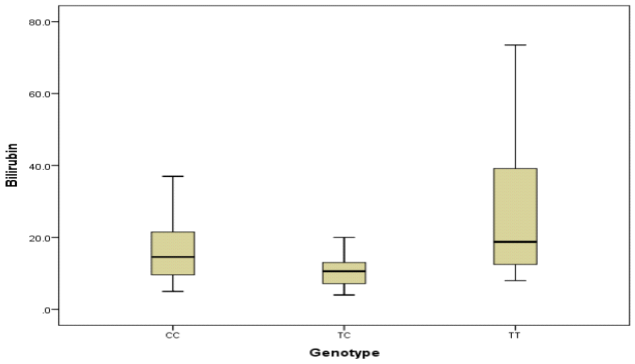


Figure 2. The total bilirubin values (mol/L) in patients with different rs4880 SOD2 polymorphism (CC, TC and TT subgroups with 23, 40 and 24 patients, respectively); for the sake of clarity outliers are removed; asterix - p=0.001 comparing to carriers of TC genotype



There were no significant differences of the ALT, AST, total bilirubin, OCT and TBA concentrations between the two control groups (p>0.05).

DISCUSSION

Our study reveals significant association between different SOD2 Val16Ala genotypes and amiodarone-associated hepatotoxicity in hospitalized patients. Carriers of Val/Val (TT) genotype who used amiodarone experienced acute liver injury more frequently than the carriers of Val/Ala (TC) or Ala/Ala (CC) genotypes. To the best of our knowledge, this is the first study investigating and reporting relation among

amiodarone use, rs4880 SOD2 polymorphism and hepatic damage in the clinical setting.

Within the family of superoxide dismutase enzymes, which is known to modulate oxidative stress response, manganese-dependent isoform (MnSOD or SOD2) is particularly important due to its presence in mitochondria (21). Decades of research conducted so far have evidenced the role of SOD2 in different metabolic, cardiovascular and neoplastic diseases, which is affected by SOD2 genetic polymorphism and epigenetic control mechanisms, as well as by life-style habits such as diet and exercise (22). The published data support our finding related to the importance of Val16Ala polymorphism in amiodarone-associated hepatotoxic symptoms and signs. In addition, there are evidence of rs4880 SOD2 polymorphism contribution to liver injury associated with anti-tuberculosis drugs (12), modification of methotrexate cytotoxic effects (23) or attenuation of rosuvastatine cholesterol-lowering action (24).

It has been observed that SOD2 polymorphism rs4880 could influence the protein trafficking across mitochondrial membranes and, consequently, the oxidative stress response based on this enzyme activity. Namely, during experimental conditions SOD2 protein that contains alanine (Ala) was freely transported into the mitochondria matrix, while the valine (Val) containing form is confined within the inner mitochondrial membrane (25).The cellular consequences of these events are much higher concentrations of mature protein and SOD2 activity in the presence of Ala than Val (26). In addition, the Ala variant of rs4880 SOD2 gene polymorphism, together with the diet rich in antioxidant substances, decrease DNA damage in healthy, young people despite high inter-individual variations in basal and oxidant-triggered responses (27). Other researchers also reported importance of essential nutrients for antioxidative defense system and body function in both experimental and clinical conditions (28, 29). Finally, the augmentation of inflammatory signaling pathways could contribute to the effects of adverse, prooxidative state in the presence of SOD2 polymorphism rs4880. It has been recently reported that the human peripheral blood mononuclear cells from the carriers of Val/Val SOD2 genotype produced more proinflammatory cytokines (e.g. IL-1, IL-6,TNF-α, IFN-γ) than their Ala/Ala counterparts (30).

The majority of studies so far suggested that Val at position 16 of SOD2 reduced activity of the enzyme and increased oxidative stress, but there were opposite findings as well - higher enzyme activity in the carriers of the variant in comparisons with homozygous Ala/Ala (CC) carriers (31). In addition, the effects of the polymorphism on drug actions were not absolutely consistent. For example, patients with SOD2 rs4880 T allele taking dopaminergic anti-parkinson drugs had less nausea and vomiting, but the motor adverse reactions remained unchanged (32). Further, clozapine response in patients with schizophrenia had not been affected with Val16Ala SOD2 polymorphism (33). In our study, patients experiencing amiodarone-associated liver damage (the group where Val16Val (TT) SOD2 genotype was the most frequent) had, in general, more disease burden and somewhat more frequent use of other drugs known to have hepatic adverse effects. In addition, there were no gradual increase in serum concentration of hepatic damage biomarkers (ALT, AST, bilirubin total) from the CC, across the TC to the TT polymorphism carriers, and the levels of OTC and TBA did not differ among the groups with different rs4880 SOD2 genotypes. Therefore, the contribution of other factors to hepatic injury in our patients cannot be excluded (34).

Our study reports Val16Ala SOD2 gene polymorphism distribution in Serbian population and our results correspond well to the distributions across the subjects participating in two clinical trials, which have been recently conducted by two different research groups in Belgrade (35,36). Another study that included patients with bronchial asthma from the area of Nis City reported larger differences between allele frequencies (37). Such divergence seems rather a random event than the consequence of true genetic diversity of this single nucleotide polymorphism in Serbian population, as it does not conform to results of the abovementioned studies, nor to the data available from The International Genome Sample Resource 1000 Genomes Project with reference to rs4880 SOD2 gene allele distributions for other Europeans(https://www.pharmgkb.org/variant/PA166156900).

The relatively small sample size represents the major limitation of our study, as it precludes multivariable analysis of all factors contributing to acute liver disease, such as heart failure, occasional alcohol intake, burden of comorbidities and additional hepatotoxic drugs. Supplementary control sample, including patients with hepatic injury not taking amiodarone, could possibly increase final performance of statistical modeling. Detection of other important polymorphisms of genes coding for cytohromes, transferases, transporters, cytokines and other gene products associated with drug-induced hepatotoxicity would provide data for more comprehensive pharmacogenomic approach.

In conclusion, our study reports higher incidence of 47T allele of SOD2 in patients with amiodarone-associated liver injury as compared to patients on amiodarone not experiencing hepatotoxic effects. In order to evaluate SOD2rs4880 polymorphism as a potential and clinically meaningful predictive marker for DILI in patients on amiodarone treatment, additional studies involving larger number of patients and investigating additional genetic markers should be conducted.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. The study was approvedby the Ethic Committee of Clinical Center Kragujevac, the number of Ethical Approval 01/3518. Voluntary written and informed consent was obtained from each participant prior to enrollment in the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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None.

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NEUROPSICHIATRIC MANIFESTATIONS OF SYSTEMIC LUPUS ERYTHEMATOSUS: DIAGNOSIS AND TREATMENT APPROACH

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ABSTRACT

Neuropsychiatric involvement in systemic lupus erythematosus includes heterogeneous manifestations involving both the central and peripheral nervous system. A major issue in clinical evaluation is the attribution of neuropsychiatric symptoms to systemic lupus erithematosus. Antiphospholipid antibodies, immune complex, microangiopathy, early and accelerated arteriosclerosis are factors that have the main role in pathogenesis of neuropsychiatric manifestations of systemic lupus erithematosus. There are no neurological symptoms specific to systemic lupus erithematosus, but they can also occur very commonly in the general population. Lesions of nervous system can be focal or diffuse and may be due to systemic lupus erithematosus itself (primary lesions), but it also may be caused by other diseases or disbalances. Therapy of the neuropsychiatric manifestations depends on the nature of the pathological process (dominant inflammation or thrombosis). If it is result of an inflammatory neurotoxic process and in the presence of an increased activity of systemic lupus erithematosus, therapy includes glycocorticoids independently or in combination with immunosuppressives. Focal neuropsychiatric syndrome with antiphospholipid antibodies positivity should be treated with anticoagulant and/ or antiplatelet therapy. In addition, control of classical cardiovascular risk factors, stop smoking, and treatment with hydroxychloroquine is recommended.

Keywords: *Neuropsychiatric manifestations, systemic lupus erythematosus, diagnosis, treatment.*

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic multisystem inflammatory autoimmune disease with a large spectrum of clinical presentations (1). Neuropsychiatric involvement in systemic lupus erythematosus (NPSLE) includes heterogeneous manifestations involving both the central and peripheral nervous system. A major issue in clinical evaluation is the attribution of NP symptoms to SLE. Due to the lack of a gold standard, it represents a clinical challenge that obligates the strict exclusion of any other potential cause. In clinical practice, an individual multidisciplinary diagnostic and therapeutic approach based on the suspected cause and severity of symptoms is recommended (2, 3).

PREVALENCE OF NPSLE

The prevalence of NPSLE is highly variable (range 4.3-91 %) and incidence (range 8-40 %), depending on patient selection method and on the nomenclature used to classify the event as NPSLE. It is noted that the incidence of neuropsychiatric disorders is significantly lower in patients with late onset of SLE (after the age of 50). (4, 5) The meta-analysis of large number of studies reported that NPSLE prevalence was 44.5 % in prospective studies versus 17.6 % in retrospective studies. (6) Disparities in frequency have been mainly attributed to differences within the definition used and stringency in attributing the events to SLE. Most of these studies included minor, nonspecific symptoms (e.g. mild depression or anxiety) that are investigator-dependent. After exclusion of these minor events and peripheral nervous system (PNS) syndromes, Kampylafka et al. reported an NPSLE prevalence of 4.3 % and an incidence rate of 7.8/100 person years. (7) NPSLE is a severe complication of SLE that is associated with a lower quality of life over time, with poor prognosis. (8) It has been reported a tenfold increase in mortality rate in NPSLE compared with the general population. (9)

PATHOGENESIS OF NPSLE

The pathogenetic mechanism of NPSLE has not been fully clarified. Antiphospholipid antibodies, immune complex, microangiopathy, early and accelerated arteriosclerosis are considered to have the main role. The European League Against Rheumatism (EULAR) defined the main risk factors for the emergence of neuropsychiatric manifestations of SLE: 1. high activity of the disease or a large degree of damage due to SLE, 2. previous neuropsychiatric disorder caused by SLE, 3. positive antiphospholipid antibodies (aPL) (10).

Many evidence show that blood-brain barrier dysfunction may be essential to the development of NPSLE, allowing the passive diffusion of auto-reactive antibodies and cytokines. This proces involves an abnormal reaction between the endothelium and the leukocyte that allows the passage of proteins and cells into the CNS (8, 11, 12). Endothelial cells, under the influence of proinflammatory cytokines, increase the expression of adhesive molecules, which allows the entry of lymphocytes into the CNS. In conditions of increased SLE activity, the level of intracellular adhesive molecules (ICAM-1) is also increased. The damage of the brain barrier increases the risk for corticosteroid induced psychiatric disorders in SLE too (8, 11).

Primary neuropsychiatric disorders in SLE are due to direct neuronal damage. Patogenetic mechanisms are: autoantibodies against receptors for N-methyl-d-aspartate glutamate (anti-NR2), accelerated arteriosclerosis and antiphospholipid antibodies leading to prothrombotic condition (13). Although some mechanisms are common to focal and diffuse syndromes, there is a clear relationship between the presence of vasculopathy and antiphospholipid antibodies (aPL) and focal NPSLE (cerebrovascular disease, seizure, chorea, myelopathy), and between inflammatory mediators and diffuse NPSLE (14). Antiphospholipid antibodies can induce blood-brain barrier dysfunction, through their interaction with endothelial cells and induce neurotoxicity (12). There are immune mechanisms mediated by inflammation, anti-neuronal antibodies (P antibodies), cytokines, increased permeability of the blood-brain barrier and intrathecal formation of immune complexes, in most diffuse neurological syndromes (such as psychosis or acute confusion) (14).

Autopsy findings in patients with NPSLE show a very wide range of CNS changes: multifocal infarcts, cerebral cortex atrophy, major infarction, haemorrhage, vasculitis, ischemic demyelination and multiple demyelinization fields, as in systemic sclerosis. The most common histological findings are cerebral microinfarcts, while vasculitis occurres very rarely (15).

DIAGNOSTIC APPROACH OF NPSLE

There are very different data about the incidence and prevalence of NPSLE. One of the reasons for such great differences is the fact that none of the neurological symptoms is specific to SLE, but can also occur very commonly in the general population (depression, headache). Modifying the criteria for NPSLE, in order to exclude mild or more subjective manifestations, led to an increase in the specificity of the criteria from the previous 46% (Table 1.a.), up to the current 93% (Table 1b.) (16).

In addition to primary CNS lesions which are caused by the disease itself, CNS damage can be secondary, for example, in patients with uremic syndrome, electrolyte disbalance, hypertensive encephalopathy, hypoxia, infections, or medication (psychosis induced by glicocorticoid therapy, dizziness and headache caused by antimalarials, aseptic meningitis caused by ibuprofen or azathioprine).

Table 1. Neuropsychiatric disorders in SLE as defined by the American College of Rheumatology (ACR) 1999 and their modification 2001.

a. American College rheumatology (ACR) defined syndromes (1999)	
Central nervous system	*Peripheral nervous system*
Epilepsy	Cranial neuropathy
Psychosis	Polineuropathy (confirmed by EMNG)
Cerebrovascular disease	Mononeuropathy
Headache	Gillan-Barre syndrome
Demelinization	Vegetative disorders
Motility disorder	Plexopathy
Aseptic meningitis	Myasthenia gravis
Acute confused state	
Anxiety disorder	
Cognitive dysfunction	
Mood disorder	
Myelopathy	
b. Modified Objective Criteria (2001)	
Epilepsy	Cranial neuropathy
Psychosis	Polineuropathy (confirmed by EMNG)
Cerebrovascular disease	Mononeuropathy
Demelinization	Gillan-Barre syndrome
Motility disorder	Vegetative disorders
Aseptic meningitis	Plexopathy
Acute confused state	Myasthenia gravis
Cognitive dysfunction (moderate and severe)	
Depression (severe)	

Assessment by physicians, whether neuropsychiatric syndromes are a direct consequence of active SLE, or are a consequence of therapy or complications of long-lasting illness, is very important for deciding on further treatment. There are no clinical tests that can help us evaluate whether neuropsychiatric manifestations are directly related to active SLE. Factors that can help include: general activity of SLE, previous history of neuropsychiatric changes, the presence of antiphospholipid antibodies.

Different attribution models have been proposed to distinguish NP events, depending on whether or not they are due to SLE. The minor and most common neuropsychiatric events (headache, mild depression, anxiety, minor cognitive complaints, and electromyographynegative polyneuropathy) used as exclusion events in the most relevant attribution models. These minor events became known as the "Ainiala criteria" (16-19).

Studies have shown that headache does not occur significantly more often in patients with SLE than in the general population (11, 20, 21). However, it is very important that the onset of headache, in an appropriate clinical context, is examined for the possibility of developing cerebral or subarachnoid haemorrhage, aseptic meningitis, or sinus thrombosis. If there are no risk factors (temperature, infection, antiphospholipid antibodies, anticoagulant therapy, immunosuppressive therapy, focal neurological signs, mental status disorders, meningism, and increased general activity of SLE), headache in patients with SLE does not require more extensive examination than what would be done in the general population that does not suffer from SLE. One of the non-specific symptoms is depression. It is very common in the general population and there is no way to differentiate it from depression within the NPSLE (21).

Demyelinization of CNS and transverse myelitis occur in about 5% of patients with SLE and usually associated with positive antiphospholipid antibodies (20, 21). Demyelinization of the CNS can be difficult to differentiate from multiple sclerosis in differential diagnosis. SLE and multiple sclerosis rarely occur together. So if there are no clear criteria for SLE, it is considered as the case of multiple sclerosis. Transverse myelitis is characterized by the appearance of paraplegia or quadriplegia, depending on which level of the spinal cord the

pathological process occurs. It can progress to a higher level with time (20, 21).

Peripheral nervous system disorders include the most common polyneuropathy, and less frequently monoeuropathy, acute demyelinizating polyradiculoneuropathy, myasthenia gravis, plexopathy and muscular weakness and atrophy. Sensomotor peripheral neuropathy is the most common form of peripheral nervous system involvement in SLE (21).

Acute psychosis occurs very rarely in SLE (about 2%) and is presented by the occurrence of delusions (incorrect beliefs that are maintained by using objective evidence, contrary to and in spite of cultural norms) or hallucinations (perceptions that occur in the absence of external stimulus) (20, 21). Corticosteroids induced psychiatric disorder occurs in about 10% of patients who are at high doses of prednisolone (≥1 mg / kg) and are more likely to experience mood disorders (93%) rather than psychosis(21). In clinical practice, it is important to exclude psychosis induced by high doses of corticosteroids or psychoactive drugs.

Cognitive dysfunction involves disturbance at the level of memory, learning, processing of information and expression. Cognitive dysfunction in NPSLE is manifested by disturbance of attention, visual memory, verbal memory, executive functions and psychomotor speed. Most SLE patients have mild to moderate levels of cognitive dysfunction. Severe degree of cognitive dysfunction occurs in a small number of NPSLE patients (2-3%). Special neuropsychological tests are used in cognitive dysfunction diagnosis (11, 20, 21).

When evaluating SLE patients with new neuropsychiatric symptoms, the same diagnostic algorithms should be applied as in all other patients with the same symptomatology. When suspected of infection, examination of a cerebrospinal fluid should be done. In patients with epileptic seizures, it is necessary to do electroencephalography. The occurrence of confusion and psychosis requires examination of the metabolic status and history of drug use. Neuropathies require the examination of vitamin B12 status. Magnetic resonance with angiography is used most often to exclude structural changes.

Magnetic resonance imaging (MRI) has become the gold standard tool for NPSLE assessment, replacing computed tomography (CT) in the evaluation of brain pathology among these patients. The average sensitivity of MRI in active NPSLE is 57% (22, 23). The MRI changes described in NPSLE patients range from small focal lesions in white matter, defined as white matter hyperintensities, to severe large lesions. Abnormal findings may be divided in three groups, according to their pathophysiology and imaging features: small-vessel disease, large-vessel disease, and inflammatory-like lesios

(24) Small vessel disease (30%- 75% of MRI findings in NPSLE), includes white matter hyperintensities, cortical brain atrophy, lacunes, small subcortical infarcts, and microbleeds. Among them white matter hyperintensities being the most commonly documented in SLE patients. The majority of small vessel lesions are often considered non-specific, as they may be related to age, hypertension, disease duration, low complement, aPL antibodies, and the presence of NPSLE manifestations, mainly cognitive dysfunction, seizures, and cerebrovascular disease.

Large vessel disease is less frequent than small vessel lesions (10%-15% of NPSLE MRI findings). It causes medium to large size vessel infarcts (single or multiple), involving both the grey and white matter (22). Inflammatory-like lesions are rather less common, accounting to for 5%-10% of NPSLE (24).

The lack of sensitivity of conventional MRI has led to the exploration of other techniques. These techniques include quantitative MRI, such as: magnetic resonance spectroscopy, magnetization transfer imaging, diffusion tensor imaging, and diffusionweighted imaging, or fluorodeoxyglucose positron emission tomography (FDG-PET) and single photon emission CT (SPECT). Nonetheless, these techniques, have low specificity, and did not show a high degree of validity in the differential diagnosis, which limits their use in clinical practice (25).

TREATMENT APPROACH IN NPSLE

Therapy NPSLE depends on the nature of the pathological process (dominant inflammation or thrombosis). In some cases, both lesion mechanisms are present at the same time. In cases where neuropsychiatric manifestations arise as a result of an inflammatory neurotoxic process (aseptic meningitis, optic neuritis, transversal myelitis, peripheral neuropathy, epilepsy, psychosis, acute confusional state) and in the presence of an increased generalized activity of SLE, therapy includes glycocorticoids independently or in combination with immunosuppressive drugs (azathioprine, cyclophosphamide) (23, 25). In the most severe forms of NPSLE, refractory to standard immunosuppressives, intravenous immunoglobulins, plasma replacement and rituximab might be effective. (23, 25).

In the LUMINA cohort hydroxychloroquine and moderate prednisolone dose delayed the first NPSLE manifestation, regardless of the type of event (27). The SALUD study showed that aspirin improved cognitive function in older patients with risk factors. In other studies, the odds of having cognitive impairment was significantly lower for patients taking hydroxychloroquine (28). When the neuropsychiatric event is acute and diffuse, it is presumably mainly inflammatory and almost always associated with generalized SLE activity. In this scenario, SLE global activity should be controlled at the same time as NPSLE is assessed, and if NPSLE is severe (acute confusional state, seizures, encephalitis) it should be treated with immunosuppressive drugs. Steroids have been used in several different doses (prednisolone 0.5- 1 mg/kg/day or bolus of intravenous methylprednisolone 3-5 days of 500 mg to 1 g/day (29). Cyclophosphamide can be used in two regimens. The low-dose EuroLupus regimen

intravenous 500 mg every other week for six cycles and the USA National Institutes of Health regimen of 1 g/monthly for 6 months (30, 31).

In maintenance treatment after induction therapy with cyclophosphamide or high-dose steroids, in severe NPSLE, azathioprine or mycophenolate mofetil can be used.(28, 32, 33) A recent revision reported the positive effects of rituximab (with high rates of response) and belimumab in severe NPSLE.(33). In severe refractory cases, intravenous immunoglobulins and plasmapheresis have been successfully used as bridge therapy, mainly when infection is not completely ruled out, in pregnant patients, or when there are life-threatening symptoms (15, 23, 26).

SLE patient with focal neuropsychiatric syndrome (ischemic and cerebral venous thrombotic events) and aPL positivity should be treated with anticoagulant therapy (15, 23, 26). In addition, control of classical cardiovascular risk factors, stop smoking, and in cases with positive antiphospholipid antibodies treatment with hydroxychloroquine is recommended. Other focal syndromes (seizures, ischemic optic neuropathy and chorea associated with antiphospholipid syndrome, as well as in myelopathy refractory to immunosuppressives) as also patterns of microvascular disease, might benefit from antiplatelet drugs or anticoagulation. If there are other clinical or laboratorial signs of inflammation, additional immunosuppression should be considered (15, 23, 26). Anticoagulant therapies have an advantage over antiplatelet for the purpose of secondary prevention of a cerebrovascular accident (CNS infarction or transient ischemic event) in patients with an antiphospholipid syndrome. In contrast, antiplatelet therapy is used for the primary prevention of cerebrovascular injury in patients with high titar of antiphospholipid antibodies.

In NPSLE patients with symptoms of psychosis, antipsychotics and / or antidepressants are indicated. Cognitive behavioral treatment has very positive effects on symptoms of depression. In psychiatric manifestations of SLE, combined therapy with glycocorticoids and immunosuppressants (induction therapy with cyclophophamide initially, and later in the maintenance phase of remission with azathioprine) in most cases leads to significant improvement of symptoms (60-80% of patients). However, there is a possibility of a relapse occurring and ranges up to 50%. In patients with psychiatric disorders refractory to therapy, rituximab can lead to significant improvement. Most psychiatric episodes are resolved within 2-4 weeks, while 20% of patients develop a chronic psychotic disorder (11, 15, 26).

In patients with seizures anticonvulsive drugs are recommended. If seizures reflect increased SLE activity and the exacerbation of the inflammatory process, glycocorticoids and immunosuppressants are recommended. The pulses of methylprednisolone and cyclophosphamide have shown good effects in cases of refractory seizures in the context of increased SLE activity (15).

Myelopathy in SLE is usually manifested as transverse myelitis, but in some cases it can be caused by an ischemic or thrombotic event. In patients with myelitis, combined therapy with methylprednisolone and pulses of cyclophosphamide can be very effective, especially at the onset of the disease. (15) After introduction of aggressive antiinflammatory therapy in a timely manner, the neurological improvement, as well as the improvement on magnetic resonance occurs within a few days to 3 weeks. Relapse of myelitis is common especially during the reduction of the dose of glycocorticoids and occurs in 50-60% of patients. In patients with ischemic myelopathy and antiphospholipid antibodes, anticoaglutination therapy might be usefull. Therapeutic plasma replacement may be a therapeutic option in severe cases of myelitis. In patients with a late diagnosis of SLE myelopathy and delay in initial therapy for more than 2 weeks, severe neurological deficits occur, followed by extensive lesions on the spinal cord magnetic resonance (15, 26).

Cranial neuropathy in NPSLE usually involves the third, fourth, sixth and eighth cranial nerves, and rarely the fifth and seventh. Pulse doses of methylprednisolone and cycofosfamide are recommended. Optical neuritis within the NPSLE is usually associated with a greater lack of vision. Immunosuppressive tearapy is also indicated, while anticoagulant therapy may have effects in patients with positive antiphospholipid antibodies refractory to immunosuppressives. (15, 26) In peripheral nervous system disorders monotherapy with glycocorticoids or in combination therapy with immunosuppressive drugs gives good results in 60-75% of patients. Intravenous immunoglobulins, plasma replacement therapy and rituximab can be used in severe cases (15, 23, 26).

Treatment of cognitive dysfunction in NPSLE involves treatment of causes of anxiety and depression worsening, as well as control of cardiovascular risk factors. Psychoeducational group treatments have shown improvement in the domain of memory and ability for daily activities. Patients with cognitive dysfunction and increased activity of SLE generally benefit from glycocorticoid and immuno-suppressives, and those with antiphospholipid syndrome of anticoagulant therapy (15, 26).

Analysis of implementation EULAR's preventive measures for the treatment of NPSLE, pointed that glycocorticoids and immunosuppressive therapy are often used not only in cases where there are clear indications (inflammation-aseptic meningitis, myelitis, cerebral vasculitis, cranial and peripheral neuropathies and psychosis), but also in thromboembolic cerebrovascular events, with no clear indications for their use (34).

CONCLUSION

Neuropsychiatric symptoms constitute an uncommon and poorly understood event in SLE patients, and pose a diagnostic and therapeutic challenge to the physician. Assessment by physicians, whether neuropsychiatric syndromes are a direct consequence of active SLE, or are a

consequence of therapy or complications of long-lasting illness, is very important treatment decision. Factors that are directly related to active SLE include: general activity of SLE, previous history of neuropsychiatric changes, the presence of high titar of antiphospholipid antibodies. Therapy NPSLE depends on the nature of the pathological process (dominant inflammation or thrombosis). If NPSLE is result of an inflammatory neurotoxic process and in the presence of an increased activity of SLE, therapy includes glycocorticoids independently or in combination with immunosuppressives. Focal neuropsychiatric syndrome with antiphospholipid antibodies positivity should be treated with anticoagulant and/ or antiplatelet therapy. In addition, control of classical car diovascular risk factors, stop smoking, and treatment with hydroxychloroquine is recommended.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ERYTHEMA NODOSUM ASSOCIATED WITH STAPHYLOCOCCUS SPECIES INFECTION IN A CHILD

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ABSTRACT

Erythema nodosum (EN) is a poly-etiological disease with an acute flow that is characterized by symmetric emergence of painful nodules often in pretibial areas. A twenty-month-old male child was admitted to hospital for evaluation of the eruptive skin changes in the lower extremities and forearms. The disease began 10 days before getting febricity and loose stools. The laboratory analysis showed an elevated erythrocyte sedimentation rate and leukocytosis. Blood cultures demonstrated the presence of coagulase-negative Staphylococcus, while Proteus vulgaris was isolated in urine cultures. After initiation of the empiric antibiotic therapy and then, the targeted antibiotic therapy according to the antibiogram, there was a significant improvement in a general condition and regression of cutaneous lesions. Erythema nodosum in the present case, is the result of staphylococcal bacteremia although Proteus vulgaris cannot be excluded as a cause.

Keywords: *Erythema nodosum, Bacteremia, Staphylococcus.*

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INTRODUCTION

Erythema nodosum (EN) is a poly-etiological disease with an acute flow that is characterized by symmetric emergence of painful nodules often in pretibial areas. The prevalence in adults is 1-5 per 100,000 while in children, it has not been established. It occurs most often between 20 and 40 years of age and is more common in women (1). It usually occurs in children between the ages of 8 and 10 and is equally present in both sexes (2). According to the etiology, EN may be primary (idiopathic) or secondary. Each EN for which the secondary etiology has not been proven, is defined as idiopathic. In most cases of children with EN, this disease is idiopathic (3). The most common causes of the secondary EN are infectious agents (streptococcal infection, Mycobacterium tuberculosis, viruses, Mycoplasma pneumoniae, parasites, fungi) and drugs (sulfonamides, amoxicillin, contraceptives). In 3-10% of cases, the first manifestation of malignancy (leukemia, non-Hodgkin lymphoma, rarely, myeloid leukemia) is EN. In the systemic autoimmune diseases (sarcoidosis, SLE), EN occurs in 11-25% (4,5). It can also occur as a part of other diseases (Crohn's disease, ulcerative colitis, M. Behcet, cat-scratch disease). In addition, there was a case of EN in children caused by Shigella flexneri (6). Also, the cases of EN are well described in a normal pregnancy.

In general, erythema nodosum represents a ductile structure inflammation of the subcutaneous tissue, septal panniculitis, followed by the eruptive changes on the skin. The changes are localized in hypodermis, but without vasculitis. The onset of the disease is usually manifested by fever, arthralgia, myalgia, the upper respiratory tract infection and abdominal pain. The pathognomonic signs of erythematous are subcutaneous nodules that are painful on palpation, sensitive and symmetrically localized in the front of the lower extremities (4). The indicated changes are not ulcers and they are usually withdrawn after 2-8 weeks without leaving scars on the skin. Sometimes, EN is the first clinical manifestation of the disease for which the diagnosis requires a multidisciplinary approach.

CASE REPORT

A 20-month-old male child is brought to hospital for examination because of the eruptive skin changes, initially on the shins and then on the forearms. The disease began 10 days before getting the fever up to 38.7 °C (the highest 39 °C). Along with the increase in body temperature, rare fluid stools occurred (3-4 per day, on the day of the admission, 10 stools) and he was treated with probiotics. The day before the admission, the skin changes occurred. During the physical examination, the child was conscious, with fever (38°C), eupneic, eutrophic. The skin of the anterolateral side of both shins was pale, with less elasticity and turgor and with present erythematous nodules, painful and warm on palpation (Figure 1A, 1B). A pair of similar skin lesions was present on both forearms (Figure 1C).

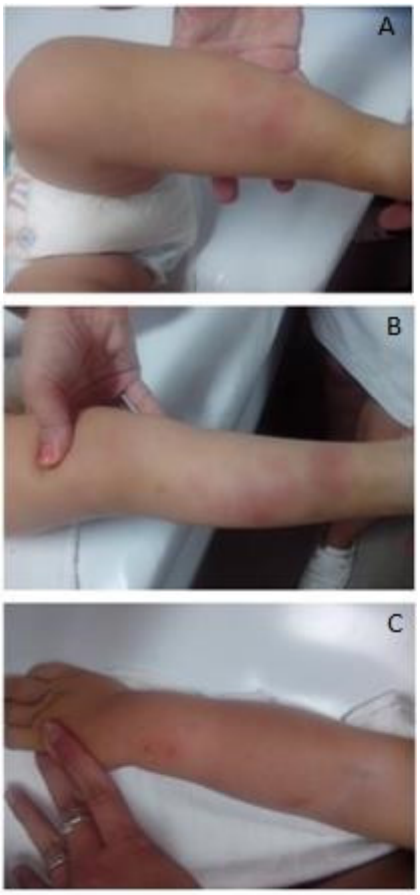

Figure 1.

The laboratory examination showed leukocytosis $14.0x10^9/L$ with neutrophilia 50.8%. The erythrocyte sedimentation rate (ESR) was elevated (45 mm/hr). The other indicators of inflammation were not increased (C-reactive protein was 3.9, procalcitonin was 0.120). The erythrocyte and platelets count as well as hemoglobin concentration were in normal range. The urinary analysis showed no pyuria but the urine cultures for bacteria and fungi showed Proteus vulgaris (100000 cfu/ml). The nasal and pharyngal smear showed negative results. The blood cultures for bacteria and fungi showed coagulase-negative Staphylococcus species (MRSA). There were no isolated pathogenic bacteria in feces. The Rotagen test showed negative results. Anti-Streptococcal (ASO) titer was normal. The serological analysis (Immunoglobulin M) of Mycoplasma pneumonia was negative. The chest radiograph showed no hilar adenopathy or pulmonary infiltrates. On the basis of the clinical presentation, medical history and laboratory indicators, the boy was diagnosed with: Erythema nodosum; Sepsis. The treatment was initiated by a parenteral administration of a broad spectrum of antibiotics

(cephalosporin's 3rd generation) and after obtaining the blood cultures findings, the anti-staphylococcal drug (Vancomycin) was administered. The therapy led to the improvement of clinical symptoms described by the regression of skin lesions and afebrility. After ten days of the therapy, the skin lesions were repaired without occurrence of ulceration and scarring and with normalization of the laboratory parameters (Figure 2).

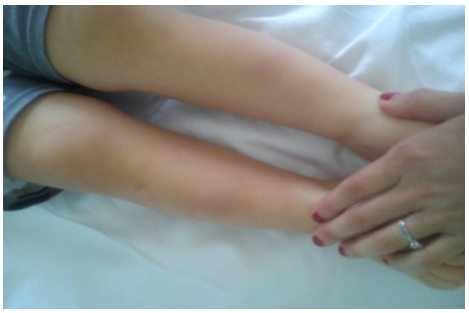

Figure 2.

DISCUSSION

In this study, a less typical case of EN for the age is described, as well as causes and localization of the reported changes. Most of the published studies show that the most common age for EN is 8-10 years whereas in our case, it was quite early, in the 20th month of life (2,4). This was the earliest detection of EN in our clinic in the last couple of years. Although many studies have shown that the most common cause of EN in children is beta hemolytic streptococcus group A (50-77%), in our case, the cause was Staphylococcus species. Taking into consideration that the patient had frequent loose stools, the Rotagen stool culture test was made and the result was negative. Often, a gram-negative bacillus Yersinia enterocolitica, as the cause of acute diarrhea with occurrence of the abdominal pain, is associated with EN. Also, salmonella infection can be the cause of erythema nodosum. These two possible causes were not found in feces, and the stools were normalized by the antibiotic treatment. Although the patient did not have signs of the respiratory infections, the lung radiological examinations and serological analysis excluded pneumonia caused by Mycoplasma pneumoniae (as an atypical cause that can lead to EN) (7). Since BCG scar was clearly visible (the child was vaccinated at birth), there was no evidence of tuberculosis infection in the family, the chest X-rays were normal and there was no need for PpD3 (Protein phosphatase D3) test. Moreover, the hypersensitive reaction of subcutaneous tissue to the presence of mycobacterium tuberculosis is manifested in the form of subcutaneous nodules (localized mainly on the back side of the lower extremities), which are prone to ulceration and scarring after the rehabilitation process. Since the common cause of EN is an infectious agent, the laboratory diagnosis usually showed the elevated parameters of inflammation. In the study of Litwin and coworkers, ten of total twelve children had the elevated CRP values, while two children had the CRP value below 5 mg/L (8). In 75% of children with EN, the diagnosis can be set on the basis of a medical history of patient, laboratory tests and clinical symptoms (2). In patients with an undefined etiological cause or atypical forms of EN, the diagnostic procedure should be extended for the detection of serious diseases, such as systemic lupus erythematosus (SLE), Crohn's disease and ulcerative colitis (3). Also, EN could be a very rare manifestation of Behcet disease and sarcoidosis in children (9). Based on the clinical presentation, laboratory and other analyses and due to staphylococcus that was isolated in the blood cultures, the established diagnosis was
erythema nodosum probably caused by infection of staphylococcus. Since the urine cultures showed Proteus vulgaris infection, it is possible that both agents caused occurrenceof EN. The literature describes the so-called double diagnosis that caused EN in children, such as streptococcal infections plus mycoplasma pneumonia or streptococcal infection plus latent tuberculosis infection or streptococcal infection plus chlamydia pneumonia. However, only 56.4% children had a confirmed infectious agent as the cause of EN (10). Very often, EN represents the first manifestation of tuberculosis infection (11). Staphylococcal septicemia and subcutaneous inflammatory nodules are well described in patients with multiple brain abscesses (12). The first case of EN associated with coagulase-negative staphylococcus infection was described in 2012, in young women (13). Our case represents the first described case of EN in children caused by staphylococcus infection.

The predicative sites for the formation of skin lesions in EN are pretibial regions of the shins (14) as shown in Figure 1A and 1B. The skin lesions are most commonly localized on legs bilaterally, unilaterally and very rare on hands (15). In our 20-month-old patient, the changes were localized in addition to the shins and forearms, especially around the wrists. In the differential diagnosis of EN, in children, the allergic skin diseases, infections, insect bites, trauma and many other conditions should be excluded. We need to look for the most common infectious causes that provoked EN as well as for those less frequent. When there was EN with an unknown trigger, a possible connection with other diseases should be taken into consideration and in this sense, further investigations should be performed. As the infectious agent in children is the most common cause of the disease, the laboratory tests of blood, urine and bacteriological cultures are conducted in order to find the possible cause. The elevated inflammatory parameters, ESR and leukocyte count with neutrophilia showed a bacterial infection. The general condition of the child was in this favor. We got the confirmation that in the blood cultures, Staphylococcus was isolated and Proteus, in the urine culture. Because the disease represents the hypersensitive reaction of the subcutaneous tissue structure of the infectious agent in the twentymonth-old boy, the final diagnosis was erythema nodosum.
By giving the antibiotic therapy, at the beginning, the initial broad-spectrum antibiotic and targeted antibiotic, with the symptomatic treatment, there was a rehabilitation of the skin lesions with clinical recovery and normalization of the

laboratory parameters. After two weeks of the treatment, there was a complete recovery.

CONCLUSION

Erythema nodosum is a rare infectious disease, atypically associated with coagulase - negative staphylococcus, especially in young children. The microbial spectrum of EN may be much greater than we know, since all microbes can potentially induce the activation of immunological events in their host through complex mechanisms.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013. Voluntary written and informed consent was obtained from the patient prior to enrollment in the study

CONFLICT OF INTEREST

There are no conflicts of interest.

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RADIAL FLAP IN RECONSTRUCTIONS OF THE HAND - CASE SERIES STUDY

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ABSTRACT

Radial forearm flap is an axial flap that has become an important technique in reconstructive surgery as a free flap or as a pedicled flap. Defects of the skin and other tissues on the hand are very common and most often they are work-related injuries. In deep defects, flap must be used, as well as in reconstruction of the amputated thumb. There are many flaps that provide adequate soft tissue coverage for the hand. Therefore, the aim of this paper is to present the results of the use of radial forearm flap in various reconstructions on the hand. The retrospective study included 35 patients who underwent reconstruction with radial forearm flaps due to defects of the skin and other tissues on the hand, in the period 1997-2019. Results and complications of the surgery were analyzed. The functional and aesthetic outcome was assessed using Michigan Hand Outcome Qusteionnarire. All patients were followed for a minimum of 1 year. Fasciocutaneous flap was the most commonly used (65.71%), followed by adiposofascial (20%) and osteocutaneous (14.29%). The size of the fasciocutaneous flap ranged from 2.2x3.1cm to 9x13.5cm. The majority of donor sites were closed with split-thickness skin grafts (56.52%), and less frequently with direct suture, local skin flap, and full-thickness skin graft. There were no complete flap losses in the study. Marginal necrosis was noted in 8.57%, and graft failure at the donor site in 14.29%. Secondary surgical procedures were performed in 13 patients. The functional-aesthetic result of the operation, based on the MHQ score, ranged from 31 (1/35) to 130 points (3/35). The ultimate aim of hand reconstruction is to restore sufficient function and form, also, closing the wound within three days following the injury is desirable. Meticulous intraoperative dissection of radial flap and early physical therapy after surgery are mandatory. The radial flap is a suitable method, especially for large and more distal skin defects on the hand, and a very good method for thumb reconstruction.

Keywords: *Flap, surgery, reconstruction.*

INTRODUCTION

A flap is a segment of skin or other tissues that has its own vascularization, used for soft tissue coverage. It is most commonly performed when direct suture and skin grafting are not possible, due to lack of circulation in the area of the defect, or it is undesirable for functional or aesthetic reasons. The flap can be random or axial, which has the clear defined vascular basis.

The radial flap is one of the well known flaps in plastic and reconstructive surgery. It is also called Chinese flap because it was used for the first time in China in 1978. (1-2). It is an axial flap containing a radial artery with accompanying veins. Radial forearm flap (RFF) may be fasciocutaneous, adiposofascial, but may also contain other tissues, such as bone, muscle, tendon and nerve. According to the transfer, the RFF can be pedicled or free-microvascular. According to the direction of blood flow, there are a proximally and a distally based or reverse radial flaps. A proximally based RFF is used as a pedicled flap for defects in the proximal part of the forearm, elbow area and distal part of the upper arm, or as a free microvascular flap. A distally based flap is used for hand defects. Retrograde blood flow through it originates from the ulnar artery and the deep palmar arterial arch. The perforant branches of the radial artery are numerous, constant, and extend through the lateral intermuscular septum of the forearm, ending on the superficial side of the deep fascia of the forearm. The vascular cutaneous territory of the RRF is therefore large. Constant anatomy, reliable vascularization, large arch of rotation, thin and pliable skin, make this flap widely used, not only on the hand and upper extremity, but also on almost all parts of the body, as a free flap (defects in the face, lip, oral cavity, pharynx, esophagus, penis reconstruction, vagina, lower extremity defects) (3). For defects in the hand, the forearm flap was first used by Stock, Mühlhauer and Biemer (4, 5). Due to its long vascular pedicle and large vascular cutaneous territory, the radial flap can be applied to various skin defects in the hand.

Skin defects of the hand are common and sometimes affect other tissues. Traumatic etiology is most common. Sometimes it is necessary to use a flap for coverage of the defect. For smaller defects, local skin flap is appropriate. Various methods (6-9) have been used for larger defects in the hand, with two-stage distant direct flaps from the anterior chest and abdomen, the most commonly inguinal flap. Also, microcurgical transfer of distant free flaps is used (10). Most of these methods are associated with prolonged surgeries, multi-stage procedures, undesirable patient positions, prolonged and difficult rehabilitation. In addition to the radial forearm flap, other flaps from the forearm can be used: ulnar artery flap or interoseus posterior flap (11, 12). For medium-sized defects, flaps with radial or ulnar artery perforators may also be used (13, 14).

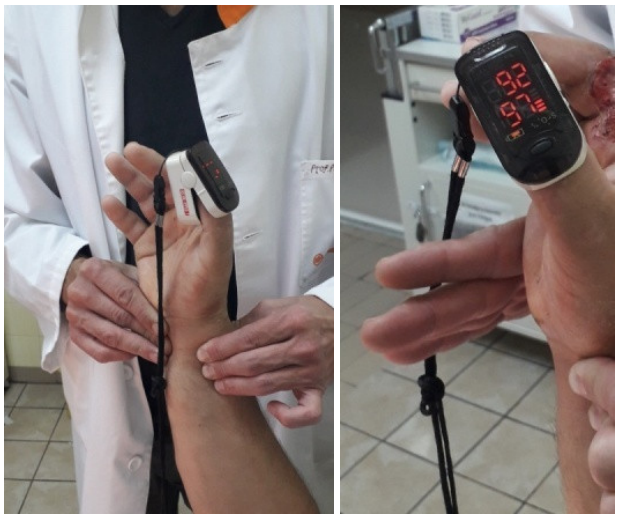
Since defects of the skin and other tissues on the hand are quite common, primarily due to injuries, but also other pathological conditions (tumors, infection, iatrogenic), in many situations it is necessary to use the flap as a method of reconstruction. So, the aim of this study is to present experiences with the use of radial forearm flap (RFF) in various skin defects of the hand.

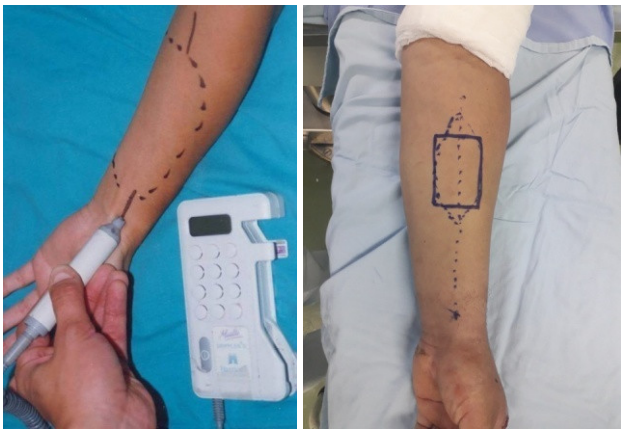
METHODS

A case series study in the period 1997-2019. included patients who had a skin defect in the hand that had to be closed with a flap, or skin defect could not be covered by a free skin graft for other reasons (need for secondary surgical procedures on deeper structures, functional or aesthetic reasons). Excluding criteria were: age under 18, injury or more extensive scar on the anterior forearm, positive Allen test (weak anastomosis between radial and ulnar artery), incomplete medical records (insufficiently long observation period) and severe formes of systemic deseases.

Allen test and Doppler ultrasonography were performed preoperatively (Fig.1). The operations were performed under general or regional anesthesia, under the tournique control (Fig.1). Block anesthesia was used in a smaller flap when donor site was suitable for closure by direct approximation. Longitudinal lazy "S" incision at the forearm was used in all patients, and flap dissection was performed subfascially (Fig.2, Fig.3), with the preservation of the superficial branches of the radial nerve (Fig.2). According to anatomical conditions, a cephalic vein or other superficial vein of the forearm was incorporated into the flap to improve venous drainage within the flap. Anticoagulant prophylaxis was not used. All patients underwent intensive physical therapy as early as possible. The test parameters were race, age and gender, side (right/left), hand dominance, etiology, localization and size of defect, type and size of flap, type of anesthesia, length of surgery and length of hospitalization, success of flap transfer, donor site, presence and type of complication of surgery, and functional-aesthetic result.

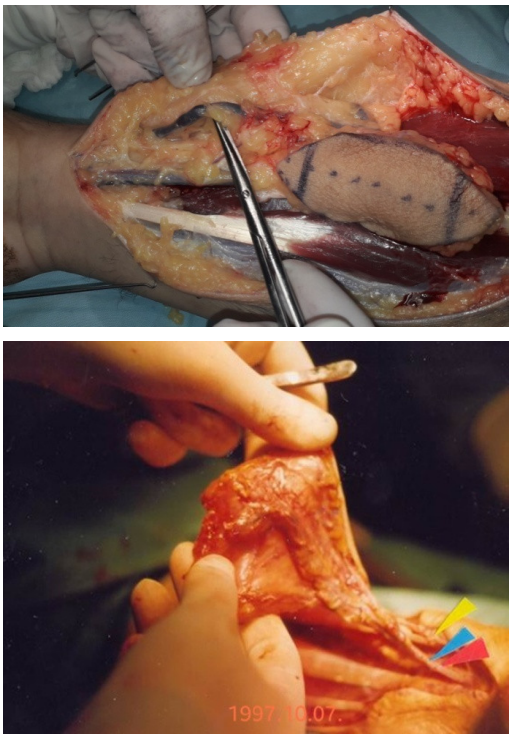
Figure 1. Allen test with pulse oximeter before surgery (picture first left and second left).





Doppler ultrasonography -10MHz probe placed in the course of radial artery (image on the right). Image at the right end: Marking the flap at the beginning of the surgery under the tourniquet control

Figure 2. Radial forearm flap dissection under tourniquet control.



In the picture on the right (analog photography), radial blood vessels and superficial branch of the radial nerve are marked with arrows.

Figure 3. Completed elevation of distally based radial forearm fasciocutaneous flap

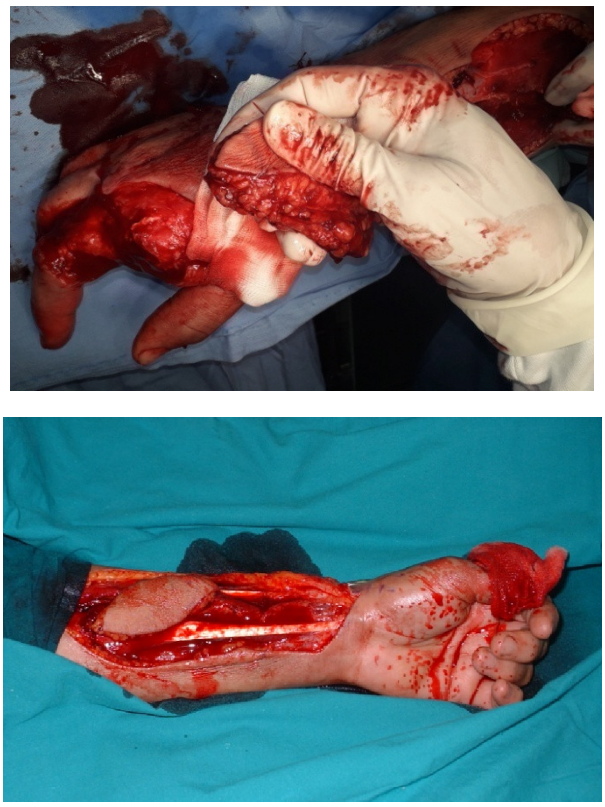


Figure 4. On the left: massive hand defect after injury. On the right: giant hand squamocellular carcinoma

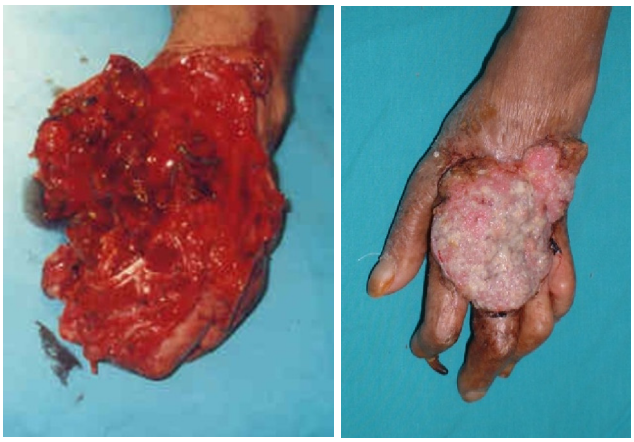
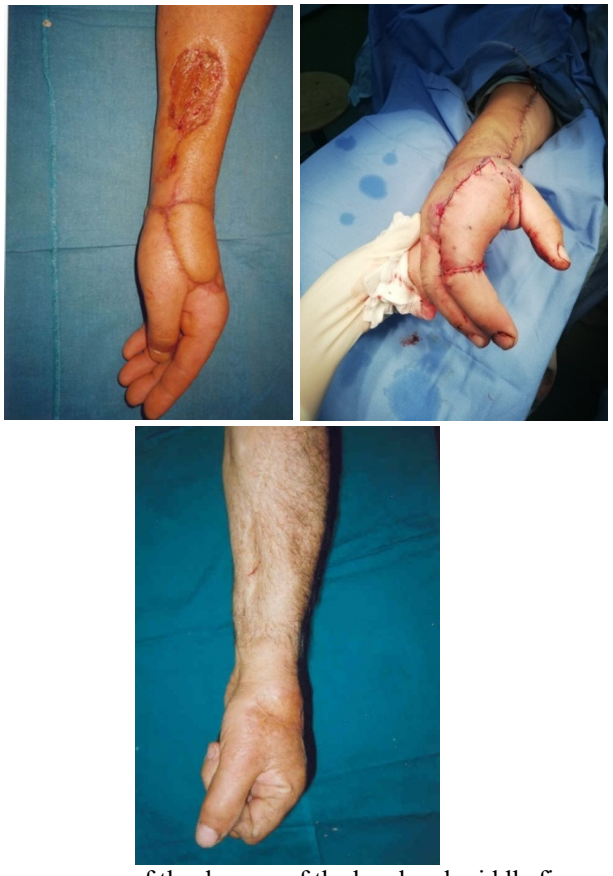


Figure 5. Fasciocutaneous flap with donor defect closed by split-thickness skin graft (picture left).



Flap coverage of the dorsum of the hand and middle finger (picture in the middle). Right: adipofascial flap radial flap, with direct closure of the donor site.

Figure 5. Small Chinese flap in thumb reconstruction

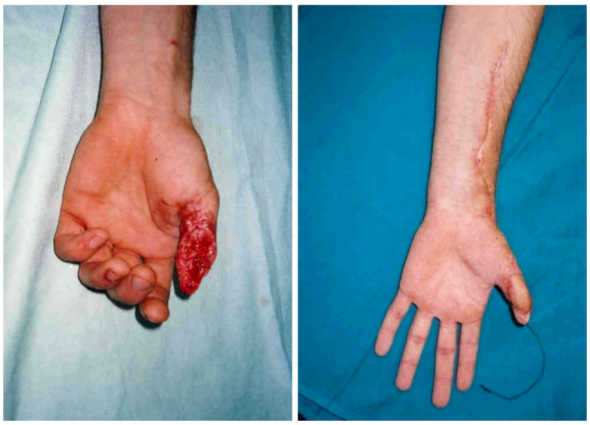
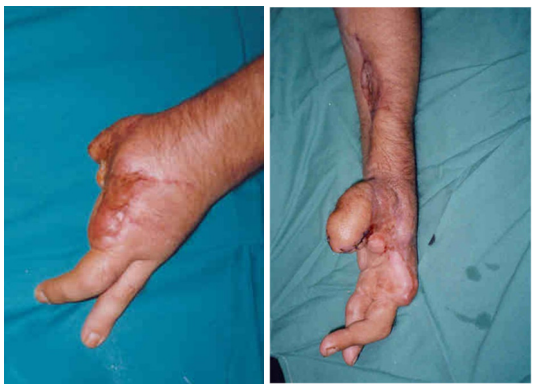
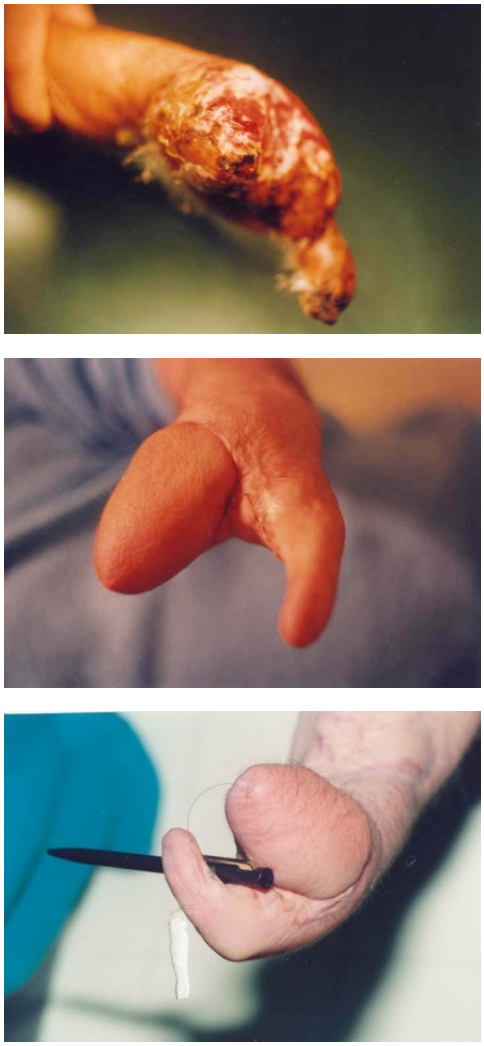


Figure 7. Small Chinese flap in thumb reconstruction

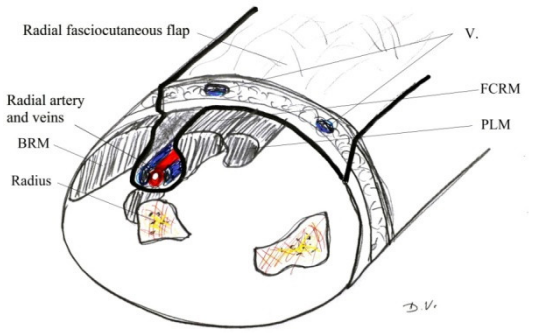


Total thumb reconstruction with osteocutaneous radial flap.

Figure 8. Hand reconstruction with osteocutaneous forearm radial flap. Picture at the end: thumb grip after surgery: note the hair growth at the tip of the flap (analogous photos).



Scheme 1. Schematic cross section through the forearm. Radial blood vessels in the intermuscular septum between the brachioradialis muscle (BRM) and the flexor carpis radialis muscle (FCRM). Marked: segment of skin, adipose tisse and fascia in the flap. Vv.: cephalic and basilic veins; PLM: Palmaris longus muscle.



The functional and aesthetic result of a surgery was assessed using a modification of the MHQ Score (Michigan Hand Outcome Qusteionnarire)[15] which included six groups of questions: general hand function, quality of normal and frequent daily life activities, work ability in major aspects, presence and intensity of pain, subjective assessment of aesthetic results of surgery and general patient satisfaction with surgery. The overall function of the hand was estimated by answering 5 questions: How does your hand work/How well do your fingers move/How well does your wrist move/What is your fist strength/How do you feel your hand? Responses were rated from 1 to 5 (very bad, bad, medium, good and very good). Daily life activities were evaluated by answering questions related to the use of certain items and frequent activities (doorknobs, keys, glasses, buttons, pads, forks and knives, washing dishes and hair, picking up small items, carrying a bag). Work ability was examined by answering questions related to the working status of the patient, the amount of work load, interruptions at work due to difficulties, etc. The fourth category of the questionnaire refered to pain, in terms of whether it exists (always, often, sometimes, rarely) or not, as well as the intensity of the present pain (very strong, severe). The aesthetic aspect was assessed by the question: What impression did you get when you looked at your fist in the last 7 days?; with answers: it interferes with my social activities; I am depressed;, sometimes I am uncomfortable when I am in the company; I am satisfied). The last question was about the satisfaction with the operation, with the answers: very dissatisfied, somehow dissatisfied, neutral, somehow satisfied, very satisfied. The MHQ score totaled 41 questions, with a minimum of 24 points and a maximum of 137, with a higher score for better functional and aesthetic results. Period of opservation was one year, or more. In the statistical data processing, a descriptive approach was applied whereby categorical variables were presented as absolute and relative frequencies of individual categories, and continuous variables were summarized as the mean and the associated range of variation.

RESULTS

All patients were caucasians, mostly male (82.86%), and aged from 23 to 67 (mean: 44.2). Men ranged from 23 to 67 years and women from 28 to 44 years. The right hand (60%) and the dominant hand (71.43%) were injured more often. The etiology of the defect (Fig.4) was different: injury in 30 patients (85.71%) excision of skin tumors in five patients (14.28%) and infection in two patients (5.71%). Most injuries were mechanical in nature (25/35). In three patients, there was explosion wound (3/35) and in two patients, a deep electrical burn (2/35). Most patients with traumatic skin defects were from rural areas (22/30). Localization of skin defect was most common on the dorsum of the hand (18/35). Other sites of the defect were: palm of the hand (3/35), finger (6/35), and combined (8/35). Surgery was performed in all cases as primary delayed or secondary closure of the defect. Five patients underwent excision of a previously placed skin graft because of the need for secondary surgical procedures on deeper tissues. In 27 patients, the surgery was performed under general anesthesia and in eight patients under regional anesthesia.

According to compose, fasciocutaneous flap (Fig.5) was most commonly used (23/35). Adiposofascial flap (Fig.5) was used in seven patients (20%) and osteocutaneous in five patients (14.29%). The size of the skin segment in the flap ranged from 2.2x3.1cm to 9x13.5cm (average: 8x4.5cm). The donor site of fasciocutaneous flap (Fig.5, Fig.6) was closed by a split thickness skin graft in 13 cases (56.52%), direct suture in five patients (21.73%), local skin flap in three patients (13.04%) and a full thickness skin graft (FTSG) in one patient (4.35%). The Z-plasty was used in two patients (8.69%), a rotational flap in one (4.35%) and a bilobar flap in one patient. The length of the operation ranged 75 to175 minutes (mean 145 minutes). There were no cases of complete flap necrosis. In three patients, partial necrosis occurred (8.57%) with no significant consequences. Wound dehiscence in the flap was noted in five patients (14.29%), infection in three (8.57%), hematoma and seroma in five patients (14.28) and gaft failure in the donor site in five patients (14.29%). Three patients underwent total lysis of the graft, and two patients had partial lysis. In one patient, the skin graft was partially lysed on the fascioadipose flap. The length of hospitalization was 8-31 days (mean 17). Secondary surgical procedures were performed in 13 cases (37.14%). Tendoplasty (17.14%) was performed in 6 patients and osteoplasty (11.43%) in four patients. In six patients, flap debulking was necessary (17.14%).

The functional-aesthetic result of the operation based on the MHQ score ranged from 31 (1/35) to 130 points (3/35) and averaged 95.5 points. There were no minimum (24) or maximum points (137), with the average value of MHQ scores being more than half of the maximum.

DISCUSSION

The idea of transferring forearm skin as a cross-forearm flap (16) was not widely accepted because it is a two-stage procedure, with a forced upper extremities position and possible functional impairments. An insular, axial skin flap with a radial artery and veins was first used in 1978 in China as a microvascular free flap for neck reconstruction in extensive scar contraction after deep burn. Three years later Chinese authors published the results of large series of this method in various complex defects of the face (3). Inspired by this technique, European surgeons, after their visit to China, began to use this flap, not only as microvascular, but also as pedicled flap, first for reconstructions on the hand (4,5). Extensive anatomical studies were performed (19, 20). The radial flap has become widely used for defects in other localizations, making it one of the most used flaps. The main reason was its reliable vascularization.

The radial artery is lateral terminal branch of the brachial artery and extends from the neck of the radial bone to the styloid process. It is located in the intermuscular septum, between flexor carpi radialis muscle and brachioradicalis muscle. Numerous branches of radial artery (about 13) enter the forearm fascia, the largest of which is the inferior cubital artery. There are many fasciocutaneous perforant vessels (is 9-16), and the most proximal of them is the largest one (about 0.8mm). Vascular cutaneous territory of the RFF envelops almost the entire anterior aspect of the forearm, proximal to the antecubital fossa and distally to the flexion crease of the wrist, except small ulnar skin segment of forearm, about 3 cm wide. Agenesis or congenital absence of the radial artery is very rare (27), as are anatomical variations: high origin or high terminal branching of the brachial artery and presence of the superficial radial artery (28, 29). These cases are usually seen during the angiography. Two committing, radial veins usually join in the proximal third of the forearm. In the distally based flap, retrograde blood flow through the radial veins, contrary to the valves, is explained by the presence of anastomoses in the form of a ladder or loop, or by vein denervation.

The radial flap, originally fasciocutaneous, was used as adiposofascial and also with other tissues as a composite flap: neurocutaneous, osteocutaneous, myocutaneous (brachioradial muscle, palmaris longus), and tendocutaneous. Also, to provide a thinner flap and a direct suture in the donor region of the flap, an adiposofascial flap covered with a skin graft is used (17). This method shows good results in the early surgical treatment of deep burns on the dorsum of the hand, especially electrical burns. The superficial branch of the radial nerve may be included in the flap for the renervation in the recipient site, especially in the thumb. Segment of radius in the RFF for thumb reconstruction was first used by Biemer and Stock (4). Radius fracture after osteocutaneous RFF has an incidence of up to 25%. However, this percentage may be lower due to the keel shape technique of taking the bone segment of the radius with beveling the proximal and distal cuts or prophylactic placement of the plate. Considering the fact that the thumb accounts for about 40% of the hand function, there is importance of osteocutaneous RFF in subtotal or total thumb reconstruction (22-25) (Fig.7, Fig.8). The radial forearm flap can be used in flap prefabrication (30,31), such as cartilage transfer for reconstruction in the face.

During dissection of the RFF, it is important to preserve paratenon in order to avoid complications when skin graft is placed to the donor site. Also, attention should be paid to ulnar vessels and ulnar nerve that sometimes may be quite superficial. In fact, toward the dissection of the soft tissues of the flap (except for blood vessels), we agree with Lister's recommendation: "The skin hook and the knife are more precise and less damaging than the forceps and the scissors and should be used in preference." (6) For more extensive hand defects, the pivot point of the flap should be at least 5 cm proximal to the wrist crease, for safer hand vascularisation. If possible, one subcutaneous vein should be included in the flap, and if there are suitable conditions, an anastomosis should be performed with one of the veins in the area of the defect (32,33). This is important in reducing postoperative edema of the RFF. Identification of superficial radial nerve branches in RFL dissection is very important.

The donor site of the RFF may be closed by direct suture, or, in larger flaps, by some of the additional surgical procedures, usually split thickness skin graft (STSG). The complications of this method are relatively common, and the aesthetic result is sometimes pure. Therefore, a number of alternative methods have been used: full thickness skin graft, local skin flap, tissue expansion, vacuum assisted closure (VAC), cell cultures, synthetic skin substitutes (35-44). No one of these is ideal and many researches are still focused on the donor site of RFF (32, 34). If STSG was used, secondary transplantation of adipose tissue into the donor site of the flap is advised (45).

When considering a defect in the hand and the method of reconstruction, several points should be considered: localization, diameter and depth of the defect, types of tissue missing (skin, muscle, tendon, bone), age, sex, profession, hobby, as well as psychological profile of the patient in terms of accepting the course of the whole treatment (including intensive physical therapy) and possible complications. Reconstructive surgeons are always looking for the best surgical technique in terms of the better functional and aesthetic result, easier surgical technique, shorter operative time and the whole course of treatment, more economical treatment, minimization of complications, reliable vascularization of the transferred tissues, better quality of these tissues in relation to the characteristics of the recipient region, etc. If the skin defect on the hand is small, a local skin flap may be used. Medium-sized defects can be covered by the interoses posterior flap, such as the first commissure, and medium-sized defects in the proximal or medial part of the palmar region may be closed by the dorsal ulnar flap. When choosing a reconstructive method, one should consider from simple to complex, with the best possible functional and aesthetic result. Due to the great importants of the hand, single-stage surgery has the

advantage of early physical therapy. One of the most important factor for successful flap transfer is a good blood supply of the flap. If we look at these two important things, radial forearm flap is a resonable solution. As any flap, the radial forearm flap has advantages and disadvantages. In general, there are more advantages (46). However, current studies are based on using a flap with radial artery perforators, wherever possible. Unfortunately, the size of the flap and the arc of rotation are limited (47). Also, there are opinions that it is better to use the fascial radial flap which is thinner and the flap donor site complications are less frequent. However, postoperative monitoring of this flap is more difficult and there is a potential failure of the skin graft placed on the fascia.

According to the size of the island fasciocutaneous RFF, there are a few literature data about the minimal skin segment that can survive. Here we emphasize the dimensions of the RFF we used for reconstruction of the tip of the thumb: 2.2x3.1cm (Fig.6).

The complications of radial forearm flap transfer are varoius. Acute ischemia of the hand is the most dramatic, but only one case has been described (48). Other complications are of local, non-specific: edema, hematoma, seroma, dehiscence and infection There are also specific complications: partial or total necrosis of the flap, lysis of the skin graft, tendon exposure in the donor site, bulky flap, donor site sensory disruptions, lymphedema of the hand (49). Our results regarding the complications of RFF are generally similar to those of other studies.

Alternative methods for RFF are ulnar flap, interoseus posterior flap (IPF), forearm perforator flaps and free flap. The advantages of the ulnar flap are primarily the absence of hairs and thinner skin, but the disadvantages may be more significant: a more dominant artery, more difficult dissection, and the possibility of an ulnar nerve lesion. Any additional injury of the forearm may be fatal to the hand. The interoseus posterior flape and the perforator forearm flap have a great advantage in preservation of the main blood vessels, but also the disadvantage that they can be applied only to smaller and more proximal defects. Free flap needs special equipment, personal training, longer surgery time and possible functional and aesthetic consequences in the donor region. Various free flaps (temporal fascial, parascapular, radial flap from the opposite forearm, etc.) allows fast rehabilitation of the hand, but sometimes they are undesirable, as with more extensive injuries of the hand and forearm due to difficulties with the choice of recipient blood vessels in the recipient region. Sometimes, the only solution is distant pedicled flap, preferable inguinal flap.

There are a few reports of late vascular complications in the hand and fingers after the use of RFF. This may be important in patients suffering from some peripheral vascular disease, as well as chronic renal disease with the need for hemodialysis. In general, RRF is not recommended for children, and because of the possible presence of hair on the flap, it is not recommended for many women. That should be evaluated preoperatively by considering the degree of hairiness on the forearm.

The results obtained with functional outcome and aesthetics are similar to those of other authors (50, 51). MHQ does not say that all patients were satisfied or dissatisfied, but on a scale ranging from few points (indicating a worse subjective impression) to more points (indicating a better one), there is the statistical significance that more patients scored more than half (80.5).

The advantages of the radial flap are numerous: constant vascular anatomy, long vascular pediclee (up to 20 cm), large diameter of blood vessels (radial artery: 2-3 mm, radial veins: 1-3 mm and cephalic vein: 3-4mm), vascularization of skin and other tissues, large vascular cutaneous territory, relatively easy dissection, transfer of different tissues (skin, bone, tendon, nerve), thin and elastic skin with the potential to provide sensibility, one operative field, one-stage surgery, early physical therapy, more cost-effective treatment. Due to the fact that the operative method is in one operative field, as well as that it is a one-stage operation, it can be concluded that there are lower risks for postoperative infections. The main disadvantages of RFF are loss of the magistral artery and the scar on the donor site. The incidence of graft failure at the donor site ranges up to 30%, with tendon exposure in about 14% of patients. Another disadvantage of RFF is hair growth on the flap (Fig.8). It is recommended to cover the tendon of the flexor carpi radialis muscle with surrounding muscles (suturing flexor pollicis longus and abductor pollicis longus muscles to flexor digitorum superfitialis muscle), as well as to immobilize the wrist in dorsiflexion to improve skin graft survival.

We conclude that the radial flap is a suitable for covering various tissue defects in the hand, as a result of constant vascular anatomy, thin and pliable tissue and its ease of harvest. We emphasize the importance of this flap in thumb reconstruction (Fig.7, Fig.8). However, it is imperative to choose the optimal reconstructive method which provides appropriate functional and aesthetic result in the recipient and donor site. Pedicled or free radial forearm flap should be in the “repertoire” of plastic surgeon as a method of choice, or as an alternative method.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) and the Helsinki Declaration of 1975, as revised in 2013.

CONFLICT OF INTEREST

There are no conflicts of interest.

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Example of Statement of Animal Rights: "All research procedures were carried out in strict accordance with the European Union Directive for the welfare of laboratory animals (No. 2010/63/EU) and approved by the Ethics Committee of *Name of the Institution* (No. *number of approval*)."


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