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INTERPLAY OF BRAIN-DERIVED NEUROTROPHIC FACTOR AND CYTOKINES IN SCHIZOPHRENIA

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MEĐUSOBNA DEJSTVA MOŽDANOG NEUROTROFIČNOG FAKTORA I CITOKINA U SHIZOFRENIJI

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and plays an important role in neuroplasticity, differentiation and survival of neurons, as well as their function. Neuroinflammation has been explored in the pathophysiology of many mental disorders, such as schizophrenia. Cytokines representing different types of immune responses have an impact on neurogenesis and BDNF expression. Cross-regulation of BDNF and cytokines is accomplished through several signalling pathways. Also, typical and atypical antipsychotic drugs variously modulate the expression of BDNF and serum levels of cytokines, which can possibly be used in evaluation of therapy effectiveness. Comorbidity of metabolic syndrome and atopic diseases has been considered in the context of BDNF and cytokines interplay in schizophrenia.

Keywords: *brain derived neurotrophic factor, cytokines, schizophrenia, metabolic syndrome, atopic diseases.*

SAŽETAK

Moždani neurotrofični faktor (Brain Derived Neurotrophic Factor-BDNF) pripada porodici neurotrofina i ima važnu ulogu u neuroplastičnosti, diferencijaciji i funkciji neurona. Neuroinflamacija je izučavana u etiopatogenezi mnogih mentalnih poremećaja, pa i u shizofreniji. Citokini različitih tipova imunskih odgovora utiču na neurogenezu i ekspresiju BDNF-a. Unakrsna regulacija BDNF-a i citokina se ostvaruje posredstvom nekoliko signalnih puteva. Pokazano je da tipični i atipični antipsihotici mogu drugačije da utiču na ekspresiju BDNF-a i serumske nivoe citokina, što može biti korisno u proceni efikasnosti terapije. Komorbiditet metaboličkog sindroma i atopijskih bolesti je razmatran i u kontekstu uzajamnog dejstva BDNF-a i citokina u shizofreniji.

Ključne reči: *moždani neurotrofni faktor, citokini, shizofrenija, metabolički sindrom, atopijske bolesti.*

ABBREVIATIONS

AD- Atopic dermatitis
BDNF- Brain Derived Neurotrophic Factor
CREB- Cyclic adenosine monophosphate Response Element- Binding
IFN-γ- Interferon-gamma
IL- Interleukin
MetS- Metabolic Syndrome
p75 NTR- p75 NeuroTrophin Receptor
PANSS- Positive and Negative Syndrome Scale
ST2- IL-1 receptor- related protein
TNF-α- Tumor Necrosis Factor-alpha
TrkB- Tropomyosin receptor kinase B

INTRODUCTION

The Brain Derived Neurotrophic Factor (BDNF) is a member of the nerve growth factor family (1). It plays an important role in neuronal survival and growth, modulates neurotransmission and participates as a crucial mediator in all aspects of neuroplasticity (2), such as neurogenesis (3), synaptogenesis (4) and vasculogenesis (5). BDNF participates in learning and memory organization, food intake and motor behaviour (6).

It was shown that BDNF is widely distributed in various regions of the brain, such as the olfactory bulb, cortex, hippocampus, basal forebrain, mesencephalon, hypothalamus, brainstem and spinal cord (2, 7, 8). BDNF promotes the survival of dopaminergic neurons of the substantia nigra (9), cerebellar granule neurons (10), motor neurons (11) and retinal ganglion cells (12).

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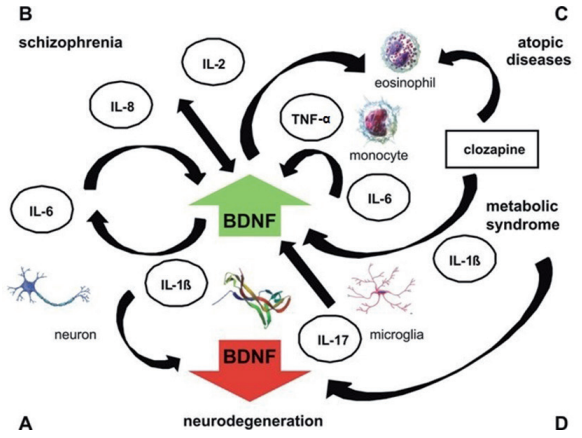

Figure 1. Brain-derived neurotrophic factor (BDNF) - cytokines interactions in schizophrenia and comorbid somatic states
In animal models application of IL-1β decreases BDNF levels in the nerve tissue and lead to degeneration. BDNF- IL-6 communication could be bidirectional. IL-17 up-regulates BDNF microglia production. (A) In schizophrenia was established significant positive association between BDNF and IL-2 and IL-8 levels. (B) TNF-α and IL-6 increase BDNF production in peripheral blood monocytes, that also underlies neuronal hyperreactivity in asthma. BDNF induces eosinophil increase in patients with atopic dermatitis and that could be a side effect of clozapine, an antipsychotic with BDNF- elevating properties. (C) In metabolic syndrome increase of inflammatory cytokines IL-6, TNF-α and IL-1β could reduce BDNF expression. (D)

Microglia and activated macrophages produce BDNF in the brain (13, 14). Human monocytes, but not lymphocytes, are the major cellular source of BDNF in peripheral tissues (15). Furthermore, it is found in the nonneuronal tissue such as lungs, heart, spleen, gastrointestinal tract and liver (16-18) and it is expressed in fibroblasts, vascular smooth muscle cells, and thymic stroma (5, 19, 20). BDNF is involved in activation of endothelial cells (21) and has an influence on the hematopoietic stem cells and thus supports haematopoiesis (22).

The BDNF gene is located on the short (p) arm of chromosome 11 at position 13 (23). As a basic dimeric protein (1), BDNF has a complex genomic structure, with sophisticated organization in terms of transcriptional, translational and post-translational regulatory mechanisms (24). Pro-BDNF is produced in endoplasmatic reticulum, transported to the Golgi apparatus and converted into mature BDNF (25). The receptor for BDNF is Tropomyosin receptor kinase B (TrkB), which exists in two isoforms: the full length receptor glycoprotein (gp145TrkB) with high affinity and truncated form gp-95TrkB lacking tyrosine kinase domain; and the Low affinity Nerve Growth Factor Receptor, also known as p75 NeuroTrophin Receptor (p75 NTR) (2).

BDNF levels in the cerebrospinal fluid in healthy controls were measured in the range of 98.1±83.99 (26). In healthy volunteers, mean plasma BDNF level was found to be ~92.5 pg/ml (8.0–927.0 pg/ml) (2). In schizophrenia, drug-naïve patients had a significant correlation between BDNF protein levels in plasma and cerebrospinal fluid (27). The BDNF can freely pass through blood-brain barrier and all of this suggests that BDNF blood levels can substantially reflect its levels in the central nervous system (28).

The role of BDNF and cytokines in central nervous system and peripheral tissues

Neuroinflammation has an important role in the pathophysiology of mental disorders and pro-inflammatory cytokines and BDNF may have a detrimental effect on neurogenesis, reviewed by Calabrese et al. (29). The impact of the pro-inflammatory cytokines on neurogenesis depends on their concentration, activation of specific cells and neurogenic factors (30). Different stimuli regulate BDNF production in neurons, such as Cyclic adenosine monophosphate Response Element-Binding protein (CREB) - dependent depolarization (31) and cytokines (32).

In study of Lapchak et al. (1993) the mRNA levels of BDNF were significantly decreased in the rat's hippocampus after 4 hours of intraperitoneal injection of interleukin (IL)-1β (33). Application of IL-1β reduces the expression of BDNF through suppression of apoptosis repressor with caspase recruiting domain, CREB and insulin receptor substrate 1 response or by increasing the phosphorilation of p38 mitogen-activated protein kinase (34). Similarly to IL-1β pathways insulin resistance in peripheral tissues (35), the "neurotrophic factor resistance" may be associated with elevation of proinflamatory cytokines, predisposing neurons to dysfunction and increased risk for degeneration (36), as shown in Figure 1A.

Some findings suggest that cytokines could not only inhibit, but also stimulate BDNF expression in neurons and thus improve neuronal survival. Intrathecal infusion of IL-6 in rats increases the concentration of BDNF mRNA in rat lumbal dorsal root ganglia (32) (Figure 1A). Tumor Necrosis Factor-alpha (TNF-α) and IL-6 increase BDNF production in human peripheral blood monocytes (Figure 1C), but IL-1β, Interferon-gamma (IFN-γ), IL-2, IL-4, IL-5, IL-13 and IL-15 did not have any effect on BDNF monocyte production (15). Treatment with IL-17 up-regulated the microglia production of neurotrophic factors (37) (Figure 1A).

The BDNF-cytokine communication seems to be rather bidirectional (presented in Figure 1A). In study of Lin et al. (2016) has been shown that application of BDNF in human Schwann cells increased the secretion of IL-6 through the Janus kinase- signal transducer and activator of transcription pathway activation (38), crucial for the development and function of the immune system (39). Also, wild type mice ($BDNF^{+/+}$) display higher IL-10 secretion in response to low-level stress compared to BDNF-deficient mice (40).

The role of BDNF and cytokines in schizophrenia

The BDNF ability to augment neuroplasticity implies that low BDNF levels may have a role in cognitive declining

observed in Alzheimer's disease, dementia (41, 42), schizophrenia (43), depression (44), and bipolar disorder (45). The influence of BDNF on dopaminergic neurons may be relevant to the pathogenesis of schizophrenia and reflect the severity or the neuronal degeneration in schizophrenia (9). BDNF increases neuronal dopamine content: modulates dopamine release relevant for neuronal plasticity in the ventral tegmental area (46) and also decreases dopamine in mesolimbic dopaminergic system (47). Reduction in BDNF expression was observed in the prefrontal cortex, striatum and hippocampus in animal models and patients with schizophrenia (48-50).

The microglia appeared to be activated in schizophrenia and have provided the main source of pro-inflammatory cytokines (51) and BDNF secretion (13, 14). The etiopathogenesis of schizophrenia is obviously related to the inflammatory responses mediated by microglia (52, 53).

Our previous results in early phases of schizophrenia suggested that type-1 and type-17 immune responses are decreased (54), type-2 response is increased and could be corrected with antipsychotics (55, 56), and higher serum levels of Transforming growth factor beta and IL-23 were measured regardless of applied therapy (57, 58).

BDNF regulation has been shown in many different types of inflammation and seems that it is not type-1 or type-2 restricted: serum levels of BDNF in schizophrenia are not in correlation with serum levels of IL-1β, IL-6, IL-8, IL-10, IL-12 and TNF-α (59), but Zhang (2016) showed a significant positive association between BDNF and IL-2 and IL-8 levels (60) (see in Figure 1B). These data are in correspondence with lower level of IL-2 in patients with early onset disease and predisposition for negative form of schizophrenia (61). Also, higher levels of IL-8 in second trimester have been linked to a higher risk for schizophrenia development (62).

The correlation between serum BDNF levels and Positive and Negative Syndrome Scale (PANSS) scores was not established in patients with schizophrenia (59, 63), but recently low BDNF levels were associated with impairment on the PANSS cognitive factor (60) and also with low cognitive scores on the Repeatable battery for the assessment of neuropsychological Status (64).

A recent meta analysis of Green at al. (2011) point out that blood levels of BDNF are reduced in both medicated and drug-naive patients with schizophrenia (65). Some studies have found a different regulation of BDNF mRNA expression in the rat hippocampus and neocortex after typical and atypical antipsychotic administration (66), where the typical antipsychotic drug haloperidol down-regulated BDNF mRNA expression and atypical antipsychotics olanzapine and clozapine up-regulated BDNF mRNA expression (67) (Figure 1C). Serum BDNF levels were higher in patients with chronic schizophrenia on clozapine or typical antipsychotics, than risperidone (68). Others showed significant decrease of IL-1β plasma levels and increase of TNF-α and BDNF after risperidone treatment (49). Some longitudinal studies have reported that lower serum BDNF levels did not increase after several weeks of antipsychotic treatment (63, 68).

Schizophrenia is associated with metabolic syndrome, expressed by type-2 diabetes and insulin insensitivity (69). This could be discussed as a consequence of unhealthy lifestyle or as a genetic predisposition that is potentiated with antipsychotic therapy (51). In schizophrenia, as in obesity, there is an imbalance between adiponectin and the pro-inflammatory cytokines TNF-α and IL-6 (70). Also, BDNF deficiency has been reported in increased food intake, hyperphagia and obesity (71). Obesity itself does not affect the value of BDNF in the serum in adults (72), but BDNF levels in plasma were decreased in patients with Metabolic Syndrome (MetS) compared with control subjects (73) (Figure 1D). For the first time, Zhang et al. (2013) tested the relationship between the BDNF Val66Met polymorphism and MetS in patients with schizophrenia under long-term clozapine treatment. BDNF serum levels appeared to be associated with clozapine-induced MetS, and that effect is only evident in male patients (71).

BDNF and cytokines in schizophrenia and atopic diseases

Results of Pedersen et al. (2012) indicated comorbidity of schizophrenia and atopic diseases, both with predominance of type-2 immune response (74). In humans, as well as in mouse models of asthma, allergen challenge increases BDNF levels in bronchoalveolar lavage fluid (75). Such increase may be a result of enhanced BDNF secretion by resident airway cells, immune cells including B-lymphocytes, eosinophils, mast cells and macrophages (75). BDNF in the presence of cytokines TNF-α and IL-13 increases proliferation of human airway smooth muscle in asthma (75). In vitro, increase of BDNF in sputum, in response to IL-13, suggests that type-2 cytokines regulate BDNF levels and activity in asthma (76). After antipsychotic treatment significant decrease of IL-13 was observed, suggesting that the BNDF- IL-13 interaction could be more thoroughly explored in schizophrenia (77). Also, IL-33, recently discovered IL-1 family member, plays a role in induction of airway contraction by stimulating expansion of IL-13-producing innate lymphoid cells (78). IL-33/ST2 signalling pathway regulates T cells, differentiation and activation of dendritic cells, activation of macrophages and mast cells and production of type 2 cytokine producing innate lymphoid cells, showed to be important in type-2 immune response in development of spontaneous obesity (79) and atopic disorders, particularly asthma (80).

As it is seen in asthma, BDNF levels are increased in serum, plasma and eosinophils in patients with atopic dermatitis (AD) (81). Patients with AD had higher eosinofilic expression of p75 NTR and TrKB in comparison with nonatopic control subjects (81). Furthermore, it has been shown that BDNF induces chemotaxis and inhibits apoptosis of eosinophils in patients with AD, indicating its role in proinflammatory actions (81). Increase in eosinophilic

count was also observed as a side effect of clozapine, an antipsychotic with BDNF- elevating properties (82). A common genetic variant of BDNF gene was associated with increased risk for developing allergic rhinitis (83). Mechanism of IL-6 and TNF-α induced neuronal hyperactivity in the allergic asthmatic patients is mediated by BDNF-secreting monocytes (15) (shown in Figure 1C).

CONCLUSION

Intriguing concept of neuroplasticity has introduced new approaches in basic and clinical research. The role of BDNF, as a member of nerve growth factor family, has been explored not exclusively in the central nervous system, but also in other peripheral tissues. Low BDNF levels or "neurotrophic factor resistance" associated with elevation of pro-inflammatory cytokines could have an impact in schizophrenia cognitive declining. The BDNF-cytokines interactions are not only restricted to type-1 or type-2 immune response. The role of IL-33/ST2 signalling pathway has been observed in MetS and atopic disorders, which are closely related to schizophrenia. This could be a rational for further research of IL-33 and BDNF interplay in schizophrenia, aiming to improve cognitive functioning and prevent metabolic dysfunction in patients with schizophrenia.

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Contributors

All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors report no conflicts relevant to this paper.

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CLINICAL CHARACTERISTICS OF ROTAVIRUS-INDUCED GASTROENTERITIS IN INFANTS

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KLINIČKE KARAKTERISTIKE GASTROENTERITISA KOD MALE DECE ČIJI JE UZROČNIK ROTAVIRUS

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ABSTRACT

Rotavirus is the most common cause of acute infectious diarrhea in infants and young children up to the age of five. The disease is characterized by profuse watery diarrhea, vomiting and fever. The major complications of rotavirus gastroenteritis (RVGE) are dehydration, middle ear inflammation and upper respiratory tract infection. The basis of treatment is compensation for fluid loss and administration of probiotics. The aim of this study was to analyze the clinical characteristics of rotavirus gastroenteritis in infants. The study was conducted by the type of retrospective-prospective clinical study on infants with rotavirus gastroenteritis diagnosed on the basis of a positive Rotalex test (Orion Diagnostica Finland) and exclusion of other etiological factors at the University Children's Clinic in Belgrade, from April 2005 to December 2010. In addition to the detailed medical history and clinical examination, relevant laboratory analyzes were performed in all patients. Descriptive and analytical statistical methods were applied in the study. Among the descriptive methods, we used grouping, tabulation, graphing, calculating measures of central tendency, calculating measures of variability and calculating relative numbers. Of the analytical statistical methods, distribution normality testing, χ2 test, Mann-Whitney U test and T test were used. Statistical significance will be taken to mean $p < 0.05$. The average infant mortality was 6.7 ± 3.7 months. All respondents were divided into two groups according to the age. The first group consisted of infants aged 0 to 5 months (46%), the second group consisted of infants aged 6 to 12 months (54%). The incidence of aqueous diarrhea (100%), vomiting (84%) and fever (74%) in infants suffering from rotavirus gastroenteritis was analyzed. The significance of the age on the symptomatology of rotavirus gastroenteritis as well as on the importance of using probiotics has been demonstrated.

***Keywords**: rotavirus, gastroenteritis, infant.*

SAŽETAK

Rotavirus je najčešći uzročnik akutne infektivne dijareje u dobi odojčeta i malog deteta uzrasta do pet godina. Bolest karakteriše profuzna vodena dijareja, povraćanje i povišena telesna temperatura. Osnovne komplikacije rotavirusnog gastroenteritisa (RVGE) su dehidracija, upala srednjeg uha i infekcija gornjih respiratornih puteva. Osnovu lečenja čini nadoknada gubitka tečnosti i primena probiotika. Cilj ove studije bio je analiza kliničkih karakteristika rotavirusnog gastroenteritisa u dobi odojčeta. Sprovedena je studija po tipu retrospektivno-prospektivne kliničke studije na odojčadima sa rotavirusnim gastroenteritisom dijagnostikovanim na osnovu pozitivnog Rotalex testa (Orion Diagnostica Finland) i isključenju drugih etioloških činilaca u Univerzitetskoj dečjoj klinici u Beogradu, u periodu od aprila 2005. godine do decembra 2010. godine. Pored detaljne anamneze i kliničkog pregleda, kod svih bolesnika sprovedene su relevantne laboratorijske analize. U studiji su primenjene deskriptivne i analitičke statističke metode. Od deskriptivnih metoda korišćeno je grupisanje, tabeliranje, grafičko prikazivanje, izračunavanje mera centralne tendencije, izračunavanje mera varijabiliteta i izračunavanje relativnih brojeva. Od analitičkih statističkih metoda korišćeno je testiranje normalnosti raspodele, χ2 test, Mann-Whitney U test i T test. Pod statističkom značajnošću podrazumevaće se vrednost $p < 0,05$. Prosečna starost odojčadi izosila je 6,7 ± 3,7 meseci. Svi ispitanici su bili podeljeni u dve gupe u odnosu na starosnu dob. Prvu grupu su činila odojčad starosti od 0 do 5 meseci (46%), drugu grušu činila su odojčad starosti od 6 do 12 meseci (54%). Analizirana je učestalost vodene dijareje (100%), povraćanja (84%) i povišene telesne temperature (74%) kod odojčeta obolelog od rotavirusnog gastroenteritisa. Dokazan je značaj uzrasta na simptomatologiju rotavirusnog gastroenteritisa kaoi značaj upotrebe probiotika.

***Ključne reči:** rotavirus, gastroenteritis, odojče.*

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INTRODUCTION

Rotavirus is the most common single cause of acute infectious diarrhea in infants and young children up to five years of age (1, 2, 3). The incidence rate of hospitalized infants and children under 5 years of age due to rotavirus gastroenteritis in Europe is 300 per 100.000 children of the same age (1, 4). Rotavirus infections occur more frequently in winter and spring (5, 6). The disease is transmitted by feko-oral route, also thought to be a drip infection (6, 7). The virus is present in a very high concentration in the stool (1011 viral particles in only one gram) of the diseased person and is excreted the day before and 8-10 days after the manifestation of the clinical picture of the disease, and in immunocompromised persons, even 2-3 weeks after the manifestation of the clinical picture. The duration of virus excretion does not correlate with the severity of clinical picture of the disease.

The clinical picture of rotavirus gastroenteritis (RVGE) can range from a subclinical form to a very severe form that can lead to the death of a patient. Whether the infection will proceed symptomatically or asymptomatically depends on the characteristics of the virus itself as well as the susceptibility of the host. The most important factor is the age of the host (8). The clinically manifested form of the disease is rare within the first 3-6 months of birth due to passive immunity acquired prenatally (2, 9). The disease is characterized by a profuse watery diarrhea, vomiting and fever (1, 2, 3, 10).

The conditions associated with chronic rotavirus infection are severe immunodeficiency, X-linked agamaglobulinemia, acquired immunodeficiency syndrome (AIDS), and DiGeorge syndrome (11).

The complications of RVGE are dehydration, upper respiratory tract infection, middle ear inflammation, febrile convulsions, bowel invaginations while other complications are rare. Expressed dehydration is the most common reason for hospitalization of the diseased children and the cause of more than 90% of deaths due to rotavirus infection.

The ELISA test and latex agglutination are most commonly used in the diagnosis of rotavirus infection (2).

There is no specific antiviral treatment for rotavirus gastroenteritis. The basis of treatment is a quick and effective compensation for the loss of fluid, water and electrolytes, as well as the use of probiotics.

MATERIALS AND METHODS

The study is a retrospective-prospective study conducted at the University Children's Clinic in Belgrade from April 2005 to December 2010. The study included 50 infants who had a confirmed diagnosis of rotavirus gastroenteritis.

The aim of this study was to analyze the clinical features of infantile rotavirus gastroenteritis and to determine the significance of the age on the expression of this pathological condition.

The criteria for inclusion in the study were: infants with gastroenteritis signs referred to the University Children's Clinic for the treatment and proven to have Rotalex test infection and infants diagnosed with Rotalex test hospitalized at the Department of Gastrointestinal and Nutritional Disorders primarily due to another pathological condition (intolerance to cow's milk proteins, gluten enteropathy, urological tract infection, etc.). The exclusion criteria were: infants with chronic diarrhea, infants with acute diarrhea who demonstrated bacterial and / or parasitic antigens in a stool sample, infants with a positive Adenolex test, infants with previous gastroenterological operations, immunodeficiency antibodies, infants with a probiotic or any other medication for diarrhea at least 7 days before the admission to hospital and infants with other infections (sepsis, pneumonia). All patients included in this study were thoroughly analyzed for their anamnesis (heteroanamnesis) data, clinical findings, as well as relevant laboratory parameters and therapeutic procedures. A latex agglutination test, the Rotalex test (Orion Diagnostica Finland), was used to demonstrate rotavirus infection or presence of rotavirus in the stool. In heteroanamnesia, the attention was paid to infant problems. The following symptoms and signs of the disease were investigated: diarrhea, vomiting and fever.

Relevant laboratory blood tests were performed in each infant to assess the severity and follow-up of the disease, such as: the acid-base status, urea level, creatinine,) and the leukocyte count with a differential formula. The use of relevant clinical-laboratory parameters in all infants has registered presence of dehydration. Dehydration, as a basic complication, was followed by presence of other complications. Finally, the analysis of the patient's rehydration (intravenous, oral) and probiotic administration was made.

The data were analyzed using modern descriptive methods (grouping; tabulation; graphical representation; calculation of central tendency measures: arithmetic mean, median, mode; calculation of variability measures: standard deviation; calculation of relative numbers: percentages) and analytical statistics (distribution normality testing, $\chi 2$-squared test, Mann-Whitney U test, T test) with computer support and SPSS 12.0 software package. Statistical significance was taken to mean $p < 0.05$.

RESULTS

Rotavirus infections occurred throughout the year as shown in Figure 1. The clinical characteristics of infants suffering from rotavirus gastroenteritis are shown in Table 1. We divided all respondents by the age into two groups. The first group consisted of 23 infants aged 0 to 5 months, which is (46%), and the second group consisted of 27 infants aged 6 to 12 months, which is (54%). The clinical characteristics

of infants with rotavirus gastroenteritis with the age were analyzed and the results are shown in Table 2.

Based on the results of the test, we proved that there is a statistically significant difference between the age groups of infants considering the average values of fever for a period of 24 hours (p=0.002), but that there is no statistically significant difference between the age groups of water diarrhea for a period of 24 hours (p=0.571) or considering the average number of vomits over a 24-hour period (p=0.788).

Analyzing the influence of age on the use of probiotics in the treatment of rotavirus gastroenteritis, it was found that there was a highly statistically significant difference between the age groups of infants with rotavirus gastroenteritis considering the use of probiotics (p=0.006). More probiotics were used in the older age group

Figure 1. Monthly occurrence of RVGE

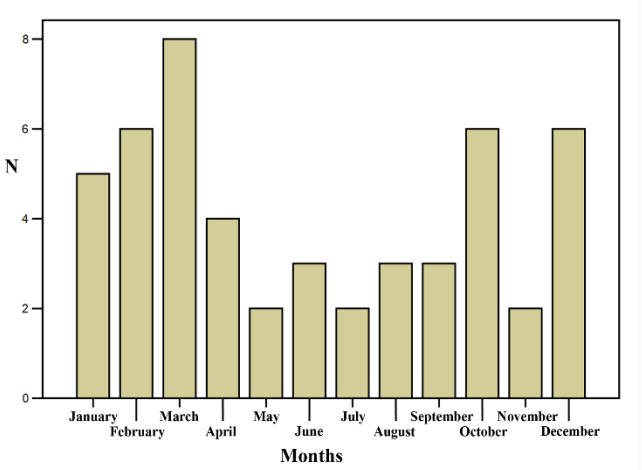

Table 1 - Clinical characteristics of infants with rotavirus gastroenteritis

Clinical characteristics of infants	infants with rotavirus gastroenteritis
average age	6,7±3,7
sex	
M	30 (60%)
F	20 (40%)
type of infection primary	
primary	34 (68%)
intrahospital	16 (32%)
clinical symptoms	
diarrhea	50 (100%)
vomiting	42 (84%)
fever	37 (74%)
average number of diarrhea in 24 hours	6,5±3,9
average number of vomiting in 24 hours	5,1±2,5
average fever for 24 hours	38,6±0,5
dehydration	48 (96%)
metabolic acidosis	29 (58%)
serum potassium value	
physiological range	37 (74%)
hypokalemia	10 (20%)
hyperkalemia	3 (6%)
serum urea value	
physiological range	24 (48%)
increased value	14 (28%)
reduced value	12 (24%)
serum creatinine value	
value physiological range	50 (100%)
blood leukocyte value	
physiological range	43 (86%)
leukocytosis	7 (14%)
complications	24 (48%)
respiratory tract infection	17 (34%)
middle ear inflammation	7 (14%)
rehydration	
i.v.	35 (70%)
per os	15 (30%)
probiotic	40 (80%)

Table 2 - Clinical characteristics of infants with rotavirus gastroenteritis in relation to age

Clinical characteristics of infants	age groups of infants with RVGE		
	0-5 months	6-12 months	p value
N	23 (46%)	27 (54%)	
clinical symptoms			
diarrhea	23 (100%)	27 (100%)	1,000
vomiting	18 (78,3%)	24 (88,9%)	0,526
fever	15 (65,2%)	22 (81,5%)	0,191
average number of diarrhea in 24 hours	6,3±4,1	6,7±3,9	0,571
average number of vomiting in 24 hours	5,0±3,6	5,2±3,2	0,788
average fever for 24 hours	38,3±0,3	38,7±0,5	0,002
dehydration	22 (95,7%)	26 (96,3%)	0,446
metabolic acidosis	12 (52,2%)	17 (63%)	0,400
serum potassium value			
physiological range	3 (13,0%)	7 (25,9%)	
hypokalemia	18 (78,3%)	19 (70,4%)	0,204
hyperkalemia	2 (8,7%)	1 (3,7%)	
serum urea value			
physiological range	10 (43,5%)	14(51,9%)	
increased value	7 (30,4%)	7 (25,9%)	0,966
reduced value	6 (26,1%)	6 (22,2%)	
blood leukocyte value			
physiological range	20 (87%)	23 (85,2%)	1,000
leukocytosis	3 (13%)	4 (14,8%)	
complications	14 (60,9%)	13 (48,1%)	0,982
rehydration			
i.v.	16 (69,6%)	19 (70,4%)	0,976
per os	7 (30,4%)	8 (29,6%)	
probiotic	14 (60,9%)	26 (96,3%)	0,006

DISCUSSION

In the United States, about 50.000 patients report having RVGE hospital treatment each year, with 20 to 40 patients dying from complications during the year, while the number of deaths in some European countries ranges from 7 to 87 (2).

A systematic review of RVGE in children under the age of five in Asia has determined that rotavirus is associated with approximately 145.000 deaths per year (12).

RVGE is the cause of 25 million doctor visits, 2 million hospitalizations and 180.000-450.000 deaths in children under five years of age, annually. In the European Union, rotavirus is estimated to cause about 3.6 million episodes of acute gastroenteritis, 700.000 doctor visits, 87.000 hospitalizations and 231 deaths in children under five (13).

In our study, Rotavirus infection occurred throughout, with statistically significant occurrence in March. The results of compatibility with ours were obtained by Shrestha et al. who conducted his research in the territory of Nepal (12), Aristegui et al. in the territory of Spain (14), as well as Nahari et al. in the territory of Saudi Arabia (15). Different results from ours were reported in Thailand, where the highest incidence was recorded in the summer months (16) as well as during the summer rains in Ethiopia (17).

Gladstone et al. in his prospective study, which lasted for three years, monitored the occurrence of RVGE from birth to the third year of life and concluded that the distribution by gender was of no statistical significance (18).

In the study of Shresth et al., the infection was more prevalent in male (31%) than in female (24%) but without statistical significance (12). The results obtained by the RVGE analysis in London are compatible with our results. RVGE was more frequent in the male population (66%) than in the female population (44%), but without statistical significance (13). In the study by Konda et al., conducted in the territory of Japan, the gender distinction is also without a statistically significant difference (19). The compatible results were obtained in the study by Aristegui et al., where there was a higher male representation with 51.9% (14), as in the study by Nahari et al., where there was a higher male representation with 66.6% (15).

Some authors in the youngest age group of 0-5 months either do not have the disease or their number is very low. Gurwith et al. do not have patients at all at that age (20). Holdaway et al. have a frequency of RVGE in the youngest age group of 4.7% (21), while in the study of Nahari et al., the incidence of less than 6 months among the patients with RVGE was 38.2% (15).

Kamia et al. examined the incidence of RVGE in children under 5 years of age in three Japanese cities and concluded that only 4.2% of the total number of children surveyed, were under six months of age and 24.8% of children < 1 year old, ie. infants (22).

The average infant age in our study is 6.7 ± 3.7 months, which is in line with Western European countries. The average age in the territory of Saudi Arabia in a study conducted by Nahari et al., was 9.8 ± 10.2 months (15).

In the multicenter prospective study covering Western European countries, the largest number of hospitalized children due to RVGE was registered at the age of 6 to 11 months (Belgium, France, Germany, United Kingdom) with the exception of Italy, Spain and Sweden, where the age of hospitalized children was in range from 12 to 23 months (23).

Diarrhea in our study was present in all infants 100%, which is identical with the study of Shresth et al. (12). In the study of Karampatsas et al. in London, diarrhea was reported in 86% of cases (13), and in the study by Arístegui et al., the incidence of diarrhea was 97% (14).

The average number of water diarrhea obtained in our study was 6.5 ± 3.9 over a 24-hour period, which is higher than the average number of diarrhea over a 24-hour period recorded in the study by Kondo et al.,which was 3.6 ± 3.2 (19).

In our study, vomiting, as a symptom of the disease, was present in 84% of infants with RVGE. The results similar to ours were obtained in their study by Nahari et al., where vomiting was reported as a symptom in 81.3% (15). A slightly lower incidence of vomiting was observed in the study of Karampatsas et al., where it was 74% (13) and in the study by Arístegui et al., where it was 69.1% (14). The lowest incidence of vomiting registered in the study of Shresth et al., was 36% (12).

The average number of returns within 24 hours in our study, was 5.1 ± 2.5 times, which is higher than in the study by Kondo et al., where it was 3.6 ± 3.8 times for a 24-hour period (19).

In our study, fever was recorded in 74% of infants. An identical incidence of fever was registered in the study conducted by Karampatsas et al. (13). In the study of Nahari et al., fever was observed in 81.3% of cases (15), while slightly lower incidences were reported in the study by Arístegui et al., 69.1% (14), and a significantly lower incidence of fever was registered in the study of Shresth et al., where it was present in 25% of RVGE cases (12).

In our study, dehydration was present in 96% of infants. In the study of Shresth et al., dehydration was present in all cases of RVGE (12). The results different from ours were obtained by Karampatsas et al., where dehydration was present in 44% (13). A lower incidence of dehydration was registered in the study of Arístegui et al., where it was present in 20% of RVGE cases (14) and in the study of Nahari et al., where it was present in only 11.7% of cases (15).

In our study, metabolic avidosis was reported in 58% of RVGE cases while in the study of Nahari et al., it was reported in 68.6% of RVGE cases (15). In our study, complications of rotavirus gastroenteritis were present in 48% of infants. The upper respiratory tract infection was common in 34% of infants while 14% of infants had the middle ear inflammation. Febrile convulsions, intestinal invagination and other possible complications were not recorded in our study. In the study of Karampatsas et al., a higher incidence of neurological complications was reported, whereas the upper respiratory tract infection complications were present in 27% of RVGE cases (13).

Rehydration, as the primary treatment for RVGE patients, was present in all infants in our study. In 70% of cases, it was intravenous, while in 30% of cases, it was oral.

In the study of Karampatsas et al., intavenous rehydration was present in 50% of cases (13), and in the study by Arístegui et al., intravenous rehydration was present in 68.3% of RVGE cases (14).

Many studies have attempted to demonstrate the importance of probiotic use in RVGE therapy. Grandy et al. demonstratedin his study that the use of probiotics in RVGE therapy in infants and young children significantly reduced the duration of diarrhea by 31.4%, as well as the duration of fever by 73%. The effect of probiotics on vomiting has not been clear yet and the exact mechanism of the effect of probiotics on vomiting length cannot be determined (24). Some studies have reported that the use of probiotics does not affect the length of vomiting, while other authors believe that the use significantly shortens the length of vomiting (25).

In our study, because of the same opinion about the beneficial effect of probiotics on the length of the problems and their alleviation, there is a statistically significant difference in the use of probiotics, ie. a significantly higher number of infants received probiotics, 80%. More probiotics in our study were present in infants of the older age group.

A more severe clinical picture of RVGE is commonly seen in intrahospital infections. In our study, of all registered RVGEs, 32% of RVGEs were due to the intrahospital infection. Different results from ours were obtained by Shrestha et al., where statistically significant was a higher incidence of non-tachospitular RVGE infections compared to the primary rotavirus infections (12). The results obtained by

Karampatsas et al. showed that the incidence of intrahospital RV infection was prevalent in 93% of RVGE cases (13).

CONCLUSION

Based on the results obtained in the study period, the following can be concluded:

- The mean age of infants with RVGE was 6.7 ± 3.7 months.
- Most infants (68%) were hospitalized for RVGE, while in other infections, they were acquired intrahospitally.
- Infection was more common in March compared to other months of the year.
- RVGE clinical imaging was based on aqueous diarrhea (100%), vomiting (84%) and fever (74%).
- Dehydration is found in 96% of infants.
- Complications (the upper respiratory tract infection and middle ear inflammation) have been reported in 48% of infants with RVGE.

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CONTEMPORARY DISTRIBUTION OF HIGH-GRADE PROSTATE CANCER IN THE CIRCUMSTANCES OF OPPORTUNISTIC TESTING

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SAVREMENA DISTRIBUCIJA VISOKOGRADUSNIH KARCINOMA PROSTATE U OKOLNOSTIMA OPORTUNOG TESTIRANJA

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ABSTRACT

Screening has dramatically changed the distribution of the mean age, stage and grade of prostate cancer (PCa) at diagnosis. However, regional-level data that characterize contemporary PCa patients are limited. The aim of the study was to ascertain main clinical and pathological characteristics of PCa at the present time in the circumstances of opportunistic testing.

High-grade PCa according to age, serum prostate specific antigen (PSA), volume prostate, PSA density (PSAD), digital rectal examination (DRE) number of positive cores biopsies and the average percentage of cancer in biopsy at diagnosis has been retrospectively evaluated in 100 men with biopsy-proven PCa, at Clinical Centre Kragujevac, from September 2016 until September 2017. PCa were stratified according to Gleason score (GS) into low/intermediate-grade (GS ≤ 7) and high-grade (GS ≥ 8). To identify the determinants associated with high-grade PCa, we performed univariate and multivariate logistic regression.

The most prevalent PCa were the low/intermediate-grade (65%), followed by high-grade (35%). The mean age of the patients was 71.5 (range: 56–88) years and median PSA was 14.6 (range: 1.4–935) ng/ml. There were significant differences in age, PSA, PSAD, DRE, number of positive biopsy and average percentage of cancer in biopsy between patients with or without high-grade GS. Logistic analysis demonstrated the PSAD and age have strong prognostic value of high-grade PCa.

In conclusion, our study has shown the worrying frequency of high-grade PCa in the circumstances of opportunistic testing. Older men and higher level of PSAD had a much higher probability of high-grade PCa.

Keywords: *prostate cancer; age; Gleason score; biopsy;*

SAŽETAK

Skrining karcinoma prostate (PCa) je dramatično promenio distribuciju srednje starosti, stadijuma i gradusa tumora bolesnika pri postavljanju dijagnoze. Međutim, regionalni, savremeni podaci bolesnika sa PCa su veoma oskudni. Cilj studije je da proceni glavne kliničke i patološke karakteristike bolesnika sa PCa u sadašnje vreme u okolnostima oportunog testiranja.

Retrospektivno su ispitivani visokogradusni PCa na 100 biopsijskih dokazanih PCa u odnosu na starost, serumski nivo prostata specifičnog antigena (PSA), volumena prostate, gustine PSA (PSAD), digitorektalni pregled (DRE), broja pozitivnih iglenih biopsija i prosečnog sadržaja karcinoma u biopsijskom materijalu, u Kliničkom centru Kragujevac, u periodu od septembra 2016 do septembra 2017 godine. PCa su klasifikovani u odnosu na Glison skor (GS) na nisko/umerenogradusne (GS ≤ 7) i visokogradusne (GS ≥ 8). Univarijantna i multivarijantna logistička regresija je sprovedena radi utvrđenja determinati povezanih sa visokogradusnim karcinomima.

Najučestaliji PCa su bili nisko/umerenogradusni (65%), a potom visokogradusni (35%). Prosečna starost bolesnika bila je 71,5 (u opsegu: 56–88) godina, a medijana PSA vrednosti bila je 14,6 (u opsegu: 1,4–935) ng/ml. Utvrđena je značajna razlika u starosti, PSA, PSAD, DRE, broju pozitivnih iglenih biopsija i prosečnog sadržaja karcinoma u biopsijskom materijalu između bolesnika sa ili bez visokogradusnih karcinoma. Logistička analiza je pokazala da su PSAD i starost najmoćniji prediktori visokogradusnih PCa.

U zaključku, naša studija je pokazala zabrinjavajuću učestalost visokogradusnih PCa u okolnostima oportunog testiranja. Stariji muškarci i više vrednosti PSAD imaju višu verovatnoću prisustva visokogradusnih PCa.

Ključne reči: *karcinom prostate; starost; Glison skor; biopsija;*

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ABBREVIATIONS

AUC - area under the receiver operating characteristic curve;
CI - confidential interval;
GS - Gleason score;
DRE - digital rectal examination;
IQR - interquartile range;
LR - logistic regression;
ORs - odds ratios;
PCa - prostate cancer;
PSA - prostate-specific antigen;
PSAD - PSA density;
SD - standard deviation;
SEER - The Surveillance Epidemiology and End Results;
TRUS - transrectal ultrasound;

INTRODUCTION

Prostate cancer (PCa) remains the most common cancer in men in Europe (excluding skin cancer) (1). With the introduction of the prostate-specific antigen (PSA) testing, PCa incidence rate increased drastically, and peaked in 1992. The rate subsequently declined, and then appeared to stabilize from 1995 to 2005 (2). PCa is usually suspected on the basis of digital rectal examination (DRE) and/or an elevated PSA. Definitive diagnosis depends on histopathologic verification. Abnormal DRE is an indication for biopsy, but as an independent variable, PSA is a better predictor of cancer than either DRE or transrectal ultrasound (TRUS).

Age at diagnosis, cancer stage, and grade are among the most important factors used to determine the PCa treatment modality such as prostatectomy, radiation, or active surveillance. With the widespread use of the PSA test, the mean age at diagnosis dropped substantially and the distribution of PCa stage and grade has also dramatically changed, with localized and moderately differentiated tumors becoming predominant (3). In USA, among newly diagnosed patients in 2004 – 2005, the majority (94%) had localized (ie, stage T1 or T2) PCa and a median serum PSA level of 6.7 ng/mL. The average age at PCa diagnosis decreased over time from 72.2 to 67.2 years (3).

The Gleason grading system remains one of the most powerful prognostic predictors in PCa. High-grade PCa, also called poorly differentiated PCa, has Gleason scores (GS) from 8 to 10, is a deadly disease that needs aggressive treatment (4). The incidence of a biopsy GS of 8 – 10 among newly diagnosed patients also decreased over time. Recent Surveillance Epidemiology and End Results (SEER) data show that nearly half of all PCa diagnosed in recent years are of low grade (GS 2–6), and there is about 14% of poorly differentiated PCa (5).

Cancer registry of central Serbia of Institute of Public Health of Serbia gives the epidemiological parameters of malignant neoplasms in the territory of central Serbia, however, demographic and clinical factors were not examined in this study (6). To our knowledge, a comprehensive examination of recent PCa incidence rates and trends in the Serbian population is lacking, especially by cancer stage and grade.

Based on these considerations, the aim of the study was to ascertain main clinical and pathological characteristics of high-grade PCa at the present time in the circumstances of opportunistic testing, and to identify the determinants associated with high-grade PCa. We hypothesized that older age and higher PSAD would be associated with an increased risk of aggressive disease.

PATIENTS AND METHOD

This is a retrospective study carried out using the database of 239 patients at Clinical Centre Kragujevac, who had undergone ultrasound-guided prostate biopsies, from September 2016 through September 2017. Patient referrals were obtained in the course of routine clinical care, regardless of PSA level or clinical findings, and not as part of a population based screening trial. After obtaining institutional review board approval, the data were collected about clinicopathological characteristics for each patient as regards prebiopsy assessment and included following: age, PSA, volume of prostate, PSAD, DRE, total number of cores taken, GS, number of positive cores biopsies and average percentage of cancer in biopsy. Exclusion criteria were patients with incomplete data, and medical therapy known to affect PSA levels. PCa were stratified according to GS into the following groups: low/intermediate-grade who has GS≤6 or GS=7 and high-grade who have GS≥8 (7). Also, the cohort was stratified into 10-*year age* groups (less than 60, 60–70, 70–80 and more than 80 yr old) and into three groups according to PSA level (PSA 1.4–10 ng/mL, 10.1–20 ng/mL and >20 ng/mL) to investigate the increasing effect of age and PSA on outcome.

A member of the urology team performed a DRE or prostate biopsy on all patients. The DRE was classified as normal, or suspicious/positive. At presentation, the serum PSA measurement (UniCel DxI 600 Access Immunoassay System, Beckman Coulter, USA) was performed. All patients underwent prostate biopsy according to protocol. A Toshiba (Aplio 300) ultrasound device with 5-10-MHz probe was used to obtain ultrasound data and prostate biopsy. All patients underwent ultrasound-guided prostate biopsies performed us-

Table 1. Baseline patients' clinicopathological characteristics **(N**=221).

Characteristics		All	BPH (n=121)	PCa (n=100)	P value
Age	mean ± SD, years	69.8 ± 7.3	68.5 ± 7	71.5 ± 7.3	0.002
PSA	median (IQR) ng/ml	11.2 (15.1)	9.9 (8.9)	14.6 (40.8)	0.000
Volume prostate	median (IQR), ml	49 (32.5)	55 (37)	44.5 (29.7)	0.008
PSAD	median (IQR), ng/ml/ml	0.24 (0.41)	0.19 (0.68)	0.38 (0.68)	0.000
DRE	abnormal n, (%)	53 (24)	13 (10.7)	40 (40)	0.000
Number of biopsy cores	median (IQR)	10 (0)	10 (0)	10 (0)	0.056

BPH–benign prostatic hyperplasia; PCa–prostate cancer; SD–standard deviation; PSA–prostate-specific antigen; IQR–interquartile range; PSAD–prostate-specific antigen density; DRE–digital rectal examination;

Table 2. Baseline clinicopathological characteristics in patients with different Gleason grade prostate cancer **(N**=100).

Characteristics		Low/intermediate grade PCa n=65	High-grade PCa n=35	P value
Age	mean ± SD, years	69.5 ± 6.9	75.1 ± 6.7	0.000
PSA	median (IQR) ng/ml	10.5 (13.25)	59 (136.4)	0.000
Volume prostate	median (IQR), ml	43 (27)	46 (27)	0.303
PSAD ml	median (IQR), ng/ml/	0.29 (0.39)	1 (2.56)	0.000
DRE	abnormal n, (%)	23 (35.4)	17 (48.6)	0.208
Number of positive biopsy		3 (4)	5 (6)	0.028
Average percentage of cancer in biopsy		33.4 ± 27	50 ± 23.3	0.000

PCa–prostate cancer; SD–standard deviation; PSA–prostate-specific antigen; IQR–interquartile range; PSAD–prostate-specific antigen density; DRE–digital rectal examination;

ing an 18-gauge biopsy instrument (Md-Tech, Pro-Mag I 2.5, USA). A median of ten biopsy cores was obtained (range, two to 12 cores), and evaluated per each hospital's standard procedure and by local pathologists. Prostate volumes were obtained by measuring the gland in three dimensions, and volume was estimated using the following formula: 0.52 [length (cm) × width (cm) × height (cm)]. The PSAD was calculated by dividing the serum PSA by the calculated prostate volume.

Statistical Analyses

Descriptive statistics was used for demographic and baseline characteristics. We expressed continuous variables as the mean and standard deviation (SD) when normally distributed or as the median and interquartile range (IQR) if their distribution was skewed. Categorical variables in different groups were expressed as frequencies and percentages, and were compared using the Chi-square test. Continuous numerical data were analyzed using t-test or the Mann-Whitney U test when the data are not normally distributed.

Univariate and multivariate logistic regression (LR) was used to identify and quantify the potential and independent determinants associated with high-grade PCa with Backward–Wald stepwise. The results of regressions were expressed in odds ratios (ORs) with 95% confidential interval (CI). For model derived from LR analysis and the strongest predictor we calculated area under the receiver operating characteristic curve (AUC). The SPSS (version 23.0) software package was used for all analyses. Statistical significance was set at $p < 0.05$.

RESULTS

Patients' characteristics

A total of 221 patients were analyzed. Prostate cancer was detected in 100 (45.2%) of patients. Table 1 shows the clinicopathological characteristics of patients with/without PCa included in the study. There were significant differences in age, PSA levels, volume of prostate, PSAD and DRE findings between patients with or without PCa. DRE was positive in 40% of patients with PCa, and median PSA was 14.6 ng/ml (range: 1.4–935 ng/ml): 34 (34%), 23 (23%), and 43 (43%) had a PSA included between 1.4 and 10 ng/ml, between 10.1 and 20 ng/ml, and greater than 20 ng/ml, respectively. The rates of prostate cancer patients were 6%, 44%, 35% and 15% at the 6^{th}, 7^{th}, 8^{th} and 9^{th} decades of life, respectively.

The majority of tumors (40%) were determined to be GS 6 or less, followed by high (35%), and then intermediate grade group (25%). There were significant differences in age, PSA levels, PSAD, DRE, number of positive biopsy and average percentage of cancer in biopsy between patients with or without high-grade GS (Table 2). A significant correlation between GS grade and age decades was demonstrated (p = 0.017), and high-grade cancer was detected in more than two-thirds (68.6%) of patients older than 70 years, and for no one under the age of 60 years. Figure 1 shows the distribution of high-grade PCa according to age decades. Also, a significant correlation between GS and PSA level was demonstrated (p = 0.000), and about three-fourths (74.3%) of patients with high-grade cancer has PSA level above 20 ng/ml.

Table 3. Logistic regression analysis of high-grade prostate cancer predictors.

Factor	Univariate analysis		Multivariable analysis	
	OR (95% CI)	P value	OR (95% CI)	P value
Age	1.124 (1.052–1.201)	0.001	1.111 (1.025 – 1.205)	0.011
PSA	1.025 (1.012–1.038)	0.000		
Volume prostate	1.011 (0.997–1.026)	0.118		
PSAD	3.693 (1.766–7.720)	0.001	2.988 (1.504–5.940)	0.002
DRE	1.725 (0.748–3.976)	0.201		

OR–odds ratio; CI–confidence interval; PSA–prostate-specific antigen; PSAD–prostate-specific antigen density; DRE–digital rectal examination;

Figure 2 shows the distribution of high-grade PCa according to PSA ranges. Overall, the probability of high-grade PCa increased significantly with increasing age decades and PSA ranges. Low/intermediate grade and high-grade PCa were present in 25 (35.4%) and 17 (48.6%), respectively, of the DRE positive PCa patients, but difference was not statistically different (p = 0.208). The median number of positive biopsy cores and average percentage of cancer in biopsy were more pronounced in high-grade PCa patients indicating a higher tumour volume (Table 2).

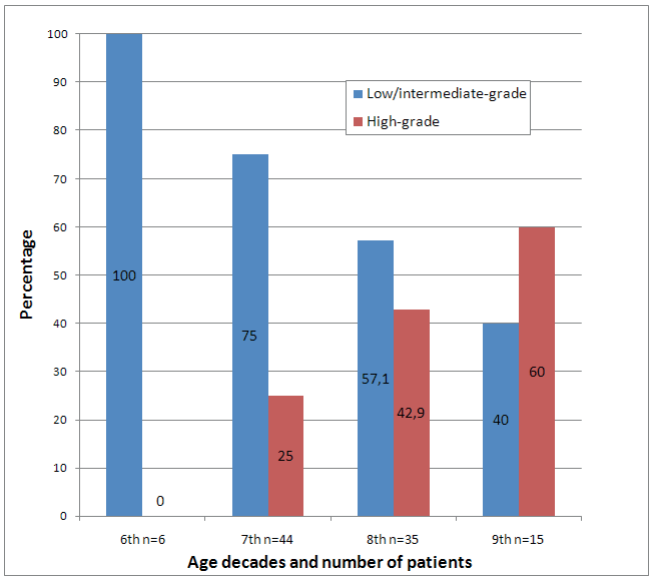

Figure 1. Distribution of high-grade prostate cancer and number of patients according to age decades.
Percentages are expressed in relation to the total number of patients in the age decade's group.

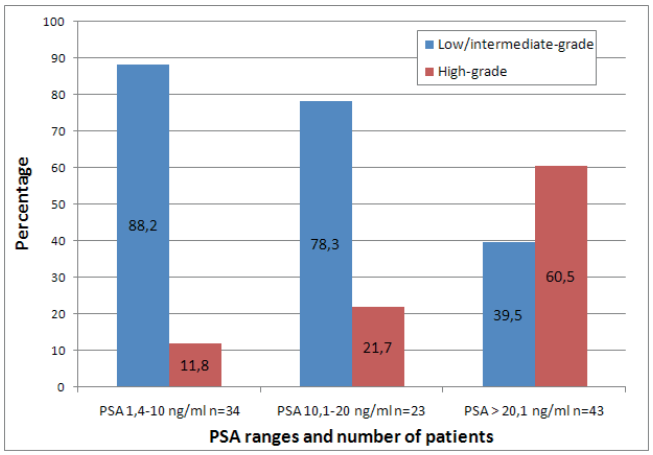

Figure 2. Distribution of high-grade prostate cancer and number of patients according to PSA level.
Percentages are expressed in relation to the total number of patients in the PSA level group.

The logistic regression analysis

In a univariate analysis, 3 risk factors displayed significant correlation with high-grade PCa (Table 2). During multivariable analysis two sustained their prognostic significance (Table 2). The analysis demonstrated that the age and PSAD have strong prognostic value of high-grade PCa (Table 2). A global metric of test accuracy (AUC) for model and individual predictor are showed in Figure 3. AUC for the model and the strongest predictor was shown to have good discriminatory ability (84%, 95% CI 75.9–92%, and 77.5%, 95% CI 67.3–87.8%, respectively), and in pairwise comparison of ROC curves difference between areas LR model and PSAD (6.48%) was significant (p = 0.043).

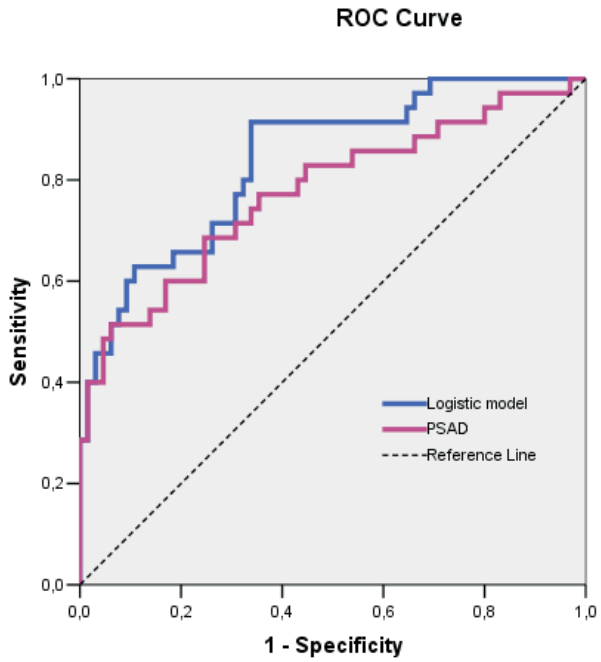

Figure 3. ROC curves analysis.
A global metric of test accuracy (AUC) for model and the most significant predictor (PSAD).

DISCUSSION

There are several findings from this study to emphasize. First, these regional-level data show the worrying frequency of high-grade PCa at the present time in the circumstances of opportunistic testing; secondly, our study has shown that older men have a higher probability of being diagnosed with more aggresive disease; and thidrly, high-grade disease may be predicted using PSAD and age with good discriminatory ability.

Previous studies have recognized criteria associated with high-grade PCa. They included age, family history, genetics, race/ethnicity, obesity, and others (8). In line with previous studies, several of those predictors have reached statistical significance in the univariate or multivariate analysis in our study. We found that the probability of high GS increases with increasing age and PSA, which is in line with numerous previous reports (9, 10). However, in some studies it has been shown that younger (men aged ≤55 years) and more elderly male (>75 years) patients are more likely to have a more aggressive disease (11). There are several possible explanations for this observation, including a higher chance of Gleason grade progression and changes in biopsy technique. Before all, older age may be associated with decreased frequency of screening, allowing tumors more time to grow and possibly dedifferentiate before diagnosis, although Gleason grade progression is controversial (12). Futhermore, it is possible that tumor biology changes with age, such that tumors that develop in older men tend to be more aggressive (13). Biological reasons for progressive dedifferentiation with aging can be the result of common disease mechanisms. The underlying mechanism linking both processes are the time dependent accumulation of cellular damage, such as the role of genomic instability, telomere attrition, epigenetic changes, loss of proteostasis, decreased nutrient sensing and altered metabolism, but also cellular senescence and stem cell function.

Gleason score, which was introduced in 1974, represents a significant histopathological parameter commonly used to assay the prognostic outcome of PCa. Gleason scores 8–10 are often considered as one group corresponding to high-grade disease. Major Gleason scoring revisions were adopted in 2005 (7). However, in 2013, a new grading system was proposed by the group from Johns Hopkins Hospital (14). The grading system includes five distinct Grade Groups based on the modified GS groups. These Grade Groups were shown to be more accurate in predicting progression than the Gleason risk stratification groups (≤6, 7, 8–10) (15). It has been reported that high-grade tumours are significantly larger than tumours which are low/intermediate grade. Our results are in agreement with these findings by showing a higher number of positive biopsies and more average percentage of cancer in biopsy in patients with high-grade disease.

Due to a lack of serum PSA specificity many authors have advocated normalizing the PSA by the volume of the prostate gland, yielding a PSAD (16). The use of PSAD for cancer diagnosis is controversial with studies both confirming and refuting the use of PSAD. In our analysis PSAD was the strongest predictor of tumour grade and review of the ROC curves indicates that its sensitivity and specificity are sufficiently good to be used as a single threshold test. However, given the complexity of prostate cancer risk assessment, model that incorporates data on multiple independent variables, including PSAD, is likely to be both more useful and appropriate. Although previous studies suggested an inverse relationship between prostate volume at diagnosis and the probability of high GS (17, 18), we did not confirm these findings. There are some hypoteses to explain this relation. Various authors stated that this was a result of sampling error of prostate biopsy in larger prostates. However, in some studies it has been shown that the correlation of prostate volume and GS depends on the stage of the disease (19). Futhermore, definitive prostate volume values can only be calculated with RP specimens and also a Gleason upgrade can be expected at radical prostatectomy pathology in some patients (20).

The adoption of PSA for PCa screening resulted in profound stage migration toward earlier stage, less aggressive disease, and a correspondent decrease in prostate cancer-specific mortality. However, PCa screening using serum PSA is a controversial subject. The European Association of Urology recommends screening for men with at least 10 to 15 years' life expectancy (21). In addition, they recommend a baseline PSA level at age 40 to 45 followed by screening at intervals based on the baseline PSA (22). Concerns about overscreening and overdiagnosis subsequently led professional guidelines (circa 2000 and later) to recommend against routine PSA testing (2). On the other hand, contemporary epidemiological data from the Pennsylvania Cancer Registry demonstrated that over the past 2 decades, PCa incidence rates have decreased, primarily because of the decreased detection of early-stage disease, and contrarily a corresponding shift toward more advanced disease at diagnosis (23). Unlike population screening, in this study we analyzed opportunistic testing that consists of individual case finding, which are initiated by the patient being tested and/or his physician. In the European Randomized Study of Screening for Prostate Cancer, the incidence of men with Gleason grade at least 8 was 10.6% in the nonscreened arm vs. 6.1% in the screened arm (24). Also, a SEER analysis demonstrates that a significant grade migration has occurred from the period just before the widespread of PSA screening (1984–2003) to more recent periods and high-grade disease accounted for 21% of all tumours (8, 25). However, in this regional-level cohort we found the worrying frequency of high-grade PCa (35%) at the present time, older age and higher median PSA compared to previous reports (3, 8). These results suggest that screening with serum PSA can allow early detection of disease, thereby reducing the proportion of men found to have high-risk disease at diagnosis.

The study is limited by the retrospective design, in a single tertiary centre with a relatively small patient cohort. Next, the higher percentage of older men with GS ≥ 8 could be biased by the selection criteria for biopsy (i.e. higher PSA values, suspicious DRE). Futhermore, we have not investigated the frequency and clinical factors affecting the under grading of biopsy Gleason sum, observed in other studies in about half of patients with radical prostatectomy (20). In addition, the accuracy of TRUS volumes is very user dependent. Finally, a targeted magnetic resonance/ultrasound fusion–guided biopsy technique produced better results than a standard biopsy in the detection of high-risk PCa (26). These data were not available in our cohort. Our results should be interpreted with caution given that our study design did not allow us to determine whether PSA screening in older, healthier men may improve their outcomes. Nevertheless, to our best knowledge, up to now, regional-level data that characterize contemporary PCa patients are limited. These parameters are crucial not only for monitoring on epidemiological situation regarding malignant diseases, but also for the evaluation of various preventive measures and programs implemented with the aim to prevent and reduce the burden of these diseases in our population. Physicians and patients should take into account the higher risk of more aggressive or advanced disease in older men when discussing the risks and benefits of PSA screening with healthy older men with a substantial life expectancy (10). These data highlight the continued need for nationwide monitoring of PCa incidence and trends by demographic and tumor characteristics and refinements in PCa screening and treatment.

CONCLUSION

In this regional-level cohort we found the worrying frequency of high-grade PCa at the present time in the circumstances of opportunistic testing. Our study has shown that older men have a higher probability of being diagnosed with high-grade disease. A more aggressive disease may be predicted using PSAD and age. Although our results do not imply that older men should receive more screening than they currently do, the striking correlation between older age and higher-grade disease could be considered when counseling healthy older men about the pros and cons of PSA screening.

CONFLICT OF INTEREST

None.

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FORMALIN FIXATION OF HUMAN HEALTHY AUTOPSIED TISSUES: THE INFLUENCE OF TYPE OF TISSUE, TEMPERATURE AND INCUBATION TIME ON THE QUALITY OF ISOLATED DNA

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FIKSACIJA HUMANIH ZDRAVIH AUTOPSIJSKIH TKIVA FORMALINOM: UTICAJ VRSTE TKIVA, TEMPERATURE I VREMENA INKUBACIJE NA KVALITET IZOLOVANE DNK

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ABSTRACT

Formalin fixation is a widely used method in histopathology that has certain limits. Formalin often leads to the degradation of DNA molecules in cancer tissues, which makes tissues unusable for molecular analysis. The other factors may also affect the quality of DNA isolated from fixed tissues. The aim of this study is to determine the impact of the incubation time and temperature on the quality of DNA molecules isolated from various healthy human tissues. The brain, lung and kidney tissues, excluded during the forensic autopsies of people who died of violent death, were fixed in phosphate-buffered formalin from 24h to two months. After the completion of the incubation period, the DNA was isolated using phenol-chloroform-isoamyl alcohol extraction method and the concentration and purity of the samples were determined spectrophotometrically. The degree of degradation of DNA was assessed by PCR reaction, by amplification of gene fragments which lengths were 150bp (GPD1) and 262bp (β-actin). The highest concentration, purity and preserved integrity of DNA were obtained from the brain samples. With prolonged tissue incubation times in formalin, the concentration and integrity of DNA decreased in all tissue samples, especially in the brain tissue, while the purity of DNA remained unchanged. Also, tissue fixation at +4°C contributed to a better quality of isolated DNA compared to DNA isolated from tissue fixed at room temperature. We can conclude that the type of human healthy tissue, temperature and the incubation time of formalin fixation have important influence on the concentration, purity and integrity of DNA during fixation of tissues excluded in the course of forensic autopsy.

***Keywords**: forensic autopsy, formalin, DNA isolation, PCR, spectrophotometry.*

SAŽETAK

Formalinska fiksacija je široko korišćena metoda u histopatologiji, koja ima i određena ograničenja. Formalin često dovodi do degradacije molekula DNK u tkivu tumora, što čini fiksirano tkivo neupotrebljivim za molekularnu analizu. I drugi faktori mogu uticati na kvalitet molekula DNK izolovanih iz fiksiranih tkiva. Cilj ovog rada je utvrditi uticaj dužine inkubacije tkiva u formalinu i uticaj temperature na kvalitet molekula DNK izolovanih iz različitih zdravih ljudskih tkiva. Tkiva mozga, pluća i bubrega, koja su izuzeta tokom sudsko-medicinskih obdukcija ljudi koji su umrli nasilnom smrću, fiksirana su u puferizovanom formalinu od 24h do dva meseca. Nakon isteka određenog inkubacionog perioda, iz tkiva je izolovana DNK ekstrakcijom pomoću fenol-hloroform-izoamil alkohola i spektrofotometrijski je određena koncentracija i čistoća uzoraka. Stepen degradacije molekula DNK procenjen je PCR reakcijom, amplifikacijom fragmenata gena dužine 150bp (GPD1) i 262bp (β-actin). Najveća koncentracija, čistoća i očuvan integritet DNK dobijeni su iz tkiva mozga. Sa produženim vremenom inkubacije tkiva u formalinu koncentracija i integritet DNK opadaju u svim ispitivanim tkivima, posebno u mozgu, dok čistoća uzoraka ostaje konstantna. Takođe, fiksacija tkiva na +4°S doprinosi boljem kvalitetu izolovane DNK u odnosu na DNK izolovane iz tkiva fiksiranih na sobnoj temperaturi. Može se zaključiti da vrsta zdravih humanih tkiva, temperatura i vreme inkubacije tkiva u formalinu imaju značajan uticaj na koncentraciju, čistoću i integritet DNK tokom procesa fiksacije tkiva izuzetih tokom sudsko-medicinske obdukcije.

***Ključne reči:** sudsko-medicinska obdukcija, formalin, izolovanje DNK, PCR, spektrofotometrija.*

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ABBREVIATIONS

AP – apurine or apirimidine sites
bp – base pairs
DNA - deoxyribonucleic acid
EDTA - ethylenediaminetetraacetic acid
FFPE – formalin fixed paraffin embedded
GPD1 - glycerol-3-phosphate dehydrogenase 1
NaCl - sodium chloride
OD – optical density
PCI - phenol-chloroform-isoamyl alcohol
PCR - polymerase chain reaction
RT – room temperature
SNP - single nucleotide polymorphisms
STR – short tandem repeats
TBE - Tris/Borate/EDTA buffer
TE - Tris/EDTA buffer
TNS – TE/NaCl/SDS buffer

INTRODUCTION

The post-mortem molecular analyses of the nuclear acids are becoming increasingly common in the epidemiological studies and in retrospective investigations, with the aim to identify genetic factors that cause death and rare diseases(1). Although blood samples are often used, formalin fixed paraffin embedded (FFPE) tissues are a suitable biological sample for the performance of a molecular autopsy (2). The different preserved tissues, excluded during a forensic autopsy, are often the only accessible samples in retrospective studies. However, in order for tissue samples to be used for molecular analysis, DNA molecules must be preserved. It is known that a large number of factors disturb the molecular profile of cells and tissues, so it is necessary as soon as possible to optimize the appropriate methodological approaches used in tissue preservation. The factors that affect the integrity of DNA in preserved tissues include factors on pre-fixation level (proper exclusion of tissue during autopsy and pre-fixation time), the type and characteristics of fixative and post-fixation parameters (paraffinization and deparaffinization procedure, DNA isolation method, storage) (3). These factors influence the yield and purity of the DNA obtained, but also lead to degradation of this molecule, which reduces its utilization in molecular analyses. The most frequently type of DNA degradation is fragmentation of polynucleotide chains of DNA into smaller fragments, sizes up to several hundred nucleotides or less (4). As a consequence, a small amount of usable DNA is obtained.

Formalin is the longest and most commonly used fixative in histopathology and protocols for formalin fixation of a tissue have changed over time. In previous years, the only requirement in tissue fixation was that the tissue morphology was preserved. However, with the emergence of immune-histochemical and molecular-biological analyses, and tissue formalin fixation protocols have become more stringent. More recently, the key requirement in forensic genetics is the preservation of the nucleic acid in formalin-fixed tissues, since large tissue archives would be available for profiling gene expression in order to generate new and reliable diagnostic and prognostic parameters (5, 6). It is known that the main effect of formaldehyde in the tissue is associated with the formation of methylol groups on the amino groups of nitrogenous bases in the DNA molecule and the establishment of cross-linking of methylene groups, leading to the correct fixation of a tissue (7). The formation of numerous strong covalent bonds in the DNA molecule leads to its fragmentation. Literature data show that the cross-linking of nucleic acids, between themselves and with proteins, cannot be solely responsible for the degradation of DNA and RNA that by maintaining a low temperature throughout the fixation process, the degree of nucleic acid degradation can be reduced (8).

Since there are not many studies analyzing the effects of phosphate-buffered formalin on the quality of isolated DNA, especially from healthy human tissues excluded during medico-legal autopsies, this study provides some important information on the usability of archived formalin fixed tissues in practice in molecular autopsy. Based on previous hypothesis, the goals of our research were also set. The aim of this study was to determine whether tissue fixation in formalin at low temperatures (+4°C) would contribute to preserving the quality of DNA molecules while preserving the morphological properties of a fixed tissue. We compared concentration, purity and integrity of DNA isolated from healthy, autopsy tissues fixed at +4°C or at room temperature (RT) in phosphate-buffered formalin for different time points.

Material and methods

Ethic statement

The Ethics Committee of the University of Kragujevac, Faculty of Medical Sciences (No. 01-4970), the Ethics Committee of Clinical Center of Kragujevac (No. 01-2798), Appeal Public Prosecutor's Office from Kragujevac (No. 79/13) and Higher Court in Kragujevac (SU-VIII-110/13) agreed to these investigations.

Tissue selection and processing

The healthy brain, lung and kidney tissues were taken from four people (two women and two men, aged between 23 and 42) who suddenly died a violent death. The corpses were on +4ºC up to 24h before autopsy was done. During the autopsy, the tissues were collected and immediately fixed in

phosphate-buffered formalin (Alfapanon 10%): one half of each tissue was fixed on +4ºC and the other half was fixed at room temperature (RT). The size of each tissue sample was about 5x5x3mm. The fixation was prepared in the formalin solution in a ratio of 20 parts fixative to one part of the tissue (v/v). The tissues were fixed 24h, 48h, 72h, 96h, 5 days, 6 days, 7 days, 14 days, 28 days and 2 months. The control tissue samples were prepared for experiments immediately after autopsy.

DNA isolation

The DNA was isolated from all tissue samples using phenol-chloroform-isoamyl alcohol (PCI) extraction method. The samples were digested in TNS buffer (TE buffer, 3M NaCl, 10% SDS, ampoules-deionized water) with proteinase K (Thermo Scientific) at 56ºC, overnight. In each sample, an equal volume of PCI solution (in ratio 25:24:1) was added and centrifuge at 4000 rpm at +4ºC, 5 min. Thereafter, the supernatant was transferred into a new tube, an equal volume of chloroform-isoamyl alcohol solution (in ratio 24:1) was added and centrifuged in the same manner. The DNA in the supernatant was precipitated using 100% ice-cold ethanol and 3M NaCl at -20ºC, overnight. After centrifugation at 15000 rpm on +4ºC, 30 min, the supernatant was removed. The 70% ice-cold ethanol was added on the pellet, centrifuged at 15000 rpm on +4ºC, 15 min, supernatant was removed and the pellet was dried at RT, for a few hours. The DNA from the pellet was resuspended in the 50 µl TE buffer (10mM Tris-HCl and 1mM EDTA) and stored at -20ºC.

Spectrophotometric quantification of DNA

The quantification of the concentration and purity of DNA were determined using spectrophotometer (UV–1800 Shimadzu UV spectrophotometer, Japan) and measuring absorbance on 260nm and 280nm. One OD260 unit corresponds to approximately 50 µg/ml DNA. The ratio OD260/OD280 indicates protein contamination and pure samples have values more than 1.5.

Polymerase chain reaction - PCR

All PCR reactions were prepared in duplicates (8 samples per each time point and each fixation method). Two primer pairs targeting two different human house-keeping genes, GPD1 - glycerol-3-phosphate dehydrogenase and β-actin – ACTB actin beta, were designed for the amplification of DNA fragments with length 150 bp and 262 bp, respectively (Table 1). The PCR reaction mix contained One Taq 2x Master Mix with Standard Buffer (New England Biolabs Inc.), primers (forward and reverse) (Invitrogen by Thermo Fisher Scientific) and approximately 1 ng/µl of isolated genomic DNA in sterile bidistilled water in the final volume of 25µl. The PCR reactions were performed in a PCR Techne, Eppendorf under the following amplification conditions: initial denaturation at 94°C for 30 seconds; 30 cycles of 94°C for 30 seconds, 60°C for 1 minute, and 68°C for 1 minute; final extension at 68°C for 5 minutes. The positive control (PCR reaction mix contained all PCR compounds with DNA isolated from adequate tissue immediately after autopsy) and negative control (PCR reaction mix contained all PCR compounds except DNA) were included in all PCR reactions.

Agarose gel electrophoresis

The PCR products were visualized by 2% agarose gel electrophoresis. The agarose powder was dissolved in TBE buffer (Tris-base, boric acid, EDTA in bidestillated water) in concentration of 2% and stained with ethidium bromide (0.5µg/ml). The agarose electrophoresis was performed in TBE buffer on 100V, 55 minutes. The results were visualized under the UV lamp and photographed.

Statistical analysis

The SPSS version 20.0 software package for Windows was used to perform the statistical significance of group differences. For comparing the differences between formalin fixation at +4°C and at RT the Mann Whitney test was used. The Kruskal Wallis test was applied to estimate the differences between the different tissues, while the Friedman test was applied to determine the differences between the different time points. P values below 0.05 were accepted as statistically significant.

Table 1. The nucleotide sequences of primers and length of the amplicons (bp)

Primer name	Primer sequence	Amplicon length (bp)
GPD1 -20 F	CAGATGCCCAGGTGAGTGAA	150
GPD1-20 R	ACTGCCTCACTCCTTACTCCT	
ACTB-20 F	TGCTAAAGACCGTGGGGAAC	262
ACTB-20 R	TGTGACCCCTTTCTCCCTCA	

RESULTS

In this study, we compared the quantities (DNA concentration, mg/µl) and qualities (DNA purity and amplifiability) of DNA isolated from different healthy human tissues (brain, lung and kidney) which were fixed up to 2 months using different formalin fixation methods (at RT as well as at +4ºC).

The concentrations of DNA varied depending on the type of tissue samples, formalin incubation time as well as on fixation method and these differences were statistically significant. The highest DNA concentration (6.51 mg/µl) was obtained from the brain tissue immediately after autopsy (Table 2). However, with prolonged incubation time in formalin, concentration of DNA significantly decreased in all examined tissues, especially in the brain tissue fixed at RT up to 2 months (2.70 mg/µl) (p=0.000). After prolonged formalin fixation at RT, the highest DNA concentration was measured in kidney (mean value was 4.32±0.74 mg/µl) and the worst result was obtained in the brain tissue (mean value was 3.84±0.97 mg/µl) (p=0.000). Also, statistically significant higher concentration of DNA was obtained from all tissue samples fixed at +4ºC in comparison with samples fixed on RT. With the increase of formalin fixation time at +4ºC, the concentration of DNA decreased significantly (p=0.000) in all examined tissues (Figure 1), with the highest DNA yield from the kidney tissue (mean value was 5.52±0.39 mg/µl) and the lowest from the brain tissue (mean value was 4.43±0.71 mg/µl) (Table 2).

Table 2. The concentration (mg/µl) and rate of purity (OD260/OD280) of DNA isolated from different healthy tissue samples fixed in phosphate-buffered formalin up to 2 months

		Concentration (mg/µl ± SD)		Purity (OD260/OD280)	
tissue	**Type of fixation**	**Mean ± SD**	**Range**	**Mean ± SD**	**Range**
brain	**RT**	3,84±0,97	2,70-6,51	1,50±0,14	1,44-1,95
	+4°C	4,43±0.71	3,55-6,51	1,88±0,10	1,76-2,04
lung	**RT**	4,22±0,57	3,48-5,27	1,53±0,12	1,40-1,87
	+4°C	4,59±0,43	4,03-6,02	1,95±0,038	1,87-2,01
kidney	**RT**	4,32±0,74	3,29-5,51	1,56±0,11	1,47-1,90
	+4°C	5,52±0,39	4,67-6,02	1,89±0,04	1,83-1,98

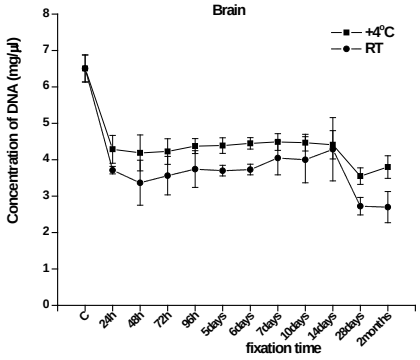
The purity of DNA extracted from human healthy tissues that were excluded 24h after death and fixed in phosphate-buffered formalin was satisfactory with range from 1.40 to 2.00. Based on the OD260/OD280 ratio, we conclude that the highest degree of purity of DNA was obtained in brain tissue samples fixed at +4ºC, but the differences among the different tissues are not statistically significant (p=0.167). Also, DNA isolated from all tissue samples has a statistically significant (p=0.000) higher purity after fixation at +4ºC in comparison with samples fixed at RT (Table 2 and Figure 1). With the increase of formalin fixation time, the purity of DNA isolated from all three examined tissues decreased, which was statistically significant (p=0.000).

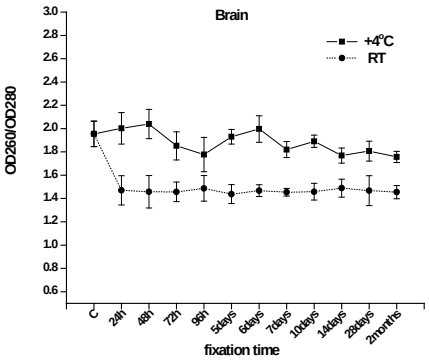
The integrity of DNA was estimated due to the amplifiability of isolated DNA. Based on PCR amplification performance, DNA isolated from tissue samples that were fixed in phosphate-buffered formalin at +4ºC had better preserved integrity than DNA isolated from tissues fixed at RT (p=0.001) (Figure 2). Amplification of the 150bp GPD1 gene fragment produced positive results in all tissue samples fixed at +4ºC up to 28 days, but only in the brain tissue fixed at RT up to 28 days. In lung and kidney tissues fixed at RT, GPD1 amplicons were visible up to 14 days. The amplification rate for 262bp β-actin gene fragment was 50% (4/8) among brain tissue samples fixed at +4ºC up to 28 days, lung tissue up to 14 days and kidney tissue up to 10 days fixation. However, after fixation at RT, reduced efficiency of the amplification of the β-actin gene fragment was obtained: 25% in brain tissue up to 14 days, 25% in lung tissue up to 10 days and 25% in kidney tissue up to only 5 days. In all examined tissue samples, the decrease of amplifiability of isolated DNA with the

prolonged incubation time in formalin (both at +4ºC and at RT) was statistically significant (p=0.015). Comparing the mean values of parameters for all three tissues studied, it has been shown that the brain tissue shows the best, and the kidney tissue the worst results.

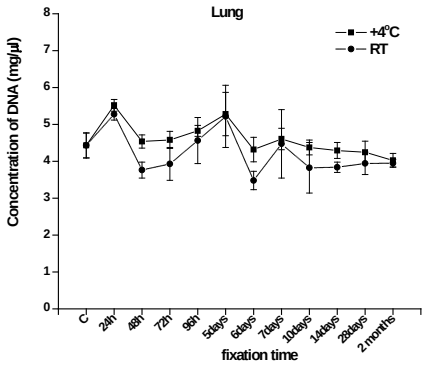
Figure 1 - Differences in concentration and purity of DNA isolated from human healthy (A) brain, (B) lung and C) kidney tissues fixed in phosphate-buffered formalin at room temperature (RT) or at +4°C. The concentration and the purity of DNA decrease with prolonged incubation time in all examined tissues (p<0.001). Statistically significant (p<0.001) higher concentration and purity of DNA were obtained from all tissue samples fixed on +4ºC in comparison with samples fixed on RT.

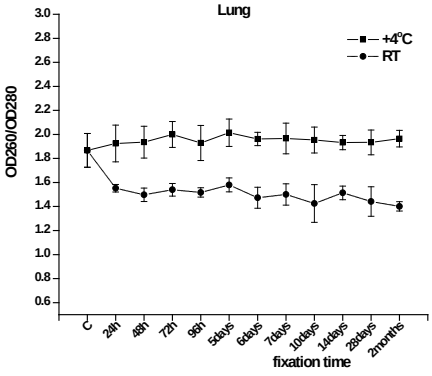
A BRAIN



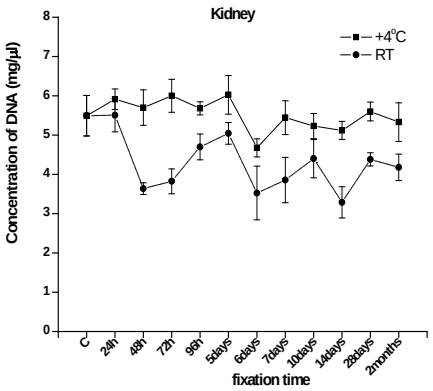


B LUNG





C KIDNEY



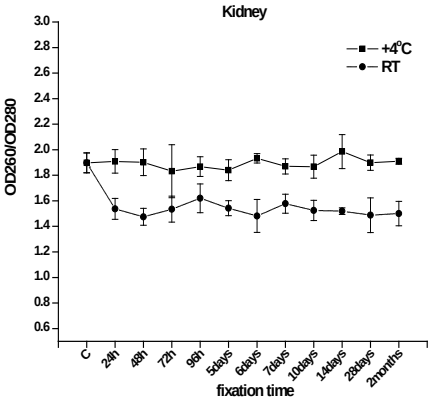


Figure 2. Number of samples in which are detected different fragments of DNA isolated from three human healthy tissues fixed in phosphate-buffered formalin for various incubation time.

		24h	48h	72h	96h	5days	6 days	7days	10days	14days	28days	2 m
Fixation on RT												
Brain												
	β-actin	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	2/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	4/8	2/8	0/8
Lung												
	β-actin	8/8	8/8	8/8	8/8	6/8	6/8	2/8	2/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	4/8	0/8	0/8
Kidney												
	β-actin	8/8	8/8	8/8	4/8	2/8	0/8	0/8	0/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	6/8	8/8	6/8	4/8	2/8	0/8	0/8
Cold fixation on +4°C												
Brain												
	β-actin	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	4/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	0/8
Lung												
	β-actin	8/8	8/8	8/8	8/8	8/8	6/8	6/8	4/8	4/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	4/8	0/8
Kidney												
	β-actin	8/8	8/8	8/8	8/8	8/8	6/8	6/8	4/8	0/8	0/8	0/8
	GPD1	8/8	8/8	8/8	8/8	8/8	8/8	8/8	8/8	6/8	4/8	0/8

DISCUSSION

The most important step in a procedure to preserve tissues is a fixation. Since 19th century, the 4% formaldehyde solution in water (formalin) is in use for tissue fixation. In addition to formalin, there are also some other fixatives such as ethanol-based fixatives, glutaraldehyde, potassium dichromate acetic acid, etc. (9). However, these fixatives are not widely used, so the formalin is the most common fixative in histopathology. In recent years, the formalin buffered with several different buffers such as calcium carbonate, magnesium carbonate, citrate, Tris and most commonly phosphate buffers (4) has been in use, which has certain advantages over the unbuffered: longer shelf life and the possibility of prolonged tissue fixation without adverse effects on DNA degradation (10).

Formaldehyde is the simplest aldehyde with one C atom, which is rapidly dissolved in water, whereby methylene hydrate (methylene glycol, $CH_2(OH)_2$) forms. The molecules of methylene glycol react with each other and form a polymer (11). The aqueous formalin solution contains 37-40% formaldehyde in the form of short polymer (containing 2-8 monomers) and 60-63% water. Addition of 10% methanol to an aqueous formalin solution prevents polymerization and the formation of paraformaldehyde (insoluble long polymers containing more than 100 monomers). In the tissue, methylene glycol is dehydrated into carbonyl formaldehyde. Both forms of formaldehyde (both hydrated and non-hydrated) fix tissue (11). When tissues are put into formalin, they are quickly penetrated by methylene glycol and a small amount of formaldehyde. Formaldehyde reacts with amines (amino acid lysine), purines and thiols (cysteine) and forms a methylol which loses the molecule of water and makes the Schiff base (imine). The Schiff base reacts with other nucleophilic molecules, such as amino groups of DNA and proteins, and creates cross-linking between the macromolecules via the methylene bridge (11). In addition to the formation of methylol derivatives and the formation of cross-linking methylene bridges, formaldehyde induces the formation of AP sites (apurine and apirimidine) and the breakdown of phosphodiester bonds in the DNA molecule (12). Formaldehyde breaks the hydrogen bonds of the dual-chain DNA molecule in regions rich with adenine and thymine, leading to the formation of new chemical reactions, protein binding for DNA, and fragmentation of DNA molecules (13). The initial phase of this reaction is reversible, while subsequent reaction phases, when a large number of covalent bonds are created, are irreversible (3).

Process of formalin fixation helps to preserve the tissue and cell structures and morphology which makes possible the histopathological analysis of samples. However, the formalin fixation has some limitation, especially due to degradation of nucleic acids. Tissue formalin fixation process has two phases: penetration and fixation. Penetration implies the

ability of the fixative to diffuse into the tissue and requires at least 1h per 1mm of tissue thickness (14) to reach the nethermost parts of cells. This process is a physical process which is determined by several physical factors: temperature, pH, volume of solution, length of incubation, pressure, surface area, concentration of formaldehyde, etc. (3). At the other hand, fixation is a process in which the initial formation of cross-linking between the macromolecules is completed. The fixation process is influenced by various factors, such as the incubation length, temperature, pH value of the formalin solution and viscosity. Penetration of formalin into tissue and initially cross-linking occurs in the first 24 to 48 hours, while the process of fixation and formation of stable covalent bonds can be confirmed for up to 30 days (15). Understanding the chemical and physical aspects of the fixation process, as well as the factors influencing these processes, contributes to the optimization of conditions and the selection of correct methodological approaches in histopathology and molecular autopsy.

The mechanism of action of formalin on DNA molecules as well as factors affecting the fixation efficiency was studied mainly on tumor tissues that, after formalin fixation, were embedded into paraffin, or on frozen tissue. There is no sufficient literature data about the effects of formalin on DNA molecules in healthy tissue excluded during autopsy. Archival fixed tissues excluded during autopsy are potentially large source for forensic testing especially or DNA analysis in clarification of cases of criminal activity, determination of paternity or in the persons identification. In our laboratory, different tissues (brain, lung, kidney, heart and liver) excluded during autopsies are fixed in formalin, embedded in paraffin and kept in the archives for many years. Therefore, it was of great importance to test how long these tissues may be fixed in formalin, optimal conditions of fixation, the influence of temperature and tissue type on DNA integrity and their usability in forensic practice.

DNA quality is measured in the context of concentration and purity as well as in terms of PCR fragment length. Concentrations of DNA in tissue samples were calculated using values obtained spectrophotometrically on 260nm, while purity was calculated due the OD260/OD280 ratio. The OD260/Od280 values more than 1.5 indicated low protein contamination in samples (16). The total amount of extracted DNA highly depended on the method used. In the previous studies, it was shown that phenol-chloroform-isoamyl alcohol (PCI) extraction gives higher yield and purity (17, 18) and successful gene amplification (18) than the commercial kit (10). For this reason, we used PCI method for DNA extraction. To evaluate the degree of degradation of DNA molecules, we used PCR amplification of fragments with a length of 150 bp (GPD1 gene fragment) and 262 bp (β-actin gene fragment). Forensic laboratories use STR (**s**hort **t**andem **r**epeats) as well as SNP (**s**ingle **n**ucleotide **p**olymorphism) markers as standard for DNA identification (19). To perform this method, DNA fragments of length from 150-450 base pairs are required. SNP markers are small genetic variations occurring in human DNA for every 100 to 300 base pairs along 3 trillion human genome bases. SNP markers appear in coding and non-coding parts of DNA as well as nuclear and mitochondrial DNA. SNP amplicons are shorter (shorter than 150 base pairs) than other markers, thus allowing the amplification of the damaged DNA. Babol-Pokora and Berent (19) used five SNP markers following the length of amplitudes 123, 99, 93, 85 and 71 base pairs and concluded that the SNP was ideal for forensic analysis of degraded samples. For this reason, the use of selected genes in our investigation is justified.

The quality of DNA isolated from different organs, fixed to formalin at different times, often varies due to the variation in the cellular composition of these tissues. Tissues that have non-homologous cellular composition (pancreas, colon, lungs) should be avoided because the concentration and purity of the DNA isolated from them are poor (20). In addition, the lung tissue is non-suitable for molecular analysis due to presence of mucus. Tissue samples isolated from organs which have high cellular density (brain, heart, liver, spleen) have been used for molecular analysis because they showed better PCR amplification of DNA molecule (21). At the other hand, DNA isolated from chest cavity organs (heart and lungs) shows better quality than DNA isolated from tissue originating from the abdominal cavity (pancreas, liver, kidney, colon, stomach), due to pancreatic enzymes and accelerated bacterial growth leading to faster tissue autolysis (22). Kidney tissue should not be taken from individuals older than 50, since the cell autolysis has probably began (21). In our study, we decided to use three different organs from different cavities. These organs are approachable for collection of samples in routine autopsy and appropriate for our study. To the best of our knowledge, there is only few literature data about the DNA integrity in the healthy tissues fixed in formalin (9, 10, 23). There are much more studies about DNA integrity isolated from healthy tissues (1, 24) or from cancer tissues (15, 25, 26) fixed in formalin and embedded in paraffin. In terms of cancer tissue and other diseased tissues which are embedded in paraffin, the results were lower probably due to permanent alteration in DNA and damage of DNA with aggressive reagents for deparaffinization (27). Also, reagents used for paraffin embedding of tissue could damage the quality of DNA molecule (21). There are no studies that analyzed DNA integrity isolated from cancer or healthy tissues only fixed in formalin, without paraffin embedding. The impact of our study is that we analyzed healthy tissues excluded during forensic autopsy which performed 24h after death. The optimal fixation time depends on the tissue type and specimen size (28). DNA isolated from brain formalin fixed tissue showed the best quality in terms of purity and integrity compared with other examined organs. The concentration of DNA isolated from the brain tissue immediately after autopsy was the highest which can be explained by the high cell density in this tissue (21). With prolonged incubation time, the concentration of DNA in the brain tissue decreased which indicates a high sensitivity of this organ to prolonged formalin exposure. The highest DNA concentration was measured in kidneys after prolonged

incubation time in formalin due to the specific cell composition in this tissue. The DNA integrity in the brain tissue was the highest in comparison with other examined tissues, both after fixation at RT and at +4C. The integrity of the DNA is sufficiently preserved in the brain tissue up to 28 days of fixation at RT as well as at +4°C. Miething et al. concluded that the brain tissue showed strong fluctuations in the test results which can be attributed to uneven penetration by fixative (9). There is no literature data about the successful amplification of DNA fragments isolated from healthy lung tissue extracted during autopsy and fixed in formalin. Funabashii et al. considered that lungs are not good for subsequent analyzing because of the presence of mucus in these organs (21). In our study, the DNA integrity in the fixed lung tissue was sufficiently preserved up to 14 days fixation at RT and up to 28 days fixation at +4°C. The worst integrity of DNA was in kidney tissue suggesting that the process of autolysis in kidney cells is intensive immediately after death (21). The temperature of formalin fixation had a significant effect on examined parameters of DNA isolated from different tissues. Better quality of DNA was obtained from samples fixed at a lower temperature. It was shown that low temperatures and short fixation time decreased the degree of DNA degradation (14). The speed of formalin fixation process depended on the rate of diffusion of formalin across the tissue and the rate of chemical reaction with cellular components (29). Formalin fixation process requires at least 1 hour per 1 mm of tissue thickness, so it is recommended that the process of tissue fixation by formalin lasts up to 48 hours (30). Fox et al. (31) have shown that for tissue samples of about 20mm the formalin penetration occurs 24h at 25°C, and 18h at 37°C. Our results have shown that the tissue formalin fixation incubation time can be longer, without significant consequences on DNA concentration, purity and amplificability up at least 10 days of formalin fixation. Moreover, increase in temperature of formalin fixation will favor the disassociation of formaldehyde from the polymers and its reaction with tissue macromolecules. A low fixation temperature reduces the activity of enzymes in the cell (including enzymes that degrade DNA and proteins), resulting in higher yields and lower degradation of isolated DNA (15).

In conclusion, the proposed method of formalin fixation of human healthy autopsied tissue results in lower degree of DNA fragmentation. Fixation at low temperatures increases the degree of preservation of molecular structures in fixed formalin tissues. The possibility of obtaining high quality DNA from archival tissues gives prospects for wider molecular analysis and profiling than presently feasible. The results of this paper will contribute to better optimization of conditions during tissue fixation after autopsy and better preservation of DNA quality in tissues that can be used for forensic purposes.

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MORINGA OLEIFERA LEAF EXTRACT AND ITS PROMISING SYNERGISTIC ANTIMICROBIAL EFFECT WITH TYPHOID FEVER VACCINE IN IMMUNIZED MICE

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EKSTRAKT LIŠĆA MORINGA OLEIFERA I NJEGOV OBEĆAVAJUĆI SINERGISTIČKI ANTIMIKROBNI EFEKAT SA VAKCINOM PROTIV TIFUSNE GROZNICE KOD IMUNIZOVANIH MIŠEVA

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ABSTRACT

Typhoid fever, a systemic infection caused by Salmonella typhi has maintained a high morbidity and mortality profile around the globe especially in developing countries. Though currently licensed vaccines are efficacious in prevention of the infection, their potency is ephemeral; hence, they require a boost by employing adjuvants that are safe and instrumental in achieving a better prolonged protective immune defense outfit. In this work, Moringa oleifera ethyl acetate leaf extract was evaluated for its possible adjuvant property to a heat-killed ST vaccine. Mice were vaccinated with typhoid vaccine and subsequently, daily weight of mice was measured. Also, post-vaccination microbial colony counts were enumerated after challenging the mice with Salmonella typhi cells. From the blood culture results, MO extract demonstrated an excellent synergistic antimicrobial effect as the mice group administered our formulated vaccine-MO extract combination had the lowest microbial load (12.25 ± 4.86) colony forming units following microbial challenge, when compared to the mice groups administered the vaccine alone (37.25 ± 4.5) and the MO extract alone (31.25 ± 9.43). Furthermore, assessment of the mice body weight of treated groups showed a growth pattern that did not deviate significantly from those of the control group. In conclusion, MO extract demonstrated a promising synergistic antimicrobial effect on coadministration with the typhoid fever vaccine against S. typhi and did not lead to adverse side effects in mice.

Keywords: *Typhoid fever, Moringa oleifera, Salmonella typhi, adjuvant, colony forming units, body weight.*

SAŽETAK

Tifusna groznica, sistemska infekcija izazvana Salmonelom tiphi održavala je visok profil morbiditeta i smrtnosti širom sveta, posebno u zemljama u razvoju. Iako su trenutno licencirane vakcine efikasne u sprečavanju infekcije, njihova moć je efemerna; stoga im je potreban podsticaj upotrebom adjuvansa koji su sigurni i instrumentalni za postizanje bolje produžene zaštite imune odbrane. U ovom radu, ocenjen je ekstrakt lišća Moringa oleifera etil acetata zbog mogućeg pomoćnog svojstva. Miševi su vakcinisani vakcinom protiv tifusa i posle toga je izmerena težina miševa. Takođe, navedeno je brojanje mikrobioloških kolonija posle vakcinacije miševa sa ćelijama Salmonella tiphi. Iz rezultata kulture krvi, MO ekstrakt je pokazao odličan sinergistički antimikrobni efekat, jer je grupa miševa koja je primenjivala našu formulisanu kombinaciju vakcina-MO imala najmanje opterećenje mikrobnog opterećenja (12,25 ± 4,86) kolonija posle izazivanja mikroba, u poređenju sa grupama miševa koji su primili samo vakcinu (37,25 ± 4,5) i sam ekstrakt MO (31,25 ± 9,43). Dalje, procena telesne težine miševa tretiranih grupa pokazala je obrazac rasta koji nije značajno odstupio od kontrolne grupe. Zaključno, ekstrakt MO pokazao je obećavajući sinergistički antimikrobni efekat na zajedničku primenu sa vakcinom protiv tifusa i nije doveo do štetnih nuspojava kod miševa.

Ključne reči: *Tifusna groznica, Moringa oleifera, Salmonella tiphi, adjuvans, jedinice koje formiraju koloniju, telesna težina.*

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INTRODUCTION

Typhoid fever is a systemic disease associated with poor hygiene and socio-economic status with high morbidity and mortality profile in developing countries [1]. This disease is caused by the bacteria *Salmonella enterica* serotype Typhi (*S. typhi*), which is transmitted orally during the ingestion of food and water contaminated with faeces or urine of an infected individual [2]. Following ingestion, the bacteria spread from the intestine via the blood (where they multiply) to the intestinal lymph nodes, liver, and spleen [3]. The incubation period of the disease is usually 10-14 days and varies considerably from 8-15 days, but may be as short as 5 days and as long as 30 or 35 days depending on the inoculum size and the state of host defenses [4]. The onset of the disease is stealthy and characterized with clinical manifestations such as the gradual inception of persistent fever, chills and abdominal pain. In some cases, patients experience rash, nausea, anorexia, diarrhoea or constipation, headache, relative bradycardia and reduced level of consciousness [5]. *Salmonella typhi* is a member of the *salmonella* genus which belongs to the Gram-negative Enterobacteriaceae family of bacteria [6]. It is serologically positive for lipopolysaccharide antigens O9 and O12, polysaccharide capsular antigen Vi and protein flagellar antigen, Hd [2]. The polysaccharide capsule Vi is responsible for the defensive outfit posed by the bacterium against the bactericidal action of an infected individual's serum [7]. Differences in the structural patterns of the lipopolysaccharide create antigenic variations that influence the virulence of different strains [6]. The Widal test is the most common diagnostic method that aids the identification of the agglutinating antibodies against the *S. typhi* antigens, O (somatic) and H (flagellar), which appear about 7 to 10 days after disease onset. Nevertheless, the high numbers of false-positive and false-negative test results limit its clinical efficacy [8]. Before now, the preferred treatment for typhoid fever was Chloramphenicol, but owing to substantial relapse rates and the development of bacterial resistance during the 1970s and 1980s, this drug was replaced by co-trimoxazole and ampicillin [3]. More recently, increasing resistance to the latter antibiotics has prompted the use of quinolone derivatives and third-generation cephalosporins [8].

As Salmonella has become a major threat to the society due to the disease severity, recurrence of disease through carrier state and the emergence of multidrug resistance, an effective prophylactic measure is essential [4]. The development of a safe and effective vaccine remains a priority for controlling the spread of the disease especially if travelers to endemic areas are considered, as it will be of immense health benefit to them. Aside the two known licensed vaccines against typhoid fever – Parenteral Vi polysaccharide (ViCPS) and oral vaccine Ty21a (live-attenuated), other vaccines that have been recommended for use include; the conjugate vaccine (Vi-TT), where the Vi antigen is coupled to a carrier protein and multivalent combination vaccines (a combination of ViCPS and hepatitis A vaccines), and are administered parenterally [3]. The licensed vaccines have limitations such as poor seroconversion after first administration, hence requires repeated booster immunization schedules; sub-optimal antibody titre production, especially the Vi polysaccharide vaccine, and a short protective period. Currently, research focus is geared towards genetically attenuating strains of S. Typhi to achieve high immunogenicity while at the same time rendering the strain nonpathogenic and secondly, the development of new parenteral Vi polysaccharide protein conjugate vaccines, which are expected to produce higher antibody titres following initial and booster immunizations [3, 8]. In addition, a promising approach to circumvent the limitations is to employ vaccine adjuvants that are bio-compactible, biodegradable and non-toxic to subjects.

Adjuvants in immunology have been defined as substances added to vaccine formulations that enhance the immunogenicity of antigens and induce protection against infection [9]. Adjuvants can act like PAMPs (pathogen-associated molecular patterns), triggering the innate immune response through a variety of mechanisms, to identify the vaccine components as a "threat", with activation and maturation of APCs (antigen presenting cells) and initiation of downstream adaptive immune activities [10]. Benefits of adjuvants include the following; they decrease the dose of antigen needed to formulate a vaccine, decrease the number of vaccine booster doses required for immunization of subjects, enhance vaccine efficacy in infacts, the elderly and immunocompromised individuals, increase functional antibody titre, induce more rapid and long-lasting immune response, induce a robust cell-mediated immunity, provide broad protection (cross-reactivity), facilitate mucosal immunity and help to overcome antigen competition in combination vaccines [11]. Classes of adjuvants that have been explored include mineral compounds, bacterial products, oil-based emulsions, immune-stimulatory complexes (ISCOMs), virosomes, phytochemicals (saponin), liposomes and glycoproteins [12].

In this work, *Moringa oleifera* (MO), a member of the Moringaceae family also known as 'Horse radish' or 'Drumstick' was evaluated for a possible adjuvant property for typhoid fever vaccine [13]. It is a fast growing drought-resistant tree native widely cultivated throughout tropical countries including Nigeria. In folk medicine it is been reported to be used in the treatment of rheumatism, cardiac and circulatory disorders and also possesses antitumor, anti-inflammatory, antihypertensive, antidiabetic, hepatoprotective, cholesterol lowering, antioxidant, antibacterial and antifungal properties [14]. Moreover, some medicinal properties associated to different parts of Moringa have been acknowledged by both Unani and Ayurvedic systems of medicines. Studies have revealed that Moringa roots possess antispasmodic activity through calcium channel blockade which is the platform for its traditional use in treatment of diarrhea. Moringa leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium, potassium and act as good source of natural antioxidants like ascorbic acid, flavonoids, phenolics and carotenoids that work mutually to strengthen immunity [15]. Hence, this work seeks to explore the plant leaves as a potential typhoid fever vaccine adjuvant.

MATERIALS AND METHODS

Plant Extraction

The leaves of *Moringaoleifera* were collected in Nsukka area of Enugu State, Nigeria and authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka. The leaves were air-dried, pulverized and the powder was stored in an air-tight container pending extraction. Cold maceration technique was employed for extraction [5] using ethyl acetate as solvent. Exactly 2500ml (2.5 litres) of ethyl acetate was used to soak the *Moringa oleifera* powder in a sterile container and the container was sealed properly and allowed to stand for 48hours before filtration. The residue was rinsed with additional 2500ml of ethyl acetate to ensure exhaustive extraction, and a solution of the extract was obtained, and the solvent allowed to vapourize at room temperature.

Test Animals

Twenty (20) young female albino mice, *Mus musculus* (6 - 8 weeks old) purchased from the Animal House, Department of Pharmacology and Toxicology, University of Nigeria, Nsukka and kept under standard pathogen-free conditions in the animal facility of the department of pharmacology and toxicology. The animals were well fed with chick's grower mesh (vital feed) and water ad libitum throughout the study period. Ethical considerations with respect to handling of laboratory animals were duly followed in accordance withthe "NIH guidelines for laboratory animal care and use" [16] and the University of Nigeria regulations for laboratory animal use.

Typhoid Fever Vaccine Preparation

The vaccine used was prepared locally by heat denaturation method. Outlined below are the steps elaborating how the vaccine was prepared.

Collection and isolation of Salmonella typhi (ST)

3.6g of the agar was dissolved in 50ml of water. The mixture was poured into two petri dishes, each containing 20ml, and the remaining 10ml was poured in two test tubes, each containing 5ml, and allowed to set or gel. ST was obtained from the University of Nigeria Teaching Hospital, Ituku-Ozalla Enugu. Pure culture of the isolate, *Salmonella typhi*, was prepared by sub-culturing using a wireloop on the Salmonella-Shigella agar in the petri dishes by streak method and then were incubated for 24hours at 37°C. After incubation, distinct colonies were again sub-cultured into freshly prepared and sterilized nutrient broth and incubated for another 24hours.

Determination of bio-load

1ml of the broth culture of the microorganism was collected and transferred aseptically into a test tube containing 9ml of sterile water and this was labelled 10^{-1} (10-fold serial dilution); this was progressively done till the ninth test tube (10^{-9}) and the last 1ml (i.e. from this test tube) transferred into a beaker to be discarded appropriately.

Nine well-labelled nutrient agar plates corresponding to the nine test tubes (10^{-1} to 10^{-9}) were each divided into 8 sections and from each test tube/dilution, one drop each on the 8 sections were made (a total of 8 drops of that same dilution) on the corresponding nutrient agar plate. The resultant plates were then incubated at 37˚C for 24 hours, after which the viable cell count (using an appropriate dilution, i.e. one that is clear enough to be counted) was done to determine the bioload or concentration of the microorganism.

Determination of death time of microorganism

The cell culture was harvested and diluted in test tubes containing 10ml sterile water, and the suspensions were evenly distributed by shaking and were sterilized by mild heating in a pressure cooker. Suspensions were diluted using normal saline, centrifuged at 3000 rev/min for 5minutes, and the supernatant decanted, leaving behind the cells. The cells were washed twice with normal saline and resuspended in fresh normal saline. 2ml of the bacterial suspension was then transferred to a sterile test tube and placed in a water bath at a constant temperature of 56ºC. Loopfuls were transferred from the selected dilution test tube at time 0, into resuscitating test tubes containing nutrient broth at different time intervals (i.e. 10, 20, 30, 40, 50, 60, 70, 80, 90 minutes), i.e. subculturing into the respective resuscitating test tubes (labeled according to the time).The test tubes with their content were then heated at a temperature of about 55-60˚C. The recovery test tubes were then incubated for 48 hours at 37˚C, after which the test tubes were examined for microbial growth (indicated by turbidity) so as to determine the death time.

ST vaccine formulation

Vaccine containing 2.5×10^{8}cells/ml was prepared. The selected broth culture was centrifuged at 3000rpm for 5 minutes. The supernatant was aspirated and the cells washed twice with normal saline by centrifugation and aspiration of the supernatant in each case. The cells were then resuspended in a specified volume of normal saline (5ml) and heated (at the same temperature) for a period of time equivalent to the predetermined death time. The "formulated vaccine", was then aseptically transferred into bijou bottles and made up with normal saline, was labeled appropriately and stored in the refrigerator.

Vaccination of mice

The experimental animal were divided into five groups named A, B, C, D and E of four mice each and vaccinated intraperitoneally as follows; Group A received 0.4ml of formulated vaccine only (which contained 10^{8} cells), Group B received 10mg of the MO extract/kg body weight only, Group C received 10mg MO extract and 0.4ml of formulated vaccine, Group D received 0.05ml of ethyl acetate only(solvent for extraction) and then Group E received 0.4 ml of

normal saline only (solvent for vaccine constitution). The vaccination procedure was repeated once after two weeks. Blood samples were collected from each mouse by intraocular eye puncture using the method described by [17] at 1 week and 2 weeks post first vaccination and 1week post second vaccination.

Weight monitoring of the experimental animals

From day 1 post second vaccination, each of the experimental animals was weighed on a daily basis using a digital weighing balance and the weight was recorded. This continued till the animals were sacrificed.

Challenge of animals with live *Salmonella typhi* (ST)

One-week after the second vaccination, the animals were challenged with 10^7 live *S. typhi* organisms contained in 0.04ml of the preparation, through the intraperitoneal route.

Blood culture

One-week post challenge with ST, blood samples were collected from each of the mice and 2-fold serial dilution of the blood samples was done by diluting 25µL of the blood sample with 25µL of normal saline. 10µL of each of the diluted blood sample was then aseptically placed on each of the 8 respective portions of the properly labeled agar plates containing freshly prepared nutrient agar. This diagnostic method was done for all the mice blood samples as described by [18]. The plates with its contents were incubated for 24 hours at 37°C and then examined for growth of microorganisms via colony count.

Statistical Analysis

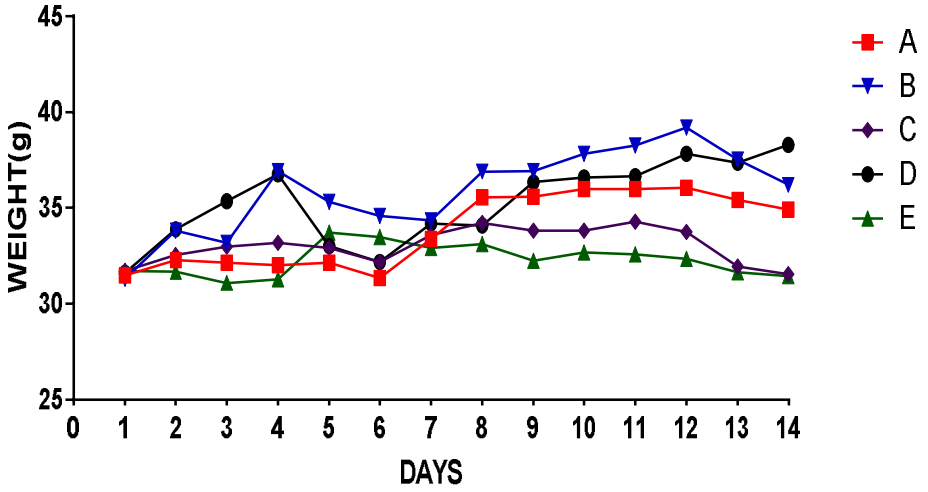
The data obtained was expressed as mean ± standard deviation (Mean ± SD). One-way analysis of variance (ANOVA) followed by Duncan post-hoc test were used to test for significance. Differences in mean were considered significant at $p < 0.05$. SPSS version 20 was used for the analysis.

RESULTS

Mice Body Weight

From the periodic mice body weight monitoring, mice body weight of treated groups showed a growth pattern that did not deviate significantly from those of the control group (Fig. 1). Fig. 1A demonstrates that the weight of mice group treated with our formulated ST vaccine only, increased progressively from day 6. Weight curve of mice group treated with Moringa leaf extract alone, showed an irregular but overall increase in mice weight throughout the period of study (fig.1B). Those treated with ST vaccine – MO combination demonstrated a slight increase in body weight from day 6, though there was a decline from day 12 through 14 (fig. 1C). There was a sharp decline in the body weight of mice group treated with ethyl acetate from day 4 to day 6; however, their body weight increased gradually from day 6 to day 14 (fig. 1D). Finally, body weight of mice in the control group was virtually uniform throughout the period of study (fig. 1E).

Figure 1. Mice body weight curve

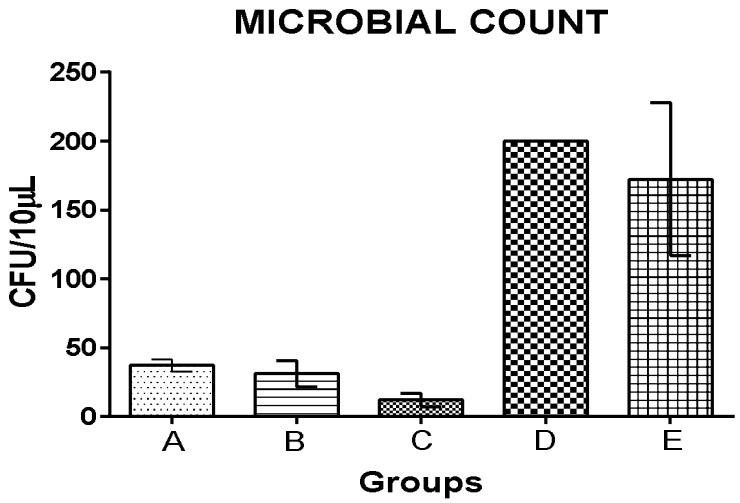


A= ST vaccine (0.4ml = 10^8 cells), B= MO extract (10mg), C= ST vaccine (0.4ml = 10^8 cells) + MO extract (10mg), D= Ethyl acetate (0.05ml), E= Normal saline (0.4ml)

Blood Culture

From the blood culture, distinct colony forming units (cfu) were observed, counted and recorded as shown in Fig. 2 and Table 1. Mice group treated with ST vaccine – MO combination demonstrateda significantly lower ($P < 0.05$) number of colony forming units than those of the control groups (group D and E). However, it was not significantly lower ($p > 0.05$) thanthe number of colony forming units in blood culture of mice groups treated with ST vaccine alone (group A) and those treated with MO leaf extract alone (group B). Interestingly, there was lower number of colony forming units in blood culture of mice groups treated with MO leaf extract alone (group B) when compared with those treated with our formulated ST vaccine alone (group A), though it was non-significant ($p > 0.05$).

Figure 2. Blood culture results



*A= ST vaccine (0.4ml = 10^8 cells) **, B= MO extract (10mg) **, C= ST vaccine (0.4ml = 10^8 cells) + MO extract (10mg) ***, D= Ethyl acetate (0.05ml) *, E= Normal saline (0.4ml) **

DISCUSSION

The immune system is able to spawn a massive variety of molecules and cells capable of specifically recognizing and eradicating virtually incessant variety of foreign pathogenic invaders [19]. Vaccination has proved to be the most efficient tool for empowering the immune system to preventa variety of infectious diseases including typhoid fever. The ultimate goal of vaccination, in addition to safety, is to generate a pathogen-specific immune response that would elicit robust humoral and cell-mediated immunity providing long-lasting protection against infection [20]. Upon recognition of the vaccine antigen, a cascade of reactions is triggered resulting in the release of inflammatory mediators which may include chemokines, activation of the complement pathway and also cellular recruitment; all these may lead to the development of signs and symptoms of local inflammation or allergy in the host [21]. These reactions would consequently result in elimination of infectious agent or pathogen from the living system. Low pH in the stomach poses a barrier to the oral route of administration of live attenuated S. typhi vaccine; hence we sought to use an alternative route of administration, the intraperitoneal route, during the study. One way of assessing the imminence of typhoid fever in a mammalian host is by determining the presence of *S. typhi* in blood samples of the host.

Table 1. Colony Forming Units

	N	Colony Forming Units
Group A	4	$37.25 \pm 4.50^{**}$
Group B	4	$31.25 \pm 9.43^{**}$
Group C	4	$12.25 \pm 4.85^{***}$
Group D	4	$200.00 \pm 0.00^{*}$
Group E	4	$172.25 \pm 55.50^{*}$

Results are expressed as mean ± S.D. (* > ** > ***)

Blood culture is said to be the most decisive method of typhoid fever diagnosis, generally after seven days of infection; more than 80% of patients are likely to test positive for ST during this period of infection [18]. Results from the blood culture test correlates relatively the potency of the treatments administered to the mice in the elimination of ST infection. Elevated levels of microbial load observed in mice

groups administered normal saline (172.25 ± 55.50 CFU) and in those administered 0.05ml ethyl acetate (200.0 ± 0.00 CFU) which are both control groups, implies that these treatment agents, expectedly, lacked antimicrobial or therapeutic potentials. This was consistent with the findings of (5), showing that there was elevated quantity of ST in blood culture of mice administered normal saline. MO-treated groups showed a significantly lower microbial load profile (31.25 ± 9.43 CFU) when compared to those of the negative control groups (D, E), indicating that MO has remarkable antimicrobial and immunomodulatory properties. This observation is consistent with the findings of [22], who reported a heightened antimicrobial activity against ST. This further supports the claim in folk medicine, about MO, as having antibacterial properties [14]. Again, there was a significant decline in the microbial load (37.25 ± 4.50 CFU) of mice treated with our formulated ST vaccine alone when compared to those of the control groups. This indicates that the ST vaccine is potent and has capacity to mediate animmunoprotective effect. This observation is consistent with the findings of (5), who reported a similarly formulated ST vaccine to have a prophylactic effect against ST infection. Furthermore, mice groups treated with our formulated ST vaccine in combination with MO extract demonstrated a much lower microbial load profile (12.25 ± 4.85 CFU), though non-significantly, when compared with those treated with the ST vaccine alone (37.25 ± 4.50 CFU). This implies that the MO leaves extract has a promising adjuvant property as its synergistic effect in reducing the microbial load of the mice was phenomenal. Moringa leaf extract was also shown to have upregulated the immunoprotective effect of a respiratory syncytial virus vaccine when administered in combination with it [23]. It also suggests that bioactive phytochemical(s) embedded in the MO extract has both immunomodulatory and adjuvant properties that could be of immense support to the development of better efficacious vaccines for myriad infectious diseases, when used in isolation or incorporated into vaccine formulations [15].

Weight curve results correlates the general physiological effect a prophylactic treatment exerts on a subject. Substantial loss in body weight of animals up to 10% of initial body weight, with the administration of extract is considered as toxic for its use [24]. Again, ST infection has been associated with weight loss [25]; hence, the need to monitor the body weight of the mice. Results showed that there was a slight progressive decline in the weight of control group administered normal saline (fig. 1E), which indicates that the S. typhi cells administered during the challenge were live and viable. This is in contrast with the observation of (5), who reported a sharp decline in the body weight of mice administered normal saline. This observation suggests that the mice model used in the experiment may have a very strong innate immune outfit which may be the reason why there was no sudden decline in the weight of mice administered normal saline. A progressive increase from day 6 was observed in the weight of control group administered ethyl acetate (fig. 1D). This observation suggests that ethyl acetate may have exerted a sterilizing effect (antimicrobial activity) which inhibited the proliferation of ST cellstemporarily and consequently, the mice having not been affected severely, gained weight progressively from day 6. However, a sharp decline was observed in the body weight of mice groups treated with the ST vaccine – MO combination from day 12 to day 14. It may be that the prophylactic effect of the ST vaccine – MO combinations led to a transient loss of appetite in the mice.Furthermore, weight curves of mice treated with the vaccine alone and those treated with MO showed a uniform weight gain pattern; there was irregular weight increase in the treated groups (fig 1A,1B).This observation also correlates the findings reported by [5, 23], which showed that a similarly formulated vaccine did not have an adverse effect on the mice as they gained weight in an irregular manner.This suggests that the prophylactic agents are relatively safe and could be used in treatment of typhoid fever.

Limitation of this study entails assessment of the humoral and cellular immune responses, especially looking out for the immunoglobulin titre levels and cytokines, to further illustrate the possible mode of action of our formulated ST vaccine in isolation and when combined with MO extract on the immune system.

CONCLUSION

The search for a more potent typhoid fever vaccine with a lasting immuno-protective effect is still on. Here, we evaluated the synergistic effect of MO leaves extract when co-administered with typhoid fever vaccine and our findings revealed that MO extract demonstrates a promising antimicrobial effect when combined with ST vaccine and may be evaluated for adjuvant properties for the vaccine in view of conferring a longer immunity. Hence, further studies on identification and isolation of the bioactive compound (s) responsible for antimicrobial and possible adjuvant properties is recommended.

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DOES THE DIFFERENCE IN LEUKOCYTE CONCENTRATION OF PRP AFFECT THE SHORT-TERM FOLLOW-UP RESULTS IN CASES DIAGNOSED WITH EARLY STAGE KNEE OSTEOARTHRITIS?

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DA LI RAZLIKA U KONCENTRACIJI LEUKOCITA PRP UTIČE NA REZULTATE KRATKOROČNOG PRAĆENJA U SLUČAJEVIMA KADA JE DIJAGNOSTIKOVAN OSTEOARTRITIS KOLENA U RANOJ FAZI?

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ABSTRACT

This prospective study was conducted for the clinical evaluation of pain severity and knee functionality following PRP injections with different leukocyte (WBC) concentrations applied to cases diagnosed with knee osteoarthritis. A total of 109 patients were included in the study. According to the leukocyte content the PRP injections were prepared as low-leukocyte content PRP (P-PRP) and high concentration leukocyte content PRP (L-PRP). Patients were divided into 2 groups. Group 1 (n=44) received low-leukocyte content PRP and Group II (n =65) received high-leukocyte content PRP. The patients were evaluated clinically with Visual Analog Scale (VAS) and Knee Society Score (KSS). The changes in the PLT levels of the L-PRP group after the procedure compared to the levels prior to the procedure were found to be statistically significantly greater than the changes in the P-PRP group. The mean VAS score of all the cases before treatment was 9.05±0.91 and this score decreased to 3.71±1.46 within 12 months. The increases in the mean Knee Society Score (KSS) values were determined as 16.92±1.97 within 6 months and 16.89±2.97 within 12 months in the P-PRP group and 19.71±1.24 within 6 months and 19.86±0.42 within 12 months in the L-PRP group. The most important aspect of this study is that, in contrast to many other studies, the results continued after the 6th month and were reported to be good in the 12th month. It was also recorded that L-PRP was clinically superior to P-PRP in the treatment of early stage knee osteoarthritis.

Keywords: *leukocyte, platelet rich plasma, knee osteoarthritisspleen*

SAŽETAK

Ovo prospektivno istraživanje sprovedeno je sa ciljem određivanja kliničke procene ozbiljnosti bola i funkcionalnosti kolena posle PRP injekcija sa različitim koncentracijama leukocita (VBC) primenjenih na slučajeve u kojima je dijagnostikovan osteoartritis kolena. U ispitivanje je uključeno 109 pacijenata PRP injekcije su pripremljene u odnosu na sadržaj leukocita kao sadržaj PRP sa niskim leukocitima (PR-PP) i visokom koncentracijom leukocita PRP (L-PRP). Pacijenti su razdvojeni u 2 grupe. Grupi 1 (n = 44) je dat PRP sa niskim sadržajem leukocita dok je II grupi (n = 65) dat PRP sa visokim sadržajem leukocita. Bolesnici su klinički procenjeni sa VASi KSS. Rezultati: Utvrđeno je da su promene nivoa PLT grupe L-PRP posle postupka u odnosu na stanje pre postupka statistički značajno veće od promena u P-PRP grupi. Prosečan rezultat VAS za sve slučajeve pre lečenja bio je 9,05±0,91 i ovaj rezultat je smanjen na 3,71±1,46 tokom 12 meseci. Povećanja srednjih vrednosti KSS određena su kao 16,92±1,97 za 6 meseci i 16,89±2,97 u 12 meseci u grupi P-PRP i 19,71±1,24 u 6 meseci i 19,86±0,42 na 12 meseci u L-PRP grupi. Najvažniji aspekt ove studije bio je da se, za razliku od mnogih drugih studija, ustanovi da se rezultati nastavljaju nakon šestog meseca i da su dobri u 12. mesecu, a zabeleženo je da je L-PRP klinički superiorniji od P-PRP u lečenju osteoartritisa kolena u ranoj fazi.

Ključne reči: *leukociti, plazma bogata trombocitima, osteoartritis kolena*

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INTRODUCTION

Current pharmacological or surgical treatment methods do not provide the desired level of good results for pathologies of the skeletal system, such as knee osteoarthritis which persists to be a clinical and societal problem (1–3). Therefore, the search for different treatment methods continues. One of these methods includes the activation of biological repair mechanisms so that damaged tissues can heal and repair themselves (1). In recent years, the application of platelet rich plasma (PRP) for biological repair has become more widely used. Platelets (PLT), as being a component of this compound, are known to contain a high amount of cytokines and growth factors which are very important in tissue healing and bone mineralisation (4). Therefore, local injections of PRP are used for biological repair in the field of orthopaedic surgery, as in all medical fields (5, 6). This method has been included in the treatment protocol of knee osteoarthritis (7). In some publications it has been reported that PRP with different content ratios is more advantageous therapy in clinical treatments (8).

However, there has been no study with a clear high level of evidence investigating the separate effects of the PLT and leukocyte (WBC) ratios in PLT concentrations included in PRP. To what extent the WBC ratio is responsible for the therapeutic positive or negative clinically observed effects has not been sufficiently clarified (9, 10).

This prospective study was conducted for the clinical evaluation of pain severity and knee functionality following PRP injections with different WBC concentrations applied to cases diagnosed with knee osteoarthritis. This research aims to obtain the data that could contribute to the literature regarding PRP applied with different concentrations of WBC content to patients with knee osteoarthritis.

MATERIAL AND METHOD

Approval for the study was granted by the University Ethics Committee with decision no 10840098-155. Informed consent was obtained from all patients. The inclusion criteria for this study were a diagnosis of primary knee osteoarthritis according to the American College of Rheumatology criteria (11) classified as Kellgren-Lawrence (12) Grade 1 and Grade 2, that had not responded to conservative treatment such as physiotheraphy and paracetamol, nonsteroidal anti-inflammatory drug treatment. A total of 164 patients in our clinic met these criteria. Patients were excluded if they had any patellofemoral or meniscal pathology determined by MRI in the knee where the injection was to be administered. As a result of the hemogram evaluation, patients with hematocrit <42% (n=9) or >52% (n=3) were excluded. Patients were also excluded if within the previous 2 weeks they had taken any nonsteroid anti-inflammatory drugs (n=18), or antithrombotic or anticoagulant drugs (n=16), if they were taking oral anti-diabetic drugs or if they had consumed alcohol or smoked within the previous 48 hours (n=6). One pregnant patient and one with an inflammatory rheumatological disease were also excluded. A total of 109 patients were included for evaluation in the study.

The PRP injections were prepared according to the leukocyte content as low-leukocyte content PRP (P-PRP) and high concentration leukocyte content PRP (L-PRP) (13). The classification according to the number of leukocytes in the PRP to be applied was made according to the Dohan Ehrenfest DM et al classification (10). The final PRP obtained from both commercial kits was prepared according to their recommended steps and guidelines. 0.5 ml of buffy coat sample was assigned for trombocyte analysis from each kit. A double-spin centrifugation process was performed at speed of 5000 rpm. Spinning time was 6 minutes for PRP preparation.

Three cycles of PRP were applied in patients who were divided into 2 groups. Group 1 (n=44) receieved low-leukocyte content PRP and Group II (n=65) received high-leukocyte content PRP. The second and third doses of the injections were applied at intervals of 10 days in both groups. During each PRP administration, the thrombocyte and white cell counts were determined from the blood samples taken from each patient. After preparation of the PRP, the thrombocyte and white cell counts were determined in the content of the PRP to be injected into the knee.

No topical or local anaesthetic pharmacological agent was applied during the injections. On the first day after injection administration, patients experiencing pain were recommended to use an ice compress and no physical rehabilitation program was recommended for any patient throughout the duration of the study.

The patients were evaluated clinically in respect of the severity of pain and knee functionality prior to the application of PRP and in 6 months and 12 months after the administration. In the determination of the severity of pain, Visual Analog Scale (VAS) scoring was used (14), and knee functionality was evaluated by the Knee Society Score (KSS) (15, 16).

Statistical analysis

The analysis of the data obtained in the study was conducted using the Statistical Package for the Social Sciences (SPSS) version 20.0 software. Descriptive statistics were shown as mean ± standard deviation (SD) and/or number (n) and percentage (%). As the research sample was formed from large volume samples (n>30), single and two sample hypotheses associated with independent parameters were formed using the Student's t-test. The Pearson Chi-square test was used to determine whether there was a correlation between variables in independent groups,where there was a conformity to specific theoretical possibility distribution of the sample results, whether the samples came from the same main mass and whether the rates of more than two main masses were equal. Homogeneity between groups, regardless of whether there was a difference between two or more groups or whether the difference between observed and expected frequencies was significant, was analysed on the

basis of the Yates Continuity Correction Test. The statistical analysis tests resulted in a yes/no response to the hypothesis (e.g., are there significant differences between the groups with $p<0.05$). A value of $p<0.01$ was considered to be highly statistically significant.

RESULTS

Evaluation of the demographic characteristics of the study participants

This study comprised 19 male and 90 female patients in the age range between 40 and 70. The demographic data of the cases and the findings of the radiological grading were reported on a standard form (Table 1).

No statistically significant difference was determined between the groups in respect of age, gender, or body mass index (BMI) ($p>0.05$).

Knee osteoarthritis was determined to be Grade 1 in 22 knee cases and Grade 2 in 87 knee cases. In 45 cases osteoarthritis was identified in the right knee, and in 64 cases in the left knee.

Evaluation according to the leukocyte and thrombocyte count of the PRP content

Following preparation of P-PRP, the mean platelet values before the 1st, 2nd and 3rd injections were found to be 971.86±292.15, 926.86±264.54 and 899.86±282.33 respectively and the WBC values were 5.02±4.65, 5.28±4.61 and 5.49±4.93. In the L-PRP group, the PLT values were determined as 1628.40±833.15, 1464.00±594.24 and 1578.60±796.32 respectively and the WBC values as 30.05±12.66, 27.14±10.36 and 28.16±10.75 (Table 2, Table 3).

Table1. Demographic characteristics of patients according to groups

		Total (n = 109)	P-PRP group (n = 44)	L-PRP group (n = 65)
		Mean ± SD	Mean ± SD	Mean ± SD
Age (years)		63.69±8.09	63.52±8.52	63.8±7.86
BMI (kg/m^2)		27.92±3.07	28.32±3.08	27.65±3.06
Track length (months)		13.76±2.46	13.07±2.25	14.23±2.50
		n (%)	n (%)	n (%)
Gender	Male	19 (17.4)	6 (13.6)	13 (20.09)
	Female	90 (82.6)	38 (86.4)	52 (80.0)
BMI level	Normal	14 (12.8)	4 (9.1)	10 (15.4)
	Overweight	68 (62.4)	28 (63.69)	40 (61.5)
	obese	27 (24.8)	12 (27.3)	15 (23.1)
Kellgren-Lawrence radiological criteria	Stage 1	17 (15.6)	6 (13.6)	11 (16.9)
	Stage 1	92 (84.4)	38 (86.4)	54 (83.1)

Table 2. Platelet counts before and after injection by groups

	P-PRP group			L-PRP group		
Platelet Count (10^3/mm^3)	Before procedure	After procedure	Difference	Before procedure	After procedure	Difference
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1st measurement	226.14±70.69	971.86±292.15	745.73±271.05	218.28±62.97	1628.40±833.15	1410.12±799.18
2nd measurement	224.82±67.95	926.86±264.54	702.05±248.86	206.65±54.63	1464.00±594.24	1257.35±573.19
3rd measurement	231.55±75.05	899.86±282.33	668.32±246.72	211.15±59.26	1578.60±796.32	1367.45±771.61

Table 3. Leukocyte counts before and after injection by groups

Leukocyte counts (10^3/mm^3)	P-PRP group			L-PRP group		
	Before procedure	After procedure	Difference	Before procedure	After procedure	Difference
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
1st measurement	5.90±1.66	5.02 ±4.65	-0.88±4.27	5.88±1.60	30.05±12.66	24.17±11.92
2nd measurement	5.90±1.40	5.28±4.61	-0.62±4.25	5.50 ±1.25	27.14±10,36	21.64±9.75
3rd measurement	5.99±1.50	5.49±4.93	-0.50±4.55	5.62±1.32	28.16±10.75	22.54±10.21

As a result of the statistical evaluation of the leukocyte counts in the P-PRP group, there was a decrease in the leukocyte measurements by mean 0.88±4.27 following the 1st PRP application compared to the mean prior to the procedure. The decrease in leukocyte measurements after the 2nd PRP was mean 0.62±4.25 and the reduction in leukocyte counts after the 3rd PRP injection was mean 0.50±4.55. The differences in all of these measurements before and after each injection were statistically significant ($p<0.01$). No statistically significant difference was determined between the leukocyte levels measured before the 1st, 2nd and 3rd injection ($p>0.05$). No statistically significant difference was determined among the leukocyte levels measured after the 1st, 2nd and 3rd injection ($p>0.05$).

As a result of the statistical evaluation of the leukocyte counts in the L-PRP group, there was an increase in the leukocyte measurements by mean 24.17±11.92 following the 1st PRP application compared to the mean prior to the procedure. The increase in leukocyte measurements after the 2nd PRP was mean 21.64±9.75 and the reduction in leukocyte counts after the 3rd PRP injection was mean 22.54±10.21. The differences in all of these measurements before and after each injection were statistically significant ($p<0.01$).

In the comparison of the two groups in respect of leukocyte count, no statististically significant difference was determined in the leukocyte level before the 1st application, the 2nd application and the 3rd application ($p>0.05$).

The PLT levels of the L-PRP group were found to be statistically significantly higher than those of the P-PRP group in the measurements taken after the 1st application, the 2nd application and the 3rd application ($p<0.01$).

The changes in the PLT levels of the L-PRP group after the procedure (compared to the levels before the procedure) at the 1st measurement, the 2nd measurement and the 3rd measurement were found to be statistically significantly greater than the changes in the P-PRP group ($p<0.01$)

Evaluation of the VAS scores

The mean VAS score of all the cases before the treatment was 9.05±0.91 and this score decreased to 3.71±1.46 within 12 months after the procedure. This tendency was observed to be similar in both L-PRP and P-PRP groups and in both the 6-month and 12-month follow-up examinations.

In the L-PRP patient group, the initial VAS score was mean 9.11±0.96 and this score decreased to 6.61±2.41 within 6 months after the procedure and to 3.58±1.54 within 12 months.

In the P-PRP patient group, the initial VAS score was mean 9.02±1.18 and this score decreased to 6.05±0.12 within 6 months after the procedure and to 3.79±1.46 within 12 months. (Table 4).

The reductions in the VAS values in both groups were determined to be statistically significant ($p<0.05$).

Evaluation of the KSS and Functional KSS scoring

In the L-PRP group, the KSS and functional KSS scores before the injection were determined to be 54.31±14.35 and 55.55±10.08 respectively and 77.83±10.69 and 74.22±10.62 in the 12-month follow-up examination. The increases in the KSS and functional KSS scores were found to be statistically significant ($p<0.05$).

In the P-PRP group, the KSS and functional KSS scores before the injection were determined to be 56.48±12.71 and 57.05±10.96 respectively and 78.98±10.52 and 73.86±13.93 in the 12-month follow-up examination. The increases in the KSS and functional KSS scores were found to be statistically significant ($p<0.05$).

The mean increases in the mean KSS measurements within 6 and 12 months after the injections compared to the mean values before the injections were 22.94±2.09 in Group I and 22.82±3.83 in Group II. The increases were determined to be statistically significant ($p<0.05$).

The increases in the mean KSS values were determined as 16.92±1.97 within 6 months and 16.89±2.97 within 12 months in the P-PRP group and 19.71±1.24 within 6 months

and 19.86±0.42 within 12 months in the L-PRP group. These increases were determined to be statistically significant ($p<0.05$). The changes in the KSS functional measurements within 12 months after the injection compared to the baseline values before the procedure showed a significant difference related to the WBC content ($p<0.05$) (Table 4).

Table4. Clinical results of the groups before and after injection

	P-PRP group			L-PRP group		
	Before injection	Last Control	Difference	Before injection	Last Control	Difference
Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Knee Society Scores	56.48±12.71	78.98±10.52	22.50±11.84	54.31±14.35	77.83±10.69	23.52±11.04
Knee Society Scores Functional	57.05±10.96	73.86±13.93	16.82±11.11	55.55±10.08	74.22±10.62	18.67±10.70
Visual Analog Scale	9.09±0.96	3.59±1.56	-5.50±1.78	9.02±0.87	3.78±1.40	-5.23±1.56

DISCUSSION

There has been wide acceptance in the scientific literature that more platelets are contained in PRP obtained after centrifuge processing from autologous full blood compared to normal blood. While the initial spin separates red blood cells from the buffy coat/plasma layer, it has been reported that more PLT can be separated in systems using a second spin. It has been emphasised that the final PLT values of PRP and the WBC concentration are affected by the number of centrifuges, the speed and the duration (17).

Some researchers have advocated that the WBC ratio should be kept at a low concentration in order to be able to obtain effective treatment by reducing the catabolic effect to a minimum (18). On the other hand, with the argument that WBCs as a source of enzymes and cytokines could be effective in the prevention of infection, it has also been stated that high concentrations of WBC should be contained in PRP (19, 20). In the current study, the mean platelet values in the prepared P-PRP before the 1st, 2nd and 3rd injection were 971.86±292.15, 926.86±264.54 and 899.86±282.33 respectively and the WBC values were found to be 5.02±4.65, 5.28±4.61 and 5.49±4.93. In the L-PRP group, the PLT values were determined as 1628.40±833.15, 1464.00±594.24 and 1578.60±796.32 respectively and the WBC values as 30.05±12.66, 27.14±10.36 and 28.16±10.75.

In the study by Yin W et al, which provided valuable data on knee osteoarthritis, pro-inflammatory cytokines at a high level in L-PRP were suggested to have a beneficial effect on growth factors in bone regeneration; therefore P-PRP and L-PRP groups were compared with molecular analyses. Histological examination of in-vivo effects was conducted by creating a calvareal defect in rats. It was observed that better histological results were obtained in the calvareal defects of the P-PRP group compared to the L-PRP group. The WBC content of L-PRP was shown to have created negative effects on bone regeneration by activating pro-inflammatory cytokines. It was concluded that P-PRP could be an effective treatment method in bone regeneration (21). In the current study, although no histological research was conducted , no statistically significant difference was determined in the clinial results between both types of PRP.

In the double-blind randomised controlled study by Duif et al, P-PRP was applied to degenerative lesions and patients were evaluated in respect of pain, functionality and quality of life. Knee arthroscopy for cartilage or meniscus degeneration was applied to a total of 58 patients, comprising 24 patients in the P-PRP group and 34 patients in the control group. At the end of 6 months, pain was significantly reduced in the group administered with P-PRP. However, it was emphasised that the same effect was not observed in 12 months. The data of the Lysholm score in the P-PRP group were reported to have significantly improved in the 6-month and 12-month period. In addition, the physical component scores of the Short Form (SF) -36 norm-based scale were reported to have significantly improved within 6 weeks and 6 months. However, it was stressed that there was no change in the results at the end of one year. It was concluded that the application of P-PRP could lead to a significant improvement in pain and knee functions over the period between 6 and 12 months (6). The results of the current study were similar to the clinical results of that study.

In the study by Patel S et al, 156 knees of 78 patients were divided into 3 groups. PRP preparates used in this clinical study had the PLT concentration 3 times higher than WBC filtered normal.

Group A comprised 52 knees administered with a single PRP injection dose; Group 2 comprised 50 knees administered with 2 doses of PRP; Group C comprised 46 knees administered with a single dose of isotonic sodium chloride (0.9% saline). Clinical evaluations were made using the WOMAC index before treatment, then in 6 weeks, 3 months and 6 months after the injections. The change in pain severity was examined by VAS. In the WOMAC scoring, a statistically significant improvement starting 2-3 weks after the treatment and lasting for up to 6 months was observed However, it was reported that this value showed a slight decrease at the end of 6 months. Similar improvements were observed in Group B, whereas the values in Group C were reported to worsen compared to the baseline. After comparison of the 3 groups, no improvement was reported in Group C compared to Groups A and B. In conclusion, it was emphasised that a single dose of PRP containing 10-fold PLT of WBC filtered normal was as effective as two injections of PRP in the symptomatic treatment of early stage osteoarthritis. In the results of that study, as in other clinical studies, the efficacy of the treatment was reported to start diminishing after 6 months (22). In the current study, no change was observed in the clinical results after 6 month post-treatment, and the treatment efficacy continued after 6 months.

In conclusion, the evidence from this study implies that within the mean follow-up period of 13.81±2.46 months there was an evident positive effect of PRP treatment on functional results and the elimination of pain in the treatment of early stage knee osteoarthritis. Clinically, in respect of pain severity and knee functionality, the KSS and KSS functional scores of the cases applied with L-PRP were observed to have increased more than those of the P-PRP group whereas the VAS values decreased. The most important aspect of this study is that in contrast to many other studies (6, 8-10, 13, 23), the results continued after the 6th month and were reported to be good in the 12th month and this was statistically significant. In addition, it was recorded that L-PRP was not only clinically superior to P-PRP, but also statistically significant in the treatment of early stage knee osteoarthritis ($p<0.01$).

CONFLICT OF INTERESTS

The authors state that there are no conflicts of interest regarding the publication of this article.

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VARIATION OF THE CYTOKINE PROFILES IN GINGIVAL CREVICULAR FLUID BETWEEN DIFFERENT GROUPS OF PERIODONTALLY HEALTHY TEETH

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VARIJACIJA PROFILA CITOKINA U GINGIVALNOJ ZGLOBNOJ TEČNOSTI IZMEĐU RAZLIČITIH GRUPA PARODONTALNO ZDRAVIH ZUBA

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ABSTRACT

Profiling of biomarkers of physiological process represents an integrative part in optimisation of diagnostic markers in order to adjust the diagnostic ranges to the potential effects of the local factors such occlusal forces in case of periodontal tissues. The objective of this study was estimation of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IL-22, TNFα and IFNγ concentrations in gingival crevicular fluid samples (GCF) between different groups of teeth. Two hundred fifty-nine systemically healthy non-smokers having at least one vital tooth without restorations, with healthy periodontal tissues, were clinically examined and the GCF sample was retrieved. The cytokine levels were estimated using flow cytometry and compared between central incisors (CI), lateral incisors, canines, first premolars, second premolars, first molars and second molars. Cytokine profiles varied between different groups of teeth with tendency of increase in pro-inflammatory cytokines from anterior teeth toward molars. Molars might be considered teeth with natural predisposition for faster bone resorption while the adjustment of diagnostic range of periodontal biomarkers for anterior or posterior teeth should be considered within diagnostic context. Cytokine profiles varied between different groups of teeth with tendency of increase in pro-inflammatory cytokines from anterior teeth toward molars. Molars might be considered teeth with natural predisposition for faster bone resorption while the adjustment of diagnostic range of periodontal biomarkers for anterior or posterior teeth should be considered within diagnostic context.

Keywords: *biomarkers, gingival crevicular fluid, cytokines, periodontal ligament, occlusal forces, periodontal disease*

SAŽETAK

Profilisanje biomarkera fiziološkog procesa predstavlja integrativni deo optimalizacije dijagnostičkih markera, kako bi se dijagnostički rasponi prilagodili potencijalnim uticajima lokalnih faktora poput okluzijskih sila u slučaju parodontalnih tkiva. Cilj ove studije bila je procena koncentracija IL-1b, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IL- 22, TNFα i IFNγ u uzorcima gingivalne tečnosti (GT) kod različitih grupa zuba. Klinički je pregledano dvesta pedeset devet sistemski zdravih nepušača sa najmanje jednim vitalnim zubom bez restauracija, sa zdravim parodontalnim tkivima, i uzet je GT uzorak. Nivoi citokina procenjeni su protočnom citometrijom i upoređeni između centralnih sekutića (CS), bočnih sekutića, očnjaka, prvih i drugih premolara, kao i prvih i drugih kutnjaka. Profil citokina varirao je između različitih grupa zuba sa tendencijom povećanja pro-upalnih citokina od prednjih zuba do kutnjaka. Molari se mogu smatrati zubima sa prirodnom predispozicijom za bržu resorpciju kosti, dok bi podešavanje dijagnostičkog raspona parodontalnih biomarkera za prednje ili zadnje zube trebalo razmotriti unutar dijagnostičkog konteksta.

Ključne reči: *biomarkeri, gingivalna tečnost, citokini, periodontalni ligament, okluzijske sile, parodontalna bolest*

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INTRODUCTION

The cytokines were widely investigated in periodontology as host response markers of periodontal diseases (1-3) The strong tendency in periodontology toward personalized medicine approach. This approach refers to the clinical decision-making supported by molecular findings for the selection of optimal treatment tailored to the individual patient. The reason for that is clinical periodontal parameters which are not sensitive enough to provide refined diagnostic information on nature of pathological process, disease activity, its magnitude and responsiveness on the performed treatment. On the other hand, cytokines being the objectively measurable regulators of periodontal inflammation are able to provide the real-time information on ongoing processes in the tissue. Additionally, the gingival crevicular fluid (GCF) is a diagnostic specimen that is easily accessible without additional invasive procedures qualitatively corresponding to the liquid biopsy (4). Briefly, GCF evolves by the serum transudation through the vessels of gingival plexus therewith skimming all biological markers on its flow from the local vessels' endothelium, junction epithelium, gingival crevices and entire gingival sulcus/ pockets. Therefore, cytokines in GCF reliably reflect ongoing processes in the periodontal tissues and provide the exact information on their nature and magnitude. However, when optimizing biochemical markers around metabolically active tissues such as periodontal ligament (PDL) and bone tissue it is of substantial importance the adjustment of the diagnostic ranges to the local physiological factors (5). In relation to that, biomarkers of normal biological processes represent the independent field in the biomarkers research (6). One of the ultimate specificities of teeth and supporting periodontal tissues is exposure to the strong occlusal forces counting about 160-240N at incisors and 490-840N in molars. The crucial role in amortization of these forces plays PDL interposed between root cement and alveolar bone while its structural integrity represents the key determinant of function. The main structural units of PDL are periodontal fibers and intercellular matrix with reach cellular content including fibroblasts, mesenchymal stem cells, osteoblasts, osteoclasts, cementoblasts and cementoclasts responsible for formation and remodeling of PDL, cement and alveolar bone (7). The activity of these cells is regulated by proprioceptors stimulated by occlusal forces via cytokine networks and corresponding autocrine/paracrine mechanisms (8, 9). In brief, stimulation of the mechanoreceptors leads to the local release of balanced concentrations of neuropeptides, growth factors and cytokines with subsequent physiological remodeling of the periodontal tissues (8, 10). Subsequently, in the case of inappropriate mechanical loading, the balance between pro-resorptive and pro-formative mediators remains disrupted leading to the structural changes in PDL and inflammatory osteoclastogenesis via receptor-activator nuclear factor kappa-B ligand (RANKL) (11). Moreover, different groups of teeth are exposed to the different intensity of occlusal forces depending of their anatomical position and primary function. However, the studies reporting the profile of cytokines around periodontally healthy teeth at different anatomical positions are very scarce.

Thus, we hypothesized that cytokine profiles in GCF are different between periodontally healthy teeth at different anatomical positions.

The objective of this study was estimation of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IL-22, TNFα and IFNγ concentrations in GCF samples of central incisors, lateral incisors, canines, first premolars, second premolars, first molars and second molars from periodontally healthy teeth.

MATERIAL AND METHODS

Study population

Two hundred fifty-nine (259) adults aged 30-60 visiting the Clinic for Stomatology, Military Medical Academy, Belgrade, Serbia were recruited from November 2013 to August 2015. Patients were selected on the basis of having at least one vital tooth without restorations, with healthy periodontal tissues including no bleeding on probing (BOP), probing depth (PD) ≤ 3mm, clinical attachment level (CAL) =0, absence of mucogingival defects and absence of traumatic occlusal contacts.

Participants were included in the study if they:

- were systemically healthy non-smokers,
- had at least 24 natural teeth,
- did not have active periodontal inflammation measured as full-mouth bleeding scores (FMBOP) < 15% and
- had good oral hygiene measured as full-mouth plaque scores (FMPS) < 25%
- and if they lacked the following exclusion criteria:
- periodontal treatment in the preceding year,
- intake of antibiotics and/or anti-inflammatory agents in the preceding 3 months
- pregnancy and/or lactation in female patients
- orthodontic treatment
- bruxism and oral parafunctions
- fresh postextractional or traumatic wounds in the area of investigated teeth.

Participants were informed about study characteristics and agreed to participate by signing the informed consent form while the study was approved by the institutional ethics committee (permission reference: VMA/10-12/A.1).

Experimental Design

This study was designed as a cross-sectional study comparing the profile of 13 cytokines in the GCF of periodontally healthy teeth at different anatomical positions in adults. The participants aged between 30 and 60 were selected in order to match the age of population affected by chronic periodontitis seeking for biomarkers for disease monitoring. In the first visit, the clinical and anamnestic parameters were recorded to verify the eligibility of the participants, while the second visit for collection of GCF specimen was scheduled

second visit for collection of GCF specimen was scheduled 24-72 hours following a clinical examination in order to avoid the contamination of the sample. In relation to the representative tooth affiliation the following experimental groups were created: 1) central incisors (CI); 2) lateral incisors (LI); 3) canines (CA); 4) first premolars (PM1); 5) second premolars (PM2); 6) first molars (M1); 7) second molars (M2).

Clinical Outcome Variables

The full-mouth periodontal measurements in six sites per tooth were performed using periodontal probe graded in mm to record the following clinical parameters:

- PD as a distance between gingival margin and the bottom of the sulcus/pocket (expressed in mm).
- CAL as a distance between the cement-enamel junction and the depth at which the probe met resistance (expressed in mm).
- Bleeding on Probing (BOP) - measured 15 s after probing and recorded as presence (1) or absence (0).
- Visible Plaque Accumulation (PI) - measured along the mucosal margin and recorded as presence (1) or absence (0).

The clinical measurements were performed by two trained and calibrated examiners (E.T., V.S. and Z.T.). Intra-examiner calibration was performed twice, before and during the study, by assessing PD and CAL with a degree of agreement within ±1mm of 95.7%. All teeth were evaluated with the exception of third molars and teeth where the cemento-enamel junction could not be accurately distinguished.

GCF sampling and storing

The GCF sample was retrieved from the bucco-mesial aspect of one representative tooth in each patient being the participant unit of analysis. The representative tooth was selected randomly using computer software, while in the case when computer selected the tooth that was not present/eligible, the process was repeated until the selection of appropriate tooth. The protocol of one representative tooth per patient was selected for the purpose of initial population screening since there is no available evidence in the published literature on cytokines profiles between different groups of teeth. Additionally, it was difficult to recruit the adults with all present teeth in the mouth; hence, to ensure the homogeneity in the protocol one tooth per patient was selected.

The samples were retrieved using previously described filter paper technique (12).

Quantification of cytokines using multiplexed bead immunoassay

Cytokines were quantified in the GCF samples with commercial flow cytometric assay (Ebioscience Human Th1/Th2/Th9/Th17/Th22 13 plex kit, Bender MedSystems GMBH, Campus Vienna Biocentar, Austria, EU) on Beckman Coulter FC500 cytometer (Brea, California, USA). The concentrations of measured markers were expressed as total amount (picograms) of each cytokine per site in 30 seconds (pg/site).

Data analysis

The primary outcome variables were GCF levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17, IL-22, TNFα and IFNγ expressed as total pg/site/30s.

Distribution of age, sex and number of the remaining teeth between the groups were compared using Fisher exact test. Distribution of biochemical data was tested for normality using Shapiro–Wilk test. Following that, the intergroup comparisons of the biochemical markers were tested with the Kruskal-Wallis test, whereas the differences were evaluated using the Mann-Whitney U test. Thereafter, the p-values were adjusted using the Bonferroni correction. Comparison of cytokines levels between maxilla and mandible was performed using Mann-Whitney test. Furthermore, the correlation between cytokine levels in the different groups of teeth were estimated using Spearman's rank correlation test.The power analysis was performed for the IL-1β levels and resulted in sample size of 28 participants per group for $\alpha=0.05$ and power of 0.95. The statistical analysis was performed using commercial software (Prism 5.0, GraphPad Software, Inc., La Jolla, CA, USA) with the significance level set at 5 % ($p < 0.05$).

RESULTS

The study sample included 112 women and 147 men, so the distribution according to the sex was similar and that was confirmed statistically. 259 investigated teeth included 32 central incisors, 34 lateral incisors, 31 canines, 36 first premolars, 47 second premolars, 38 first molars and 41 second molars, hence the quantitative composition of the groups was similar according to the statistical analysis as well. Regarding qualitative homogeneity of the groups, distribution of the age, sex, remaining teeth and periodontal status between the groups were similar as well based on the absence of any statistical significance (Table 1).

The detectability rate for evaluated cytokines between investigated teeth varied and this is outlined in Table 2. In general, the highest detectability rate for evaluated cytokines was observed in canines (10/13 cytokines) and second molars (9/13 cytokines), while in the lateral incisor no cytokine demonstrated detectability in more than 80% of samples.

Furthermore, the greatest number of cytokines with highest concentrations was observed in canines where four pro-resorptive bone cytokines including IFNγ, IL2, IL12 and IL17 demonstrated the highest levels. Further, the first premolars were the next group of teeth by the number of cytokines detected in the highest concentration including three pro-osteogenic cytokines IL-22, IL-13 and IL-4. In molars,

and IL-10 and IL1$_\beta$ in the second molars. Central incisors and second premolars showed the highest levels for one cytokine including IL-9 and IL-5, respectively while in lateral incisors no evaluated cytokines represented the highest concentration.

Table 1. Demographic and Clinical Characteristics of the Groups

Group of teeth / Parameter	Central Incisor n=32	Lateral Incisor n=34	Canine n=31	First Premolar n=36	Second Premolar n=47	First Molar n=38	Second Molar n=41
Mean age (interval)	45.2 (35-58)	46.1 (34-54)	47.8 (33-60)	46.6 (33-59)	43.9 (30-53)	45.7 (31-58)	44.2 (31-52)
Gender							
Female	18	15	15	17	21	17	17
Male	14	19	16	19	26	21	24
FMPD (mm)	2.74±2.11	2.52±1.21	2.27±0.75	2.51±1.82	2.36±1.54	2.23±0.93	2.56±1.51
FMCAL (mm)	1.98±0.77	1.65±0.89	1.56±0.75	1.65±1.15	1.89±1.25	1.75±1.45	0.98±1.01

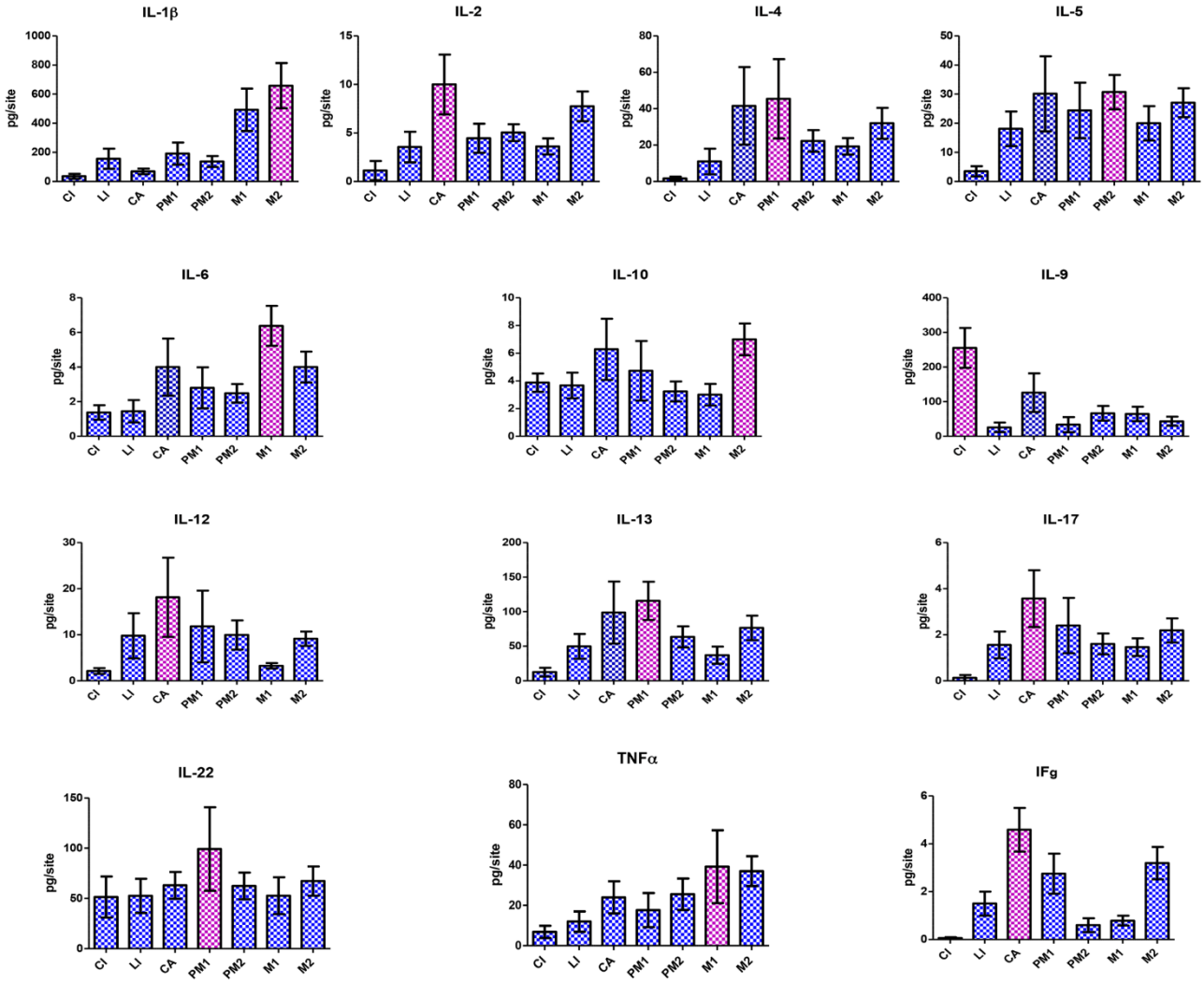
Table 2. Detectability rate and the highest GCF levels of cytokines per different group of teeth

Tooth	The highest average concentration	Detectability rate > 80%
Central incisor	IL9	IL12, IL10, IL9
Lateral incisor	-	-
Canine	IL2, IL12, IL17, IFNγ	IL12, IL2, IL10, IL9, IL22, IL13, IL4, IL5, IL1β, TNFα
First premolar	IL13, IL4, IL22	IL13, IL1β
Second premolar	IL5	IL12, IL2, IL1β
First molar	IL6, TNFα	IL12, IL6, IL4, IL1β
Second molar	IL1$_\beta$, IL10	IL12, IL2, IL10, IL9, IL22, IL6, IL5, IL1β, TNFα

Comparison of cytokine levels in GCF samples between different groups of teeth is depicted in Fig.1. Generally, the most distinct cytokine profile was observed in central incisors that demonstrated significantly increased levels of IL-9 when compared to all other groups of teeth and significantly lower levels of 10/13 cytokines when compared to canines, premolars and molars. Briefly, in the CI the following cytokines were significantly lower when compared to the corresponding groups of teeth: IL1β < PM2, M1 and M2; IL-2 and IL-12 < CA, PM2 and M2; IL-4 and IL-5 <CA, PM2, M1 and M2, IL-6 < M1; IL-13 <CA, PM1, M1 and M2; IL-17 < CA, PM1, M1 and M2; TNFα< CA and M2 and IFNγ< CA. In the CA group the following cytokines showed significantly higher concentration compared to the corresponding groups: IL-2 > M1; IL-9 > LI and PM1; IL-17 > M1 and IFNγ > CI and PM2. In the M1 group the following cytokines showed significantly higher concentration compared to the corresponding groups: IL-6 > CI, LI, PM1 and PM2 and IL-4 > LI as well as significantly lower level of IL-13 when compared to PM1. In the M2 group, the following cytokines showed significantly higher concentration when compared to the corresponding groups: IL-1β > CI, LI, CA, PM1 and PM2; IL-10 > PM2 and M1; IL-12 > CI and M1; TNFα > CI, LI and PM2 and IFNγ > PM2.

Comparison of cytokine levels between GCF samples from maxillary and mandibular teeth showed significantly higher levels of IL-4, IL-9 and TNFα in maxilla and significantly higher levels of IL-1β and IL-12 in mandible (Fig.2). IL-22 concentration was visibly higher in the GCF of maxillary teeth, but this was not statistically significant.

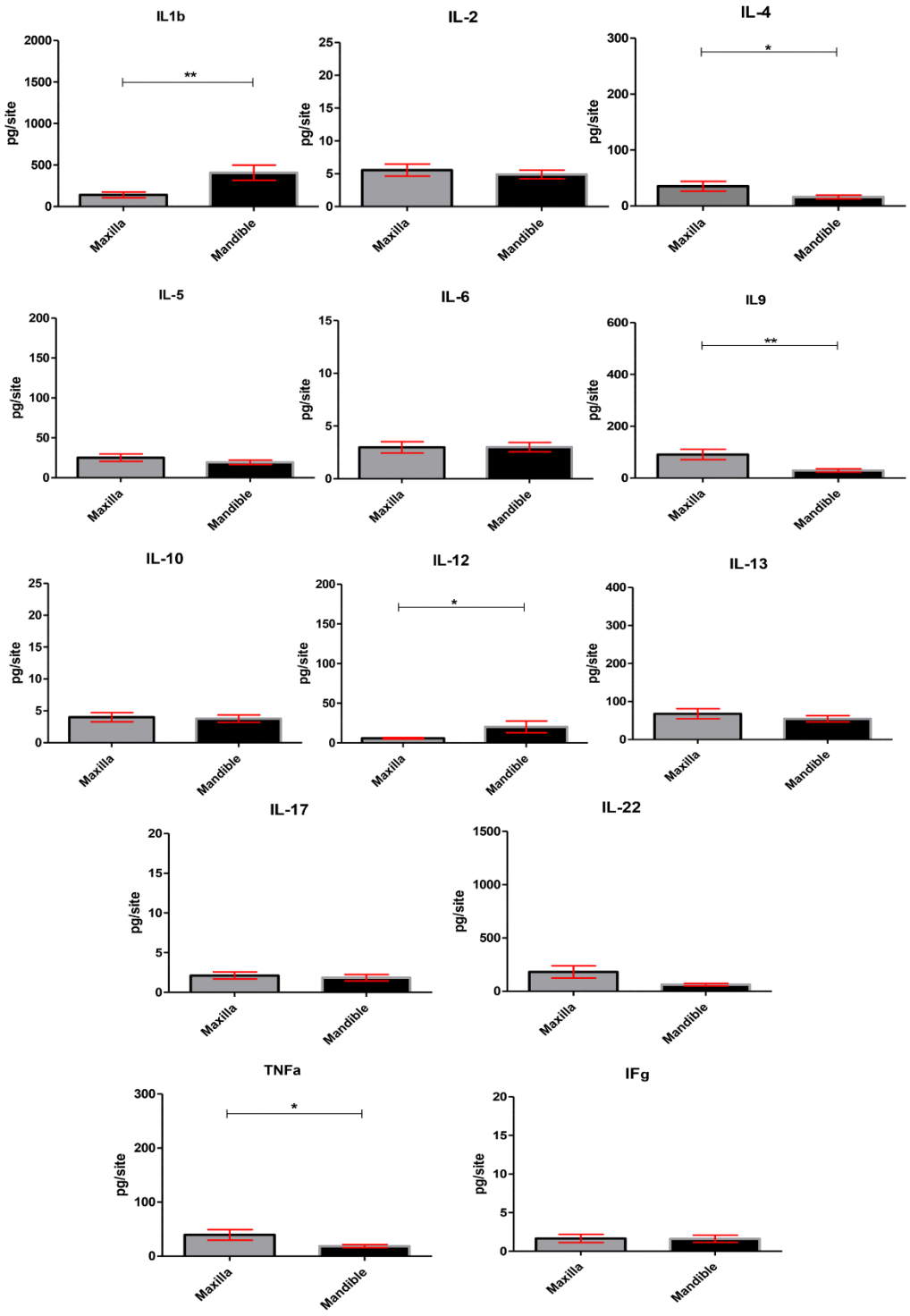
Fig.1. Cytokine levels in GCF samples between different groups of teeth



IL1b	IL2	IL4	IL5	IL6	IL9	IL10	IL12	IL13	IL17	IL22	TNFa	IFNg
PM2>CI*	CA>CI*	CA>CI*	CA>CI*	M1>CI**	CI>LI*	M2>PM2*	CA>CI*	CA>CI*	CA>CI*		CA>CI*	CA>CI*
M1>CI*	PM2>CI*	PM2>CI*	PM2>CI*	M1>LI*	CI>PM1*	M2>M1*	PM2>CI*	PM1>CI*	PM1>CI*		M2>CI*	CA>PM2*
M2>1*	M2>CI *	M1>CI**	M1>CI*	M1>PM1*	CI>PM2*		M2>CI*	PM2>CI *	M1>CI*		M2>LI*	M2>PM2*
M2>LI*	CA>CI *	M2>CI*	M2>CI*	M1>PM2*	CI>M1*		CA>M1*	M2>CI*	M2>CI*	-	M2>PM2*	
M2>CA*	CA>M1*	M1>LI*			CI>M2*		M2>M1*	PM1>M1*				
M2>PM1*					CA>LI*							
M2>PM2*					CA>PM1*							

The schema bellow the charts indicates statistically significant differences between different groups of teeth according to the Man Whitney tests following Bonferroni adjustment (*). The box plots are plotted on mean value while the read lines indicate standard error. CI-central incisor; LI- lateral incisor; CA-canine; PM1- first premolar; PM2- second premolar; M1- first molar; M2- second molar; the violet pattern indicates the group of teeth with highest level of the corresponding cytokine, **- $p<0.001$.

Fig.2. Comparison of cytokine levels between maxillary and mandibular teeth.



The chart depicts significantly higher levels of IL-4, IL-9 and TNFα in maxilla and significantly higher levels of IL-1$_\beta$ and IL-12 in mandible; *- $p<0.05$; **- $p<0.001$.

DISCUSSION

Results of this study showed that cytokine profiles in GCF varied between different groups of teeth while the teeth demonstrating the most distinctive profile were central incisors with low levels of evaluated cytokines with exception of anti-inflammatory IL-9, canines with increased levels of both pro and anti-inflammatory cytokines and first and second molars with generally increased pro-inflammatory cytokines. Hence, the active metabolic activity around canines and tendency of increase in pro-inflammatory cytokines toward posterior teeth was observed. Moreover, the comparison of cytokine concentrations between maxilla and mandible demonstrated significantly higher levels of IL-9, IL-4 and TNFα in maxilla and significantly higher concentrations of pro-inflammatory IL-1$_\beta$ and IL-12 in mandible.

Cytokines in GCF have been widely investigated in periodontology for better understanding of the physiology and pathology of periodontal tissues as well as for the purpose of identification of biochemical markers of periodontal disease (1,13,14). Surprisingly enough, although cytokine networks in the periodontal tissues orchestrate the perception and entire regulation of mechanical loading, the studies reporting the cytokine profile between different groups of teeth exposed to different intensity of occlusal forces are very scarce. Generally, the studies in orthodontics estimated cytokine levels in patients undergoing orthodontic treatment and demonstrated increase in pro-resorptive bone cytokines immediately following the application of orthodontic forces while the cytokine levels decreased gradually with time (15-19). There is only one study in orthodontics that evaluated longitudinally the MMP9, TIMP1, TIMP2, RANKL and OPG GCF levels between different groups of teeth in patients undergoing orthodontic treatment and showed clear difference in concentration of all investigated mediators between tooth with different anatomical position, canines and second molars (20). Hence, this is the first study to report cytokine levels in GCF samples within different groups of teeth.

Flow cytometry method used in this study represents the method for proteomic analysis of secreted proteins considered as a powerful platform for both the research and clinical settings concerning biomarkers and patient-stratification (21). This method allows one-shot analysis of the wide selection of biomarkers thus providing a comprehensive overview of the local cytokine profile. For the purpose of this study, the set of 13 pro and anti-inflammatory cytokines was selected from five different T-helper (Th) sub-sets including Th1/Th2/Th9/Th17/Th22. This approach allowed the functional profiling by providing information of the exact groups of Th responsible for different functions as well as by providing the overview of inter-relations between secreted cytokines. Regarding the selected cytokines, majority of estimated cytokines were already investigated in periodontology including Th1 pro-inflammatory cytokines that participate in bone resorption: IL-1β, IL-12, TNFα and IFNγ; pro (IL-2) and so called anti-inflammatory Th2 cytokines act in both soft and bone tissue related processes: IL-4, IL-5, IL-6, IL-10 and IL-17 reported (22) as an important osteoclastogenic factor. However, some recent cytokines considered of interest for periodontal physiology were also investigated in this study including IL-9, IL-13 and IL-22. IL-22 is an anti-inflammatory cytokine, member of IL10 superfamily, produced by activated DC and T cells, with the main role of regulating local antimicrobial defense (23). In addition to that, it was reported that IL-22 could have protective role in local inflammation and even regenerative function by inducing mineralization via periodontal ligament cells (24, 25). IL9 is nowadays recognized as cytokine with potent antitumor effects. IL9 induces indirectly potent antitumor response, initiating CCL20 production that mediates recruitment of dendritic cells and CD8 cytotoxic T lymphocytes expressing CCR6 (26). IL-13 secreted predominantly by Th2 cells, regulates numerous biological functions like resistance to Leishmania and Listeria species, but also processes of tissue remodeling and fibrosis (27), and could be associated with colonization of specific microbiota (28).

In this study, the parameter of cytokine detectability was followed since it is considered that, in healthy conditions, the levels of cytokines and growth factors are extremely low and frequently undetectable (due to concentrations bellow detectability threshold of the diagnostic assays). Hence, in our study we used the detectability rate >80% as an indicator of active tissue metabolism. In relation to that, canines showed the highest number of cytokines with detectability above 80% including 10/13 estimated markers. Since both pro and anti-inflammatory cytokines were in this group such an active metabolism can be explained by the role of canines as natural stress breakers (29, 30) of masticatory forces indicating their permanent exposure to the strong biomechanical stimulation (31).Moreover, it was recently indicated that proprioceptives of canine teeth are more responsive due to their role in anterior guidance, hence it seems that such hyper-responsiveness consequentially increase the levels of locally released neurotransmitters and cytokines as well (30). The next group of teeth by the number of cytokines with high detectability rate were second molars with the rate of 9/13 markers but with more expressed impact of pro-inflammatory cytokines than in canines. Such an increased detectability of pro-inflammatory cytokines in second molars as well as in first molars and second premolars who demonstrated increased detectability of three pro-inflammatory cytokines can be explained by the exposure of these teeth to the stronger masticatory forces due to their physiological function. From the biochemical aspect, stimulation of periodontal proprioceptors by mechanical loading leads to activation of transcription factors such as nuclear factor-kB, c-Fos and c-jun responsible for biosynthesis of pro-inflammatory cytokines (32, 33). Hence, such an increase in pro-inflammatory cytokines in the lateral teeth can be explained by the exposure to stronger masticatory forces that subsequently affect cytokine levels in the course of its increase. Therefore, significantly higher levels of IL-1$_\beta$, IL-6 ,IL-12, TNFα and IFNγ in molars with clear tendency of their increase from anterior toward posterior teeth in this study support this physiological rule. In relation to that, the optimal occlusal loading is a key

determinant of the periodontal homeostasis (34) since under physiological loading the amounts of pro-inflammatory cytokines are competitively balanced by anti-inflammatory cytokines. This fact explains the high detectability rate and increased levels of both pro and anti-inflammatory cytokines in canines opposite to the molars where the pro-inflammatory cytokines dominated in the profile. In fact, canines are natural stress breakers of occlusale forces but are exposed to the approximately four times lower forces than molars due to different masticatory function. In addition to that, significantly lower levels of pro-inflammatory IL1$_\beta$, IL-2 , IL-5 ,IL-6 , I IL-12 L-13 , IL-17 , TNFα, IFNγ as well as anti-inflammatory IL-4 and IL-13 in central incisors indicate the balanced periodontal metabolism in the group of teeth exposed to the lowest occlusal forces.

On the other hand, in the case of excessive biomechanical loading, the amount of secreted pro-inflammatory cytokines exceeds adaptive capacity of anti-inflammatory cytokines causing the osteoclastogenesis and the structural changes in PDL associated with occlusal traumatism. Significantly higher levels of the main pro-osteoclastogenic cytokines (IL1$_\beta$, IL-6 , I IL-12 and TNFα) in molars, compared to almost all anterior teeth, can be considered as an important pathophysiological feature of molars. In fact, such an initial physiological increase in these pro-inflammatory cytokines might facilitate achievement of their critical concentrations being the trigger of inflammatory osteoclastogenesis in periodontitis and therewith provide the natural predisposition of molars for periodontitis.

Furthermore, in this study the levels of cytokines between maxilla and mandible were compared since it was reported that the metabolic activity of cancellous bone in maxilla is about 20% more expressed when compared to mandible. This analysis revealed variation in profiles of IL-1$_\beta$, IL-4, IL-9, IL-12 and TNFα between different jaws, but further investigation in the studies with the sample size harvested in relation to the number of maxillary and mandibular teeth should be performed. In this study, the jaw appurtenance of the teeth was not considered since it is considered that there is no difference in acting of occlusal forces between maxillary and mandibular teeth of the same group of teeth.

Moreover, since this was the first study to investigate the cytokine profiles between different groups of teeth in adults, the protocol of one tooth per patient was selected for initial screening of population and this can be considered as a relative limitation of the study. Although it is considered that the inter-individual variations in cytokine networks are expressed in disease rather than in healthy condition, for the future studies the analyses of cytokine profiles around different groups of teeth in the same mouth of healthy participants should be considered. Moreover, the same analyses should be conducted in patients with periodontitis in order to establish whether different groups of teeth exhibit substantially different cytokine profile that should be considered in setting of diagnostic range of potential biochemical markers.

CONCLUSION

Results of this study indicated that cytokine profiles in GCF within different groups of teeth vary with clear tendency of increased pro-inflammatory cytokines concentration from anterior teeth toward molars. Therefore, molars might be considered as teeth with natural predisposition for faster bone resorption, while the adjustment of diagnostic range of periodontal biomarkers for anterior or posterior groups of teeth should be possibly considered.

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RELATIONS BETWEEN FAMILIAL HYPERCHOLESTEROLEMIA AND EARLY ISCHEMIC HEART DISEASE: AN ANALYSIS OF MEDICAL DOCUMENTATION DATA

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ODNOSI PORODIČNE HIPERHOLESTEROLEMIJE I RANE ISHEMIJSKE BOLESTI SRCA: ANALIZA PODATAKA IZ MEDICINSKE DOKUMENTACIJE

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ABSTRACT

Heterozygous familial hypercholesterolemia is associated with a high risk of early ischemic heart disease onset and cardiovascular death. There is almost no data about the prevalence of the disease in the Ukrainian population. The aim of the study was to assess the incidence of familial hypercholesterolemia among patients who were treated in "L.T. Malaya Therapy National Institute of the National Academy of Medical Sciences of Ukraine" due to early ischemic heart disease. Medical records data of 600 patients treated in the Institute during 2015-2017 were analyzed. Early ischemic heart disease was diagnosed in 89 patients. The disease verification has been conducted either on the basis of coronarography data, or on the basis of previous myocardial infarction with Q wave. To identify patients with familial hypercholesterolemia, the Dutch lipid clinic network criteria were used. The presence of familial hypercholesterolemia was suspected in more than 14.8% of patients with early ischemic heart disease. Among these patients, 2 (2.2%) had definite diagnosis; 27 (30.3%) were likely to have diagnosis, 26 (29.7%) had possible diagnosis and in 34 (38,2%) patients it was unlikely to diagnose them with familial hypercholesterolemia. The term "familial hypercholesterolemia" was not mentioned in the hospital diagnosis. This paper demonstrates that despite frequent occurrence of familial hypercholesterolemia, doctors' alertness towards this disease has been noted to be quite low.

Keywords: *heterozygous familial hypercholesterolemia, early ischemic heart disease*

SAŽETAK

Heterorozna porodična hiperholesterolemija povezana je sa velikim rizikom od ranog nastanka ishemijske bolesti i kardiovaskularne smrti. Gotovo da nema podataka o rasprostranjenosti bolesti u ukrajinskoj populaciji. Cilj studije bio je proceniti učestalost porodične hiperholesterolemije kod pacijenata koji su lečeni od „L.T. Malaja terapija, Nacionalni institut Nacionalne akademije medicinskih nauka Ukrajine" zbog rane ishemijske bolesti srca. Analizirani su podaci medicinske evidencije za 600 pacijenata lečenih u Institutu tokom 2015-2017. Rana ishemijska bolest srca dijagnostikovana je kod 89 pacijenata. Verifikacija bolesti je izvršena ili na osnovu podataka koronarografije, ili na osnovu prethodnog infarkta miokarda sa K talasom. Da bi se identifikovali pacijenti sa porodičnom hiperholesterolemijom, korišćeni su mrežni kriterijumi holandske lipidne klinike. Sumnjalo se na prisustvo porodične hiperholesterolemije kod više od 14,8% pacijenata sa ranom ishemijskom bolešću srca. Među tim pacijentima, 2 (2,2%) je imala definitivnu dijagnozu; 27 (30,3%) verovatno će imati dijagnozu, 26 (29,7%) - imalo je moguću dijagnozu, a kod 34 (38,2%) pacijenata nije bilo verovatno da im se dijagnostikuje porodična hiperholesterolemija. Izraz "porodična hiperholesterolemija" nije spomenut u bolničkoj dijagnozi. Ovaj rad pokazuje da je uprkos učestaloj pojavi porodične hiperholesterolemije primećeno da je doktor lekara na ovu bolest prilično nizak.

Ključne reči: *heterorozna porodična hiperholesterolemija, rana ishemijska bolest srca*

ABBREVIATIONS

HDH-C - high density lipoproteins cholesterol
LDL-C - low density lipoproteins cholesterol
TC - total cholesterol
TG - triglycerides
VLD-C - very low-density lipoproteins cholesterol

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INTRODUCTION

Familial hypercholesterolemia is an autosomal dominant hereditary disease characterized by high levels of low-density lipoproteins and early development of ischemic heart disease, as well as by early atherosclerosis of other localization (1). The homozygous form of the disease is a rare condition and occurs with a frequency of 1: 1.000.000 in the general population. Conversely, the heterozygous form is one of the most widespread genetically determined human diseases. It was believed earlier that the heterozygous form of the disease occurs in 1 out of 500 subjects; now the ratio is about 1 per 200-300 subjects (2). One of the most common causes of this disease development is a mutation responsible for low-density lipoprotein receptors functioning. Besides, the disease can be caused by other rare mutations of genes encoding apolipoprotein B, pro-protein convertase subtilisin/kexin type 9 and LDL adaptor protein 1 (3).

The disease is associated with a high risk of early atherosclerosis and cardiovascular death. Nanchen D. et al. demonstrated that patients with heterozygous familial hypercholesterolemia and those with acute coronary syndrome have two times higher risk of recurrent event during the first year than patients without familial hypercholesterolemia (4).

One of the most optimal strategies is the creation of national registries and long-term monitoring of patients' families. Such an approach will allow to identify patients with suspected familial hypercholesterolemia and initiate therapy at an early age. Besides, maintenance of state registries will furnish possibility to examine relatives, especially children, and, again, allow to start the treatment early. Currently, there is no centralized system for registering patients with familial hypercholesterolemia in Ukraine. Gold standard for this disease diagnosis is a genetic study and confirmation of mutation carriage. At the same time, this method is expensive and not affordable for routine use in many countries. Therefore, many countries use clinical criteria to make up registry and detect patients at risk. There are several options for the diagnosis as per clinical criteria, but the most common diagnostic criteria are the following: Dutch Lipid Clinic Network, Simon Broome, Make Early Diagnosis to Prevent Early Deaths and American Heart Association (1). In their work, Chan et al. matched the data of clinical scales and the results of genetic study and demonstrated quite high diagnostic value of Dutch Lipid Clinic Network, Simon Broome, and Make Early Diagnosis to Prevent Early Deaths scales. The authors state that cholesterol level before the treatment start, family history and presence of xanthomas are quite strong criteria for detecting candidates for genetic study (5).

The objective of this study was to identify subjects with familial hypercholesterolemia among patients who sought care in "L.T. Mala National Institute of Therapy of the National Academy of Medical Sciences of Ukraine" due to ischemic heart disease during 2015 – 2017, according to clinical criteria.

MATERIALS AND METHODS

Data Source

Investigators analyzed the data from medical histories of patients who were treated in "L.T. Mala National Institute of Therapy of the National Academy of Medical Sciences of Ukraine" during 2015-2017. Only medical records of patients with verified ischemic heart disease were eligible for the analysis. Early ischemic heart disease involves disease onset before the age of 60 in women and 55 in men (6). Disease verification has been conducted either on the basis of coronary angiography data, or on the basis of previous myocardial infarction with Q wave. The total of 600 medical records were analyzed.

Identification of patients with Familial Hypercholesterolemia

To identify patients with familial hypercholesterolemia the investigators used Dutch lipid clinic network criteria (Table 1).

Ethics Statement

The study was approved by the Ethical Committee of "L.T. Mala National Institute of Therapy of the National Academy of Medical Sciences of Ukraine"

Statistical analysis

Statistical analysis was performed using SPSS software, version 17.0. χ^2 test was used for comparison of clinical characteristics between groups of patients.

RESULTS

From all the medical records for the period 2015-2017, early ischemic heart disease was revealed in 89 patients. Average age of the patients included in the analysis was 49±4.5 years. Evaluation by clinical criteria allowed to suspect the presence of familial hypercholesterolemia in more than 14.8% of patients with early ischemic heart disease. Upon that, among these patients, 2 (2.2%) had definite diagnosis; 27 (30.3%) were likely to have diagnosis, 26 (29.7%) had possible diagnosis and in 34 (38.2%) patients it was unlikely to diagnose them with familial hypercholesterolemia. The distribution of patients as per familial hypercholesterolemia diagnosis criteria is shown in Fig. 1.

Investigators compared the groups with different degrees of diagnosis probability on the basis of disease course, age and risk factors (Table 2).

The patients with definite diagnosis of familial hypercholesterolemia were younger than patients with probable, possible and unlikely diagnosis. Smoking and hypertension were observed significantly more frequently among patients with unlikely diagnosis of familial hypercholesterolemia (Table 3).

It is additionally notable that among the patients included in this study “familial hypercholesterolemia” was not mentioned anywhere in their diagnosis.

Table 1. Clinical criteria for identification patients with familial hypercholesterolemia (Dutch clinics criteria - Dutch Lipid Clinics Network Criteria)

Family history	
A. First degree relatives with known premature (men < 55 y.o.; women < 60 y.o.) coronary or vascular disease or first degree relatives with known LDL above the 95th percentile	1
B. First degree relatives with tendiunous xanthomata and/or arcus cornealis or children < 18 y.o. with LDL above the 95th percentile	2
Clinical history	
A. Patients with premature (men < 55 y.o.; women < 60 y.o.) coronary artery disease	2
B. Patients with premature (men < 55 y.o.; women < 60 y.o.) cerebral or peripheral vascular disease	1
Physical examination	
A. Tendinous xanthomata	6
B. Arcus cornealis before 45 y.o.	4
LDL-C level	
A. LDL-C > 8,5 mmol/l	8
B. LDL-C 6,5 – 8,5 mmol/l	5
C. LDL-C 5 – 6,4 mmol/l	3
D. LDL-C 4 – 4,9 mmol/l (normal TG)	1
DNA analysis	
A. Functional mutation in the LDL-receptors, ApoB or PCSK9 gene	8
Use onle one score of groups, the highest applicable Diagnosis (diagnosis is based on total number of points obtained)	
‘Definite’ FH diagnosis requires	>8 points
‘Probable’ FH diagnosis requires	6 – 8 points
‘Possible’ FH diagnosis requires	3 – 5 points

Table 2. Characteristics of patients with early ischemic heart disease.*

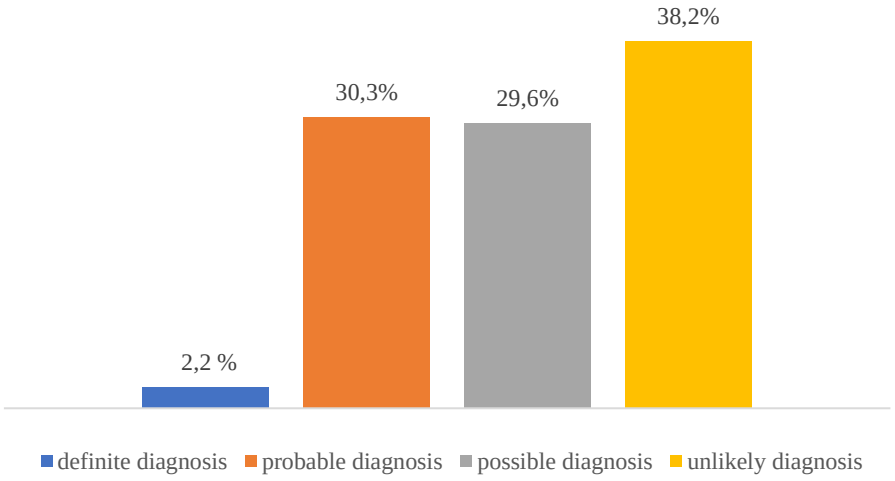
	Definite diagnosis n=2 (2,2 %) group 1	Probable diagnosis n=27 (30,3%) group 2	Possible diagnosis n=26 (29,7 %) group 3	Unlikely diagnosis n=34 (38,2 %) group 4	p
Age, years	37/39	41,2±5,7	51,4±6,3	50,3±8,7	P_{2-3}=0,001 P_{2-4}=0,001 p_{2-3}=0,588
Female, n (%)	1 male/ 1 female	17 (62,9%)	19 (73,1%)	4 (11,7%)	P_{2-3}=0,430 P_{2-4}=0,0001 P_{3-4}=0,0001
Smoking (%)	0	4 (14,8%)	5 (19,2%)	14 (41,7%)	P_{2-3}=0,669 P_{2-4}=0,025 P_{3-4}=0,070
Hypertension (%)	0	9 (33,3%)	11 (42,3%)	26 (76,5%)	P_{2-3}=0,500 P_{2-4}=0,002 p_{-3-4}=0,007
History of myocardial infarction (%)	0	12 (44,4%)	11 (42,3%)	19 (55,8%)	P_{2-3}=0,875 P_{2-4}=0,375 P_{3-4}=0,297
Clinically significant coronary atherosclerosis (%)	2	10 (37,1%)	7 (26,9%)	7 (20,5%)	P_{2-3}=0,430 P_{2-4}=0,155 P_{3-4}=0,565
Revascularisation (%)	0	4 (14,8%)	6 (23,1%)	2 (5,8%)	P_{2-3}=0,676 P_{2-4}=0,465 P_{3-4}=0,059
Stable angina (%)	2 (100%)	15 (55,5%)	15 (57,7%)	15 (44,1%)	P_{2-3}=0,875 P_{2-4}=0,375 P_{3-4}=0,297

*comparison was conducted between groups 2, 3 and 4, since in the first group there were only two patients.

Table 3. Values of cholesterol and its fractions in groups of patients with early ischemic heart disease.

	Definite n=2 (2,2 %) group 1	Probable n=27 (30,3%) group 2	Possible n=26 (29,7 %) group 3	Unlikely diagnosis n=34 (38,2 %) group 4	P
TC mmol/l	14,1/13,11	8,48±3,11	7,52±2,86	6,11±2,13	P_{2-3}=0,248 P_{2-4}=0,001 P_{3-4}=0,033
LDL – C, mmol/l	11,77/11,0	6,40±2,15	5,43±1,32	3,91±0,95	P_{2-3}=0,054 P_{2-4}=0,0001 P_{3-4}=0,0001
VLDL – C, mmol/l	0,62/0,42	1,07±0,38	0,76±0,29	1,12±0,35	P_{2-3}=0,002 P_{2-4}=0,596 P_{3-4}=0,0001
HDL –C, mmol/l	1,72/1,34	1,14±0,35	1,45±0,60	0,90±0,34	P_{2-3}=0,025 P_{2-4}=0,009 P_{3-4}=0,0001
TG, mmol/l	3,81/2,14	2,44±0,98	1,71±0,45	2,13±0,67	P_{2-3}=0,001 P_{2-4}=0,148 P_{3-4}=0,008

Fig. 1. Distribution of patients as per diagnosis of familial hypercholesterolemia based on Dutch Lipid Clinics Network Criteria



DISCUSSION

Clinical investigations have demonstrated that patients with early ischemic heart disease are also frequently diagnosed with familial hypercholesterolemia and doctors should therefore also consider this condition. Accordingly, further observation by family physicians has been conducted with slight alertness towards family members of the patients, and due to this, further examination of relatives was not performed and chances of disease were excluded. It has been shown that a diagnosis of familial hypercholesterolemia can increase patients' compliance with a healthy life style (7) and thus potentially improve outcomes.

Recent research has considered the incidence of familial hypercholesterolemia in the general population. Although studies were conducted in different ethnic groups and using different clinical criteria, most studies have demonstrated relatively high level of early ischemic heart disease among patients with known familial hypercholesterolemia. According to the United States National health and Nutrition Surveys, a personal history of early atherosclerotic disease was found in 59.1% of patients with a probable diagnosis and in 47.8% of patients with a definite diagnosis (8).

In addition, several studies have also showed that the frequency of familial hypercholesterolemia was significantly higher among the patients with ischemic heart disease than in the general population.

In our study, the incidence of familial hypercholesterolemia was 14.8% among patients with early ischemic heart disease. The reported prevalence of comorbidity varied according to the definition criteria. According the criteria of the American Heart Association, 2.5% of all patients with ischemic heart disease had familial hypercholesterolemia compared to 5.5% using Simone Broom's definition, and 1.6% patients according to the criteria of the Dutch Lipid Clinic (4). Among patients with stable coronary artery disease, 3.3% were diagnosed as having familial hypercholesterinemia (9).

The rate of familial hypercholesterolemia in our research was closer to the reported EUROASPIRE IV results. This registry enrolled only those patients with a diagnosed coronary pathology. Among 7044 patients included in EUROASPIRE IV, the prevalence of potential (considered to be both probable and possible) familial hypercholesterolemia was 8.35% in men and 11.4% in women (10). Faggiano P. et al. found that among patients that had survived acute coronary syndrome, 75% had a definite diagnosis of familial hypercholesterolemia and 60.9% of patients had a probable diagnosis. Among patients with stable coronary heart disease, a definite diagnosis of familial hypercholesterolemia was confirmed in 22.2% of the sample, and 29.1% had a probable diagnosis of the disease (11). In our study, the proportion of patients with a possible or probable diagnosis of familial hypercholesterolemia was significantly higher than these reported rates. According to our data, among patients with stable angina, 57.4% had a probable and 55.3% had a possible diagnosis of familial hypercholesterolemia. Furthermore, compared to the results of Nanchen et al. and Faggiano et al., our research suggests a significantly higher incidence of familial hypercholesteremia (4, 11). This high incidence of the disease in our study may be due to the fact that our study only included patients with early ischemic heart disease, while in the studies cited above, the data of patients with all forms of the condition were analyzed. The results reported by Cao et al. demonstrated that among patients with very early onset of coronary heart disease 38.1% had familial

hypercholesterolemia due to pathogenic mutations. According to the criteria of the Dutch Lipid Clinic Network, 26.7% of patients had a probable and 15.2% had a definite diagnosis of familial hypercholesterolemia (12). The data presented by Cao et al. are most similar to our results. Both studies included only patients with early ischemic heart diseases.

A relevant observation was presented by Pérez et al. in an analysis of a population of patients with molecularly defined familial hypercholesterolemia from the Spanish Familial Hypercholesterolemia Cohort Study (SAFEHEART). The median age of Spanish patients was similar to those of our patients who had a probable diagnosis (44,0 and 41,3). According to this study, atherosclerotic cardiovascular disease was present in 13.0% of patients with familial hypercholesterolemia and in only 4.7% of their unaffected relatives. The main difference between our study and Spanish registry was the age at which ischemic heart disease was diagnosed. The patients with confirmed ischemic heart disease included in our study were younger than patients with first manifestation of atherosclerotic events in the Spanish registry. In our study, the mean age of patients with early ischemic heart disease and probable familial hypercholesterolemia was 41,2±5,7 years old and in the Spanish study first manifestation of atherosclerotic events was at the age of 46,4±10,7 in male patients and 52±14,3 in female patients. One of the most controversial finding of the Spanish study was that familial hypercholesterolemia did not influence the rate of premature atherosclerotic cardiovascular disease. A premature atherosclerotic event was observed in 22.4% of patients with confirmed familial hypercholesterolemia and in 20.7% of their unaffected relatives (p=0,28). The authors rationalized this anomaly by suggesting that the relatively high prevalence of other cardiovascular risk factors was different from LDL-cholesterol in unaffected relatives (7).

Alternatively, the observed difference may be explained by the fact that familial hypercholesterolemia phenotype is highly variable, possibly due to both environmental and genetic factors (13, 14). Durst et al. suggested that this may be a result of the polygenic nature of the disorder, which, of course, can cause various clinical manifestations of the disease (15). Despite the recent emergence of modern methods of genetic analysis, it may still be impossible to determine the key mutation in all the cases. A pathogenic familial hypercholesteremia - causing mutation was detected in 30% of 885 patients tested. Elevated LDL-cholesterol and a personal or familial history of tendon xanthomata were independent predictors of a mutation (ORs range 5.32-15.2, P<0.001) (5).

Among patients with unlikely diagnosis of familial hypercholesterolemia, women prevailed significantly, and there were also significantly more patients with hypertension and smokers. Also, patients with unlikely diagnosis had significantly higher values of total cholesterol, LDL cholesterol and HDL cholesterol. Given that a larger number of patients with unlikely diagnosis of ischemic heart disease were females, it should be assumed that their disease had other development mechanisms, and it was not always due to high cholesterol (16). It should be noted that the prognosis in familial hypercholesterolemia may differ in men and women. Slack et al. in their study demonstrated that the risk of the first event differed in men and in women with family hypercholesterolemia. Results of this study demonstrated that the chances of fatal or nonfatal CHD for men and women were 5.4 and 0% by the age of 30, 51.4 and 12.2% by the age of 50, and 85.4 and 57% by the age of 60, respectively (17). Patients with likely diagnosis were significantly younger.

Summarizing the data, the rate of probable familial hypercholesterolemia among patients with early ischemic coronary disease was higher than 30%. These results demonstrated the needs of genetic confirmation and family members screening when suspected case of familial hypercholesterolemia is detected. Knowledge and perception of the diagnosis encourage patients to healthier life style.

CONCLUSION

Thus, this work demonstrated that despite frequent occurrence of familial hypercholesterolemia, doctors' alertness towards this disease has been noted to be quite low in Ukraine. It can be recommended to check all patients with early onset of ischemic heart disease in accordance to criteria of familial hypercholesterolemia. Development of the national register of patients with familial hypercholesterolemia is a crucial step in order to improve the situation with medical help to this group. Such register will allow to plan genetic testing in families where are members with familial hypercholesterolemia and treatment with statins and proprotein convertase subtilisin/kexin type 9 inhibitors.

CONFLICT OF INTEREST

Not declared.

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MAGNESIUM IN IDIOPATHIC MITRAL VALVE PROLAPSE

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UPOTREBA MAGNEZIJUMA KOD IDIOPATSKOG PROLAPSA MITRALNOG ZALISKA

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ABSTRACT

The aim of our research was to increase the effectiveness of the therapy administered to the patients with idiopathic mitral valve prolapse by pharmacological correction of magnesium deficiency. 79 patients (23 females and 56 males with average years of age 35.7±4.3) with undifferentiated connective tissue dysplasia and mitral valve prolapse of the 1st and 2nd degree were examined. The control group consisted of 20 healthy individuals, comparable by sex and age. A test by the UNESCO Institute for Microelements was used for the preliminary diagnostics of magnesium deficiency. Daily ECG monitoring with heart rate variability analysis, echodopplercardiography with the assessment of left ventricular diastolic function and determination of magnesium concentration in blood serum were performed. For the demonstration of autonomic dysfunction "the test for detection of the signs of vegetative changes" was used (10). For the assessment of situational and personal anxiety an "anxiety test" by Ch. D. Spielberg and Y. L. Hanin (25, 26) was used. The succeeding study was performed after 6 months. It was found that complex therapy with magnesium orotate in patients with idiopathic mitral valve prolapse helps to reduce the frequency of clinical manifestations of neurovegetative disturbances in the majority of examined patients contributing to harmonization of the autonomic nervous system function. It has a favorable effect on dysplastic changes and the state of bioelectrical activity of the heart, as well as correction of the psychoemotional state.

Keywords: *valvular disease, magnesium deficiency, treatment.*

SAŽETAK

Cilj našeg istraživanja bio je povećanje efikasnosti terapije koja se primenjuje kod pacijenata sa idiopatskim prolapsom mitralnog zaliska farmakološkom korekcijom nedostatka magnezijuma. Ispitivano je 79 pacijenata (23 žene i 56 muškaraca, prosečne starosne dobi 35,7 ± 4,3 godina) sa nediferenciranom displazijom vezivnog tkiva i prolapsom mitralnog zaliska I i II stepena. Kontrolnu grupu činilo je 20 zdravih pojedinaca, uporedivih po polu i starosti. Test UNESCO Instituta za mikroelemente korišćen je za preliminarnu dijagnostiku nedostatka magnezijuma. Vršeni su dnevni EKG monitoring sa analizom varijabilnosti srčanog ritma, ehokardiografija sa procenom dijastoličke funkcije leve komore i određivanje koncentracije magnezijuma u serumu. Za demonstraciju autonomne disfunkcije korišćen je „test za otkrivanje znakova vegetativnih promena" (10). Za procenu situacione i lične anksioznosti korišćen je Ch. D. Spilbergov i Y. L. Haninov „test anksioznosti" (25, 26). Naredna studija izvršena je nakon 6 meseci. Utvrđeno je da složena terapija magnezijum orotatom kod pacijenata sa idiopatskim prolapsom mitralnog zaliska pomaže da se smanji učestalost kliničkih manifestacija neurovegetativnih poremećaja kod većine pregledanih pacijenata koja doprinosi usklađivanju funkcije autonomnog nervnog sistema. Terapija povoljno utiče na displastične promene i stanje bioelektrične aktivnosti srca, kao i na psihoemocionalno stanje.

Ključne reči: *oboljenje valvule, manjak magnezijuma, lečenje.*

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INTRODUCTION

Mitral valve prolapse (MVP) or mitral valve prolapse syndrome is considered to be one of the most common cardiac valve anomalies. The results of population-based studies on prevalence of MVP are inconsistent. In the Framingham Heart Study (1) the prevalence of MVP syndrome in the population of 26 to 84 years of age (average age 56.7±1.5) was 2.4% with no differences in sex and age. The maximum prevalence of this pathology (17-38%) was observed in women (twice as often as men) and young people (2). Moreover, severe mitral regurgitation was observed in males with MVP over 50 years old more often than in young women with this pathology. It was demonstrated that the frequency of MVP detection with young people (18-27 years old) starts from 4.3% to 8.1% and increases with athletes up to 11-18% (2, 3).

At present, there is no universal terminology and classification of MVP. It is generally accepted to classify MVP by etiology as primary (idiopathic, congenital) and secondary one (4). Primary MVP should be considered in the context of genetically determined mesenchymal anomaly and respectively within the nosological frame of undifferentiated connective tissue dysplasia (CDT). The conventionality of the term "primary" or "idiopathic" in connection to MVP should be mentioned. Pathogenetically, it is associated with a specific cause – a congenital generalized defect of connective tissue. In addition, mitral valves in differentiated hereditary syndromes and undifferentiated CDT, differing etiologically, are virtually identical in pathogenesis. Secondary MVP is found in ischemic heart disease, chronic rheumatic heart disease, myocarditis, hypertrophic cardiomyopathy, congenital heart disease, etc.

MVP may appear as a clinically mild "phenomenon of echocardiography", as clinically significant complication occur in 2-4% of cases, and almost in the absolute case (95-100%) in the presence of myxomatous degeneration of the valves, i.e. in MVP syndrome (5). Sudden death is a rare complication of MVP, occurring in less than 2% of patients with MVP during prolonged follow-up with an annual mortality of less than 1% (6). In most cases, sudden cardiac death in MVP is of arrhythmogenic genesis and is caused by the occurrence of idiopathic ventricular tachycardia (fibrillation) or in the QT prolonged interval syndrome (7). The risk factors for sudden cardiac death in patients with MVP are the presence of severe mitral regurgitation and LV (left ventricle) systolic dysfunction.

Particular importance in the development of CTD is the deficiency of magnesium, which leads to disruption of the formation of connective tissue structures of the supporting and trophic carcass of the heart. This causes chaotic distribution of collagen fibers, disturbance of collagen synthesis and its biodegradation. Thus, in conditions of magnesium deficiency, fibroblasts produce incomplete collagen of the mitral valve flaps (8). On the other hand, magnesium deficiency leads to an increase in the total activity of matrix metalloproteinases and more aggressive degradation of collagen fibers, which also worsens mechanical resistance of connective tissue (9). We should not forget that the disturbance of the structure and function of connective tissue in MVP affects not only the chordal and valvular apparatus of the valve, but also the connective tissue stroma of the myocardium. In some cases, this leads to the disruption of the synchronicity of contractions of both separate muscle fiber groups and the whole myocardium, and possibly it leads to a decrease in its inotropic reserve, remodeling and, as a result, to the manifestation of heart failure signs. The problems of the relevant treatment and methods of correction of idiopathic MVP remain poorly studied. In connection to this, our goal was to increase the effectiveness of the therapy for the patients with idiopathic prolapse of the mitral valve via pharmacological correction of magnesium deficiency.

MATERIALS AND METHODS

The study involved 79 patients of 18-40 years of age with MVP and phenotypic signs of undifferentiated CTD (Table 1). According to echodopplercardiography, MVP of the 1st degree was diagnosed in 46 patients and MVP of the 2nd degree in 33 patients. Mitral regurgitation was not found in 12 patients. It should be mentioned that 37 patients had mitral regurgitation of the 1st degree and 30 patients had mitral regurgitation of the 2nd degree.

The initial examination was made by applying a specially prepared test including a detailed collection of complaints, anamnesis and physical examination for phenotypic signs of undifferentiated CTD.

Inclusion criteria: males and females ≥18 years old; presence of idiopathic mitral valve prolapse; informed patient consent to participate in the study.

Table 1. Clinical characteristics of the examined patients

Index	General group (n=79)
Average age, years	35.7±4.3
Males/Females	56/23
MVP I degree/II degree	46/33
Mitral regurgitation	
I degree/II degree	37/30
Smoking, n (%)	34
Family history of early cardiovascular events, n (%)	21
BMI (body mass index), kg/m²	24.3±1.6
Height, cm	171.5±1.8

Exclusion criteria: foci of chronic infection, congenital or acquired heart disease, degenerative-inflammatory myocardial lesions, hemodynamic disturbances, thyroid gland pathology, coronary heart disease, arterial hypertension, concomitant diseases of internal organs, differentiated forms of

CTD (Marfan syndrome, etc.); patients younger than 18 years of age.

The control group consisted of 20 healthy individuals without CTD, comparable by sex and age (10 males and 10 females, average age 35.3±4.6) without morphofunctional features of the heart structure according to echocardiography study.

All examined patients underwent standard clinical, biochemical and instrumental studies. For the detection of autonomic dysfunction "the test for detection of the signs of vegetative changes" was used (10). The sum of the scores, if equal to or more than 15, suggested the presence of autonomic dysfunction.

Daily ECG monitoring was performed with the "CARDIOSENS" equipment ("XAI-Medica", Ukraine). Cardiac arrhythmias and conduction disorders, coronary insufficiency and heart rate variability (HRV) were assessed (11). The registration and automated processing of ECG signals were made by the calculation of the parameters of time and spectral analysis, as well as by the indexes obtained on their basis and suggested by P.M. Baevsky (12). For the analysis of vegetative regulation, the following parameters were used:

TI is the tension index of regulatory systems (TI=AMo/2×BP×Mo), where Mo (mode) is the most frequent value of RR, AMo (mode amplitude) is the number of cardio intervals corresponding to the mode range (in %); VR (variation range) is the difference between the maximum and minimum values of RR;

SDNN is a standard deviation in the duration of normal intervals R-R; pNN 50 is the percentage of all analyzed cardio intervals; RMSSD is the square root of mean squares of the difference between adjacent RR-intervals – activity index of parasympathetic link of vegetative regulation. The higher is the value of RMSSD, the more active is the link of parasympathetic regulation (12).

LF/HF is the index of the vagosympathetic interaction, – the ratio of high-frequency and low-frequency components of the heart rhythm. It indicates the change of vegetative balance to the sympathetic or parasympathetic division.

Structural and functional parameters of the left ventricle were assessed using echodopplercardiography (EchoCG) with the ultrasound scanner "Vivid 3" (Japan) and a 3.5MHz probe in prone position on the left side from the parasternal and apical four-chamber views.

The following indices of EchoCG were assessed: aortic diameter, aortic, mitral (MV), tricuspid valve opening amplitude, the hole area of all these valves. Morphometry and evaluation of mitral valve function were performed in M-mode in the standard position II; in the mode of two-dimensional echocardiography – in the parasternal projection of the LV long axis and the LV transverse axis at the level of the mitral valve; and in the apical four-chamber position. EchoCG showed that the sign of mitral valve prolapse was the displacement of the valve (s) to the left atrial cavity by more than 3mm. The systolic deflection of one or both valves of the MV (mitral valve) in the LA (left atrium) in the parasternal longitudinal position by 3.0-5.9 mm is defined as I degree MVP, by 6-8.9 mm – II degree MVP and by more than 9 mm – III degree. Normal values of the MV anterior cusp length were taken as 21-24 mm and of the posterior one as 12-14 mm.

The degree of the severity of myxomatous degeneration was assessed on the ground of the thickness of the MV leaflet during the diastole phase in the middle part outside the chord zone, creating a false impression of its thickening. Common standards for leaflet thickness are 2-4 mm; an increase by more than 5 mm indicates a pathological change (myxomatosis, etc.) (4); by less than 5 mm – non-classical MVP and by 5 mm or more – classical MVP.

The morphology of the valve apparatus, as well as the presence and extent of regurgitation were evaluated. During the assessment of the degree of regurgitation on the mitral valve, the LA depth, the area of mitral regurgitation, the percentage ratio of the jet area, and the LA area were taken into account (13).

LV dimensions and volumes, LV stroke volume and ejection fraction, the thickness of the LV posterior wall and the interventricular septum were also measured during EchoCG examination. Disturbances in the LV local contractility were outlined by the recommendations of the American Society of Echocardiography. The type of LV architectonics was determined by the following parameters: myocardial mass, myocardial index, relative wall thickness, and sphericity index. It was mandatory to determine the size of the LA, RV (right ventricle) and RA (right atrium) cavities, pericardial condition and pressure in the pulmonary artery. The character and flow rate on the valves in systole and diastole pressure gradient were determined with Doppler echocardiography. The diastolic function was assessed by transmittal flow in the pulse-wave Doppler mode, as well as by the analysis of the motion of the fibrous ring of the mitral valve by the method of tissue Doppler imaging. In the Doppler study, LV diastolic function was evaluated according to the time of isovolume relaxation, the deceleration time for the early LV diastolic filling (DT), the maximum rate of LV early filling (peak E), the maximum rate of the atrial systole A and the E/A ratio.

For the assessment of situational and personal anxiety, an "anxiety test" by Ch. D. Spielberg (1973), adapted by Y. L. Hanin (containing 40 questions), was used. The result was assessed as follows: up to 30 points – low anxiety, 31-45 points – moderate anxiety, 46 points and more – high anxiety.

For the preliminary diagnostics of magnesium deficiency, a test by Trace Element Institute for UNESCO was used. The test results were read as follows: 0-9 points – no magnesium deficiency, 10-19 points – risk group for magnesium

deficiency, 20-29 points – moderate magnesium deficiency, 30-39 points – magnesium deficiency, 40-56 points – significant magnesium deficiency (14). The concentration of magnesium in blood serum was evaluated with the automatic biochemical analyzer "Humalyzer 2000" (Germany, the range of normal oscillations is 0.85-1.2mmol/l).

After initial screening, the patients were randomly divided into 2 groups: 39 patients (group 1) received complex therapy including β-adrenoblocker and magnesium orotate 500 mg 3 times a day for 6 months. The second group included 40 people who received monotherapy with a β-blocker. Its administration was preconditioned by the presence of clinical signs of an increase of the sympathetic nervous system tone (cardialgia, palpitations, irregular heart function, dyspnea, etc.) in all examined patients. These groups of patients with MVP were comparable by age, sex and the presence of magnesium exchange disorders. The follow-up study was performed after 6 months of observation.

The effectiveness of the therapy in each patient was assessed as clinically significant with a decrease of the severity (in points) of the analyzed parameters by 50% or more from the baseline.

Statistical processing of the results was made with the program Statistica 6.0. For the quantitative indices measured on an interval scale the mean value, standard deviation and mean error were calculated. For "qualitative" and "ordinal" indices, the index detection frequency in percents and the frequency of registration of different index rank score respectively were defined. The Student's t-test was used in the analysis of the inter-group index differences. In case of the indices measured at the nominal scale, the reliability of the differences in the frequency of index detection in two compared groups was assessed by the Student's t-test and the Fisher transform, linear correlation coefficients and rank correlations were calculated. The reliability of the relationship between the indices measured on a nominal or rank scale was further evaluated using contingency tables – with the calculation of several modifications of the Pearson chi-square test and Cramer's conjugacy coefficients. Differences in mean values and correlations were considered reliable at a significance value of $p<0.05$.

RESULTS AND DISCUSSION

Undifferentiated CTD is characterized by the polymorphism of anomalies of disembriogenesis ("stigma"), which are represented in a phenotype with different frequencies. During the analysis of external phenotypic features of the patients with MVP the most informative were CTD markers, shown in Table 2.

It has been previously observed that different clinical symptoms in MVP patients also depend on magnesium deficiency (3, 14). The study of some aspects of magnesium exchange and its influence on the dynamics of the MVP course has been of great importance. In the analysis of clinical signs of magnesium deficiency it was observed that in the patients of groups I and II, moderate deficiency of magnesium was diagnosed in 74.4% and 70%, the risk of magnesium deficiency development was recorded in 15.4% and 20%, and the signs of magnesium deficiency were absent in 10.2 % and 10%, respectively. Consequently, the majority of patients with MVP of 1st and 2nd degree exhibited clinical signs of magnesium deficiency of varying severity . Differences were statistically significant comparing to the control group – $p<0.01$. During the evaluation of magnesium concentration in blood serum, hypomagnesemia was diagnosed in 82% (32 patients) of group I and 80% (32 patients) of group II. Consequently, the values of serum magnesium were within normal limits in 18% (7 patients) in group I and in 20% (8 patients) in group II.

Table 2. Prevalence of external phenotypic markers in patients with idiopathic MVP (n=79)

Phenotypic markers	Detection frequency,%
Ectomorphy	67.0
Hypotrophy	54.4
Radial-lacunar type of the iris	54.4
The predominance of the length of the 4th hand digit over the length of the 2nd one	50.6
Varicose veins of the lower extremities, developed in adolescence	46.8
Scoliosis	41.8
Chest deformation	39.2
The predominance of the length of the 2nd toe above the length of the 1st one	32.9
Curved little fingers	29.1
Platypodia	29.1
Protruding ears	25.3

The concentration of magnesium in blood serum is the most commonly used marker of magnesium exchange in the body (15). However, the level of magnesium in the serum provides only proximate information about the presence or absence of magnesium deficiency. Hypomagnesemia clearly indicates magnesium deficiency, but its absence does not exclude significant magnesium deficiency in tissues. The concentration of magnesium in the blood serum is not associated with the content of this trace element in other biomaterials (16).

It is expected that in the pathogenesis of diverse clinical symptoms and signs in patients with primary MVP, a leading role is played by the disturbances in the function of the autonomic nervous system with an increase of the sympathetic tone. The predominance of adrenergic effects in MVP is associated both with an increase in the sensitivity of adrenoreceptors to stimulation and with an increase of their total number (17). Changes in vegetative homeostasis are so common in patients with primary MVP that most researchers consider

it an obligate manifestation of this pathology (2, 3). The manifestations of autonomic dysfunction were observed in 75 (94.9%) patients (Table 3).

Table 3. Prevalence of manifestations of autonomic dysfunction in patients with idiopathic MVP (n=79)

Symptoms	Detection frequency,%
Heartache	94.9
Heart palpitations and disturbances	73.4
Headache	74.7
Dizziness	73.4
Hyperventilation syndrome	63.3
Dysfunction of the gastrointestinal tract	45.6
Raynaud's syndrome	37.9
Disturbances of thermoregulation	27.8
Syncope	15.2

At baseline, the examined patients with MVP showed average score on the "test for the signs of vegetative changes" of 45.9±2.1 points whereas the scores of healthy individuals reached 12.3±2.3 ($p<0.001$) points. The study of vegetative homeostasis during the analysis of HRV (heart rate variability) showed the prevalence of simpathicotonia in 70.9% of the examined patients. The data obtained confirm the significant contribution of the autonomic nervous system disorders to the structure of the main clinical manifestations of idiopathic MVP.

The autonomic nervous system is currently being considered to play an important role in the emergence of various heart rhythm disorders (2). It is known that the parasympathetic link inhibits negative adrenergic effects on the heart (18). Decreased vagal activity and/or increased sympathetic activity may lead to the development of prognostically unfavorable cardiac rhythm disorders (19).

Analysis of HRV parameters at the baseline allowed us to diagnose the presence of vegetative disorders in the examined patients with MVP.

At baseline, in patients with MVP, the mode amplitude twice exceeded the results of healthy individuals, the stress index –was increased 3.5 times (all $p<0.001$), and the variation range was reduced by 1.4 times ($p<0.05$) indicating the prevalence of sympathetic activity in the autonomic nervous system. In addition, compared with the control group, a significant decrease in the total heart rhythm variability (SDNN) by 1.3 times and a decrease of the parasympathetic component of cardiac rhythm regulation (RMSSD) by 1.3 times (all $p<0.001$) were observed in patients with MVP. The predominance of sympathetic influences over vagal ones in patients with idiopathic MVP probably indicates an initially high level of adrenergic stimuli in this pathology.

One of the common symptoms of CTD is arrhythmic syndrome. Pathogenetic factors of cardiac arrhythmias are myxomatous degeneration of the conduction system of the heart and valves, especially the posterior one, as well as mitral regurgitation. In the genesis of supraventricular arrhythmias, a special emphasis is placed on the stimulation of the subendocardial areas of the left atrium with regurgitating blood stream, leading to the development of the foci of ectopic excitation. Atrial fibrillation usually develops in patients with atriomegaly caused by hemodynamically significant mitral regurgitation. Among the causes of ventricular rhythm disturbances, hypersympathicotonia, i.e. an abnormal traction of papillary muscles, (20) is taken into consideration.

The existence of a causal relationship between ventricular and atrial arrhythmias and intracellular magnesium content has been established (14). It is expected that hypomagnesemia may contribute to the development of hypokalemia (21). In this case, the membrane resting potential is increased, the processes of depolarization and repolarization are interrupted and the cell excitability decreases. The conductivity of electric impulse slows down contributing to the development of arrhythmias (14, 21). In addition, intracellular magnesium deficiency increases the activity of the sinus node, reduces absolute refractoriness and extends a relative one (22).

Detection frequency of various types of cardiac rhythm and conduction disturbances in the examined patients according to daily ECG monitoring is presented in Table 4.

The inclusion of magnesium orotate in complex therapy for 6 months in patients with idiopathic MVP of the 1st and 2nd degree led to a significant increase of magnesium content in blood serum from 0.61±0.02mmol/l to 0.97±0.03mmol/l ($p<0.001$). Moreover, magnesium concentration in blood serum in patients of group I did not differ significantly from the control group, which apparently indicates the compensation of magnesium deficiency in the studied patient population. At the same time, in the patients of group II no significant changes in magnesium content of blood serum were observed after therapy.

After the treatment, there was a significant ($p<0.05$) decrease in the frequency of clinical manifestations of neurovegetative disorders in the majority of the patients examined. Assessing the effect of magnesium therapy on symptomatology and the severity of all clinical manifestations in patients with MVP, it is necessary to emphasize the significant improvement of the general state of patients and reduction in the frequency and severity of all clinical syndromes and symptoms of the disease.

Table 4. Changes in the findings of daily ECG monitoring on treatment

Indicators	Group II (n=39)		Group II (n=40)	
	Initially	After treatment	Initially	After treatment
Heart rate, bpm	83.6±3.6	68.8±2.3**	82.7±3.2	73.2 ± 2.3*
Supraventricular arrythmia	32.8±10.7	8.9±4.9*	34.6±10.3	27.3±4.6
Ventricular arrhythmia	198±13.8	26±11.6**	179±11.3	44±10.5**
Paroxysmal supraventricular tachycardia,%	7.7	0	12.5	7.5
Blockade of the right leg of the bundle of His,%	30.7	30.7	30.0	30.0
Syndrome of early repolarization of ventricles,%	33.3	0*	35	35

* – statistical significance in comparison to the original data, $p<0.05$; ** – $p<0.001$.

At the same time, the most significant was the dynamics of asthenic complaints ($p<0.05$) of cardialgia, palpitations, cardiac disruptions, headaches, dizziness; tolerability of moderate physical activity ($p<0.05$) in comparison with the patients of group II also improved. Clinically significant decrease in the severity of vegetative dystonia syndrome was observed in 69.2% of patients during the course of magnesium orotate and in 47.5% in the comparison group ($p<0.05$).

Analysis of the effectiveness of drug therapy in the patients with autonomic dysfunction showed positive dynamics of clinical status. The data on A.M. Wayne (10) self-evaluation scale of general state (16) showed that the sum of scores in group I decreased from 45.9±2.4 to 16.8±2.1 points ($p<0.001$) and from 45.8±2.2 to 29.8±2.1 ($p<0.001$) points in group II. The reduction of the total score by 50% after the treatment was considered a positive result. The sum of scores decreased in group I by 63.4% and in group II by 34.9% (all $p<0.001$) indicating a significant decrease of vegetative signs during the administration of complex therapy with magnesium orotate.

After the treatment the patients of both groups showed a decrease in the rate of sympathetic activity. Thus, the stress index in group I decreased at 67.7% ($p<0.001$) and in group II – at 47.3% ($p<0.001$); the mode amplitude – by 33.6% ($p<0.001$) and 16.5% ($p<0.01$); the variation range increased by 64.3% ($p<0.01$) and 38.4% ($p<0.01$), respectively, indicating an improvement in vegetative tone in the patients of group II and reactivation of vegetative balance in group I. In patients with MVP, who additionally received complex therapy with magnesium orotate, a significantly better result was observed in comparison with the experimental group on the stress index (by 62.7%, $p<0.001$) and the variation range (by 21.6%, $p<0.05$). Thus, in the group which additionally received magnesium orotate, harmonization of the function of the autonomic nervous system was observed.

General heart rate variability (SDNN) and parasympathetic regulation of the cardiovascular system (RMSSD) increased simultaneously. In particular, the SDNN index, representing the overall effect of the autonomic regulation of blood circulation, increased in the patients of group I by 27.3% ($p<0.01$), and in group II – by 8.8% ($p>0.05$).. The RMSSD index indicating the activity of the parasympathetic link of vegetative regulation in group I increased by 27.7% ($p <0.01$) and in group II by 8.47% ($p>0.05$).. pNN 50 index showing the degree of the prevalence of the parasympathetic link in group I increased by 27.1% ($p<0.01$) and in group II – by 11.7% ($p>0.05$) (Table 4). Thus, in the patients with arterial hypertension and autonomic dysfunction, the combined therapy with magnesium orotate both led to a more significant increase in general rhythm variability and decreased activity of the sympathetic part of the autonomic nervous system and reactivation of the vegetative balance.

The indices of the structural-functional state of the left ventricle are presented in Table 5.

The analysis of the parameters of intracardiac hemodynamics (Echo-CG findings) showed that in patients of group I there was a significant decrease in the size of the left atrium in comparison with the initial data by 6.6% (all $p<0.05$). In the patients of both groups there was a slight increase in the end-systolic and end-diastolic size of the left ventricle,which was marginally more significant in the comparison group.. That corresponded to a slight increase in the stroke volume and ejection fraction of the left ventricle in group I patients, whereas in contrast in group II patients the stroke volume did not change and the left ventricular ejection fraction even slightly decreased but stayed within the normal range. The data obtained reflect the favorable effect of magnesium orotate on dysplastic changes, which conforms to the previously obtained results (23).

Table 5. Dynamics of the structural-geometric condition of LV in the examined patients during treatment

Index	Group I (n=39)		Group II (n=40)	
	initially	6 months	initially	6 months
LA (left atrial diameter), cm	3.51±0.07	3.28±0.05*	3.52±0.06	3.46±0.07
ESD (end-systolic dimension), cm	3.34±0.06	3.35±0.03	3.36±0.04	3.37±0.03
EDD (end-diastolic dimension), cm	4.72±0.05	4.74±0.03	4.74±0.06	4.76±0.04
ESV (end-systolic volume), ml	40.3±1.2	41.3±1.3	40.7±1.3	42.5±1.3
EDV (end-diastolic volume), ml	112.0±1.5	113.5±1.7	112.4±1.6	113.9±1.6
SV (stroke volume), ml	71.9±1.4	73.5±1.2	71.7±1.2	71.6±1.3
EF (ejection fraction), %	63.3±0.7	64.7±0.6	63.5±0.8	62.6±0.6
E/A (peak early filling (E-wave) and late diastolic filling (A-wave) velocities)	0.96±0.06	1.22±0.03*	0.97±0.06	1.08±0.04
Degree of mitral regurgitation	1.08±0.13	0.62±0.11*	1.09±0.16	1.03±0.11

* – statistical significance compared with the initial data $p < 0.05$.

The analysis of the diastolic function characteristics showed that after the treatment,the rate of LV early diastolic filling (peak E) increased by 14.9% ($p<0.05$) in group I and by 7.8% ($p<0.05$) in group II. The maximum rate of atrial systole A after the end of the treatment in group I decreased by 9.6% ($p<0.05$) and by 7.1% ($p<0.05$) in the patients of group II. As a result of the observed changes in these velocity streams in group I patients, the E/A peaks significantly increased by 27.1% ($p<0.05$), indicating improvement in left ventricular relaxation and an increase of the blood volume accepted in the first phase of diastole. At the same time, the increase in the ratio of E/A by 11.5% ($p<0.05$) exceeded the results of the comparison group, where the changes of this index became a tendency. Additionally, a significant ($p<0.05$) decrease in the degree of mitral regurgitation was observed in group I patients (Table 6). In general, the data obtained are the results of the improvement of the connective tissue diffusivity and its architectonics, determining the improvement of its elasticity and extensibility.

After the treatment the patients of group I (Table 5) experienced a significant reduction in the heart rate, in the number of ventricular extrasystoles and supraventricular extrasystole. The antiarrhythmic activity of magnesium orotate is apparently preconditioned by its component magnesium, a natural calcium antagonist, which has a membrane-stabilizing effect, prevents a loss of potassium by the cell, reduces the dispersion of QT interval and also weakens the sympathetic effect on the heart (24).

At baseline the patients with MVP showed an increase in anxiety levels on the scale by Ch. D. Spielberg and Y. L. Hanin, (25, 26) which is explained by the peculiarities of patients' response to the emergence of the disease and associated psychological changes, as well as premorbid features of the patients' personalities. Thus, the degree of reactive and personal anxiety was respectively (49.2±2.3) and (48.8±2.6) in group I and (48.2±2.4) and (47.7±2.6) in group II. High and moderate levels of reactive and personal anxiety were distinct for the majority of patients with MVP (Table 6).

In the patients of group II with low, moderate and high levels of reactive and personal anxiety at baseline no significant changes were observed after the treatment. At the same time, the patients of group I showed a significant decrease in the level of reactive anxiety by 36.8% ($p<0.001$) and personal anxiety by 38.6% ($p<0.001$). Moreover, magnesium orotate proved to be the most effective in the group with high and medium anxiety level, as indicated by the shift of 64.1% and 69.2% in patients reaching a lowlevel of reactive and personal anxiety respectively. At the same time, in the patients with a low degree of anxiety no significant changes were observed at baseline. The difference in the change of the levels of situational and personal anxiety in groups I and II was found to be statistically significant (39.1%, $p<0.001$ and 46.6%, $p<0.001$ respectively). Consequently, after the complex treatment with magnesium orotate the patients with MVP showed positive change of situational and personal anxiety, which indicated an improvement in the psychoemotional state of the patients.

Table 6. Changes of indices of reactive and personal anxiety in patients with idiopathic MVP (M ± m)

Index	Level	Group	Initially	After treatment
Reactive anxiety	low	I	28.2±1.3 (n=2)	28.1±1.2 (n=25)
		II	28.4±1.3 (n=2)	28.5±1.4 (n=3)
	moderate	I	42.6±2.5 (n=16)	33.9±2.3* (n=11)
		II	40.8±2.6 (n=17)	37.2±2.4 (n=18)
	high	I	56.3±2.9 (n=21)	46.1±2.7* (n=3)
		II	56.1±2.5 (n=21)	51.4±2.3 (n=19)
Personal anxiety	low	I	28.5±1.5 (n=2)	28.1±1.4 (n=27)
		II	28.6±1.6 (n=2)	28.2±1.3 (n=3)
	moderate	I	42.8±2.6 (n=17)	33.6±2.4* (n=9)
		II	42.1±2.7 (n=19)	39.3±2.3 (n=21)
	high	I	55.9±2.8 (n=20)	45.6±2.6* (n=3)
		II	55.3±2.7 (n=19)	53.1±2.3 (n=16)

* – statistical significance in comparison with the original data, p <0.05.

CONCLUSION

1. The majority of patients with idiopathic MVP of 1st and 2nd degree have clinical signs of magnesium deficiency of different severity and hypomagnesemia associated with the disturbances of autonomic regulation of the cardiovascular system in the form of relative increase of sympathetic influences and weakening of parasympathetic ones. Patients with MVP showed a decrease in the level of psychological health, manifested as a growth of the number of people with high and moderate levels of reactive and personal anxiety.
2. Complex therapy with magnesium orotate for 6 months in the patients with idiopathic MVP of 1st and 2nd degree led to the improvement of clinical symptoms and signs, to a decrease in the severity of the vegetative dystonia syndrome, toa decrease in the degree of mitral regurgitation and inthe size of the left atrium, as well as to the improvement in the diastolic function of the left ventricle along with the replenishment of magnesium deficiency according to the content of this trace element in the blood serum.
3. Administration of the combined therapy with magnesium orotate in the patients with MVP and autonomic dysfunction led to an improvement in HRV parameters and state of bioelectrical activity of the heart, as well as to a decrease in the level of reactive anxiety, which enables an increase of functional capacities of the body based on the improvement of the psychoemotional state.

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DRUG METHODS FOR ARTEFICIAL TERMINATION OF UNWANTED PREGNANCY

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MEDIKAMENTOZNE METODE VEŠTAČKIH PREKIDA NEŽELJENE TRUDNOĆE

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ABSTRACT

All medical and surgical procedures are carried out in order to premature termnination of pregnancy, can be divided on medicament and surgical methods, according to the way of procedure.

Medications used today in order to break unwanted pregnancy are inhibitors of the synthetics of progesterone and antiprogesterone, prostaglandini and antimetabolite.

Mifepristone is a derivate of norethidrone, binds to the progesterone receptor with an affinity similar progesterone, but it does not activate them so as to act as an antiprogestine.

Metotrexat is an antimetabolite and is used in gynecology practice for more indication areas. It is used the most often in conservative treatment of ectopical pregnancy. Because of low price and accessibility in order to mifepristone, it was used for application in drug methods of inducative abortions.

Misoprostol is an anlogue PGE1, used in peroral pills.

The complication are very rare at aplication of mifepristone and misoprostole in the aim to the termination the early unwanted pregnancy. The appearance of more efficient procedure of drugs called out abortions, it does not mean taht decision for the abortion is more modest. The ease and safety should not help to make a decision.

Keywords: *medical abortion, mifepristone, metotrexate, misoprostole*

SAŽETAK

Sve medicinske i hiruške procedure koje se sprovode u cilju preterminskog prekida trudnoća, mogu se prema načinu izvođenja podeliti na medikamentozne i hiruške metode.

Medikamenti koji se danas koriste u cilju prekida neželjene trudnoće su inhibitori sinteze progesterona, antiprogesteron, prostagladini i antimetaboliti.

Mifepriston je derivat norethindrona; vezuje se za progesteronske receptore sa afinitetom sličnim progesteronu, ali ih ne aktivira tako da deluje kao antiprogestin.

Metotreksat je antimetabolit i u ginekološkoj praksi se koristi za više indikacionih područja. Najčešće se primenjuje u konzervativnom tretmanu ektopičnog graviditeta. Zbog male cene i dostupnosti u odnosu na mifepriston, pokušavana je njegova primena i u medikamentozno indukovanim pobačajima.

Mizoprostol je analog PGE1, koji se koristi u obliku peroralnih tableta.

Komplikacije su izuzetno retke kod primene mifepristona i mizoprostola u cilju terminacije rane neželjene trudnoće. Pojavljivanjem sve uspešnijih procedura medikamentima izazvanog pobačaja, ne znači da je odluka o prekidu trudnoće jednostavnija. Lakoća i bezbednost ne bi trebalo da pomažu u donošenje odluke.

Ključne reči: *medikamentozni abortus, mifepriston, metotrexat, misoprostol*

INTRODUCTION

The drugs methods of arteficial abortion are defined as a use drugs in order to call out the expulsions of unwanted pregnancy. The successful drug abortion is defined as complete remove of products concept, so that instrumental revision is not necessary. The drugs used today in order to abortion, are inhibitors of the synthesis of progesterone, antiprogesterone, prostaglandine and antimetabolite.(1).

The abortion called out of mifepristone and analogue prostaglandine are very effective options for early abortion. Since 1988. years when antiprogesterone has been

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showed RU 486 (mifepristone), the million of women all around the world had possibility to have abortion without use surgical method (2).

Mifepristone and Misoprostole

Mifepriston is a derivate of norethodrone, binds for the progesterone receptors with affinity similar to progesterone, but it does not activate them so as to act as an antiprogestine (3). In pregnancy it works that it causes necrosis deciduae and detachment of the embryo affects the reduction of the inhibitory effect of progesterone on the uterine muscules and increases the sensitivity of the uterus to endogenous and exogenous prostaglandine (4,5). Also affects the structural changes in the cervix in terms softening and thus helps in the process of expulsion.

Mifepristone is easily absorbed into digestive tract, actives a high level of serum for 2 hours from the application (6). It acts as inhibitor of ovulation and affects the hypotalame-pituitary-ovarial axis, even in small doses (7). The peak of contrentation in serum is identical to all womwn who have taken increasing doses per os from 100 to 800mg (8). Misoprostol is an analog PGE1, that is used in the form of oral tablets. It is stable at room temperature. In France (9) and Great Britain(10) the researches of mifepristone and misoprostole showed that they increased uterine activity in early prgnancy.

Pharmacokinetic studies of misoprostole have shown that at peroral application, the concentration of drug in serum was greater than in vaginal application (11, 12). But vaginal application of misoprostole has better clinical effect, that explained by the fact that probably applied vaginae, has direct effect on cervix and uterus.

One of the first studies, that describe mifepristone (RU486) and misoprostole as aborifacient drugs, was published in France in 1993(13). Administered dose is 600mg mifepristone, after 36 to 48 hours practiced 400μg misoprostolefor the purpose of termination of pregnancy of 49 days of gestation (7 weeks gestation). If the expulsion has not occurred within 4 hours of application misoprostole, dose is repeated. Application of this protocol with 895 women induced abortion complete in 99% cases.

Immediately after the publication of these results was counducted in the USA multicenter study 1994. and 1995. in wich was investigated the application similar protocol but gestational period of 63 days (9 weeks of gestation) with only one per oral dose of mifepristone of 400μg. The results showed that successfully challenged the medicament abortion occurred in 92% cases of women with gestational age of pregnancy was less than 49 days, 83% pregnancy age 50 to 56 days (8 weeks of gestation) and 77% pregnancy age 63 gestation days (14).

Thanks to some other researches, FDA approved in 2000. the use mifepriston in the USA. It is also approved the use mifepristone in a dose of 600mg at pregnancy less than 49 days in hospital ad out-patient conditions with peroral use of misoprostole after 36 to 48 hours in a dose of 400μg. After approval FDA for usemifepristone and misoprostole in induction of abortion, where taken many studies about this drugs. Some authors showed that dose of mifepristone could reduce on 200mg and it could reach the same effects as with a dose of 600mg (15,16,17,18,19,20,21). After that it was turned out that the vaginal application of misoprostole was more effective than peroral drug aplication, specially in pregnancy age between 50-63 days gestation(22,23,24). Schaf at al. published that women were trained, in their studies to apply indipendently the tablets of misoprostole at home, and there isn`t important difference if misoprostole is used one, two or three days after application mifepristone (20). Their protocol is considered the practicing 200mg mifepristone, at pregnancy less than 63 gestation days, in ordination, and after in period of one , two or three days, women would apply in vagina four tablets misoprostole (800μg) at home. On the 4th and 14th day control was done with patient in ordination, as it could confirm the success of procedure. In researches done in the USA, it was always use ultrasound diagnostics as a confirmation of success procedure. It was showed useful application prescribed level of serum beta HCG-a.

Using recommended procedure, complete abortions are realized depending of authors in a span of 95% to 98%. In 1% cases are diagnosed intact pregnancy (25,26,27,28).It cites more factors that influence the high level of failure, as the greater gestational age of 50 and 63 days, less expirience of doctors, specially in the cases of ample bleeding from uterus or strong patients` pains (27,29). If the gynecologist is trained well to recognize and set the ample bleeding, side effects and regularly explains ultrasounds findings, than the level of instrumental intervention was less. Some studies showed that the women who had delivery and the women who had diltation and abortion, they have greater risk for faliure procedure than nulipar (27,30,31). It is possible that every pregnancy remains persistent changes in the cervix structure, in sense of the relationship of muscule and connective tissue components in favor of the latter.

Complications are extremely rare in the application of mifepristone and misoprostole for the purpose of early researching this combination of drugs in the USA, 80 000 women are exposed by drug abortion, but only 139 (0.19%) are registered to FDA that they had unwanted - 13 patients was carried out blood transfusions for bleding and 10 patients received antibiotics for an infection and 6 women had an allergic reaction. Fifty patients (0.063%) were diagnosed pregnancies intact and them was later conducted suction abortion. Also 50 patients was mode instrumental revision or suction abortion due bleeding.It is recorded 5 ectopic pregnancies, and 1 death for shattered tube with ectopic pregnancy (33). It is estimated that the level of less serious complication higher but they were not reported to official organs.

Bleeding and crampy expected consequences of a successful medically induced abortion and patiens should be advised of this, esperially during and immediately after the expulsion of products of conception. If the bleeding is

heavier and it is considered to be more abundant when in the course of an hour's blood and filled fwo standard napkins and it takes longer than 2 hours, then it is necessary to examine the patient and check the necessary parameters.

Side effects of a single dose of mifepristone are rare and more side effects registered during this procedure comes from the systemic effects of misoprostole, and they are usually transient and they do not require additional intervention. Of these symptoms are the most common nausea (34%-72%), vomiting (12%-41%), diarrhea (3%-26%), and higher temperature followed by chills (34).

Contraindications for drug abortion with mifepristone are: confirmed or suspected ectopic pregnancy, intrauterus napkins, chronic systemic corticosteroids application, insufficiency adrenal gland, coagulopathy, allergy to the mifepristone and hereditary porphyria. It is not clinically approved that mifepristone affects to bronchial asthma, but many patiens use corticosteroids in therapy, what is the contraindication for mifepristone application.

Contraindications for mizoprostole application are: alleggy drug, epilepsy and acute inflammatory diseases of the digestive tract. The special precantions are necessary with patiens who are at serious anemia, sever liver damage, kidney and lungs, uncontrolled hypertension and cardiovascular disease angina pectoris, valvular disease, arrhythmia or cardiac waekness.

Metotreksat-Misoprostole

Metotreksat is an antimetabolite and in gynecological practise is used for more induction area. IT more aften applies in the conservative treatmenent of ectopic preagnancy. Because of the low price and availability in relation to the mifepristone, it was attempled its application and in medically induced abortion. One dose of 50 mg per square meter of surface patient is administered orally or intramuscularly. After three to seven days it is applied 800μg misoprostole vaginal. Using this sheme, Creinin and associates are made a success in 1995 (32) on the level of 88% to 98 % depending on the size of pregnancy. The termination of pregnancy in slower, so that almost 10-30% women had complete abortion after three or four weeks. Wiebe an associates (33) showed, on the sample of 1042 women that there is not a significant difference in the rate of success at women who received combination of mifepristone and misoprostole and women who received combination of metotrksat and mizoprostole. The success of bolth procedures was about 96% and the frequency of side effects was similar. Buth, it is evident that the groub of womwn who received mifepristone, abortion accured more quickly and this is in any case more preferable for patients.

Misoprostole

Misoprostole is used alone to induce artefitial abortion. The first researches, made by patiens have shown the low level of success, what is about 5% to 11% if it's applied in dose at 400μg per per oral (34). Later studies in which misoprostole administered vaginally in a dose of 200-800μg, showing success in a range of complete abortion of 20% to 60% (35, 36). Unlike these first studies some autors as Carbonell and associates (37) and Esteve and associates (38) have shown the rate of 90% complete abortion repeated intravaginal application of moistened tablets misoprostole in total dose of 800 μg at pregnancy less than 56 of 63 gestation days. Ngai and associates (39) compared the effects of moistened intravaginal tablets misoprostole and dry, and theycame to the result that the moistened tablets lead to 85% success of abortion and to the 65% abortion, but that difference is not ststistically significant (p=0.43).

Randomized study of women over 250 that Jaina and associates (40) hare done, showed that vaginal aplication moistened misoprostole in daily dose of 800 μg during theree consecutive days, aachired the success rate on 88% compared to standard combination of mifepristone and misoprostole, in which the rate of success 95.7% what is statistically significant better.

Dimitrijevic and associates showed that vaginal aplication moistened misoprostole in daily dose of 1000 μg during theree consecutive days, aachired the success rate on 92,8% compared to standard combination of mifepristone and misoprostole, in which the rate of success 95.7% (42-44). And another authors showed the similar results used the same doses of misoprostole (48)

Based on an analysis, of previous studies on the use of misoprostole, it ca be concluded that repated doses od 800 μg moistened tablets misoprostole, intravaginal, at pregnancies less than 63 gestation days (9 gestation week) show the efficacy in 85% to 90% of cases (45, 46, 47). Adminis - tered in this way and these doses, misoprostol alone has lower degree of success than the standard procedure in the combination with mifepristone or metotrexat.(32,33,41)

CONCLUSION

Drug abortions are safe and effective alternative to surgical abortions.

Mifepristone in a dose of 200 or 400 μg, after which the vaginally administred misoprostole in a dose of 800 μg are the most effective combination available women who decided for abortion. These drugs can cause safely expulsion in pregnancies less than 63 gestation days (9 gestation week). The main side effects of using these drugs are gastrointestinal discomforts. The bleeding is variable, but it lasts more often two or theree weeks. Instrumental revision (suction or abortion) is rarely needed as emergency procedures. By aplication of drugs, dilatation of cervical canal is mosthy completed so that the instrumental revision facilitated.

The therapists are required to comply with the will with their patients. Howerer gynecologist who proposed manner of termination of unwanted pregnancy must be completely sure that the patient is sure of hes decision. Appearing all more successful procedures drug inducted

abortion, it does not mean that the decision for abortion easier. When the decision on the abortion is made by the patient, gynecologist proposes the ways of termination of pregnancy, but it is necessary that the patient makes a decision which of methods will be applied. Depending on each specific case, gynecologist is the on who offers alternative. The selection method is recommended by gynecologist, and a final decision is made by patient.

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FEMORAL ARTERY THROMBOSIS IN A VERY LOW BIRTH WEIGHT PRETERM NEWBORN

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TROMBOZA FEMORALNE ARTERIJE U PREVREMENO ROĐENOG DETETA VEOMA MALE TELESNE MASE

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ABSTRACT

Thromboses are considered to be rare disorders in the pediatric population. However, they occur more frequently during the neonatal period. Potential risk factors for thrombosis onset include frequent use of peripheral, umbilical or central venous / arterial catheters, inflammation, disseminated intravascular coagulation, liver disorders, hypovolemia, asphyxia, congenital heart disease, polycythemia and dehydration.

On the seventh day of life in male premature infant born in 29th / 30th gestation week, we noticed an increased level of inflammatory parameters. The patient's right leg was edematous and cold with immeasurable pulse and pressure over the femoral artery, mottled skin and no spontaneous mobility. In the following days, edema becomes generalized and abdominal meteorism with bile vomiting appeared. Vascular surgeon established a diagnosis of the acute femoral artery stenosis (CW Doppler). We initiated continuous infusion of unfractionated heparin, but due to the development of hemorrhagic syndrome, next day we replaced it with low molecular weight heparin. The hemorrhagic disease was treated with tranexamic acid, fresh frozen plasma and concentrated platelets of the corresponding blood group. Signs of recirculation were registered with a palpable femoral pulse. CW Doppler confirmed complete recovery.

In spite of the fact that antithrombotic therapy can cause numerous complications, it should be considered in vital indications.

Keywords: *newborn, arterial thrombosis, diagnosis, heparin*

SAŽETAK

Iako tromboze predstavljaju redak problem u neonatalnom dobu, imaju veću učestalost u odnosu na ostali period detinjstva. Potencijalni faktori rizika su česta upotreba perifernih, umbilikalnih ili centralnih venskih/arterijskih katetera, inflamacija, diseminovana intravaskularna koagulacija, poremećaj funkcije jetre, hipovolemija, asfiksija, urođene bolesti srca, policitemija, dehidracija i slično.

Muškom prevremeno rođenom novorođenčetu u 29/30-oj gestacijskoj nedelji sedmog dana života registrovan je porast parametara inflamacije. Desna noga bez spontane motorike, edematozna, marmarizovana i hladna. Puls i pritisak nad femoralnom arterijom nemerljivi. Narednih dana edemi se generalizuju, abdomen postaje meteoristični, povraća žuč. Konsultovan vaskularni hirurg koji CW doplerom dijagnostikuje akutnu trombozu femoralne arterije. Terapija započeta nefrakcionisanim heparinom u kontinuranoj infuziji, ali je zbog razvoja hemoragijskog sindroma sledećeg dana zamenjen niskomolekularnim heparinom. Hemoragijska bolest je tretirana primenom traneksamične kiseline, transfuzijama sveže smrznute plazme i koncentrovanih trombocita odgovarajuće krvne grupe. Desna noga postaje prokrvljena, puls nad femoralnom artrijom se palpira, a CW dopler potvrđuje kompletnu rezoluciju.

Iako antitrombotična terapija može izazvati niz komplikacija, u vitalnim indikacijama je treba primeniti.

Ključne reči: *novorođenče, arterijska tromboza, dijagnoza, heparin*

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INTRODUCTION

Thromboembolism is a rare problem in the neonatal period occurring more frequently compared to the rest of the childhood (about 2.4-6.8 / 1000 infants) (1). Irrespective of the term (on term born or before term born), critically ill infants most often develop a thromboembolic disease (TE) due to high prothrombotic activity, low levels of natural anticoagulants, and fibrinolytic disbalance of various etiology (2). TEs are representing an increasing problem in tertiary neonatal intensive care units (NICU) arising after invasive therapeutic procedures such as applying peripheral, umbilical or central venous / arterial catheters. Until nowadays, in addition to the aforementioned exogenous risk factors, the most commonly studied endogenous risk factors include inflammation, disseminated intravascular coagulation (DIC), liver dysfunctions, congenital heart disease, asphyxia, hypovolemia, polycythemia and dehydration (1,2). The influence of hereditary factors on the development of neonatal thrombosis still remains unclear, even though some authors reported the association between the thrombosis and congenital thrombophilia. The deficiency of antithrombin, protein C and S was also noticed. The diagnosis of thrombosis can be reliably established by Doppler ultrasound, venography, scanner or magnetic resonance imaging with angiography (2). Prevention of thrombosis is of paramount importance in critically ill infants, in particular those extremely immature (1, 2). Treatment includes a range of procedures such as observation, intravenous use of unfractionated or low molecular weight heparin as well as aggressive thrombolytic therapy or catheter revascularisation (1-4).

THE CASE

Here we present a case of a critically ill male newborn, the first child from the second controlled pregnancy. The first pregnancy was terminated by spontaneous abortion during the 26th gestational week. On the day before delivery, the mother was hospitalized at the Gynecology and Obstetric Clinic of the Clinical Center "Kragujevac" due to thrombophilia and the threatening eclampsia. A delivery was completed by caesarean section (sectio Caesarea) in the 29th / 30th gestation week.

Artificial rupture of membranes was performed during the intervention; the amniotic fluid was clear and the umbilical cord normal. Basic characteristics of the newborn were as follows: body weight 1330 g, body length 42 cm and head circumference 27 cm. Due to the development of moderate asphyxia (5th minute Apgar score was 6), aspiration, tactile stimulation and manual ventilation with a balloon for newborns were performed in the operating room. As the newborn was immature and developed deterioration of respiratory function, we arranged the transfer to the Center for Neonatology of the Pediatric Clinic in Kragujevac. On admission to NICU, the patient was groan-

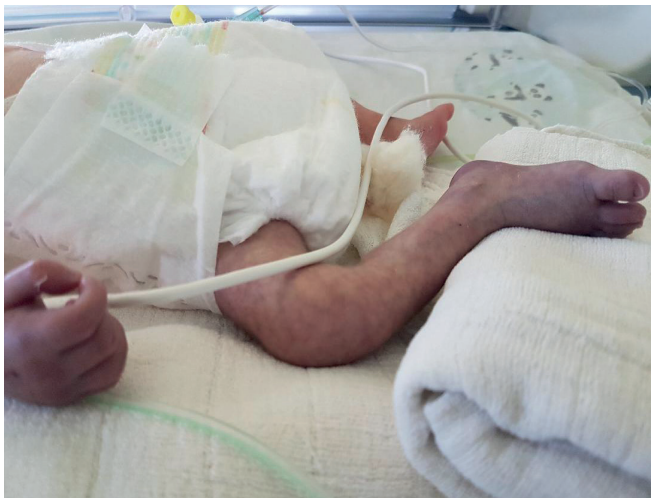

Figure 1. The appearance of the right limb at the moment of thrombosis onset

Figure 2. The establishment of the diagnosis of right femoral artery thrombosis - CW Doppler

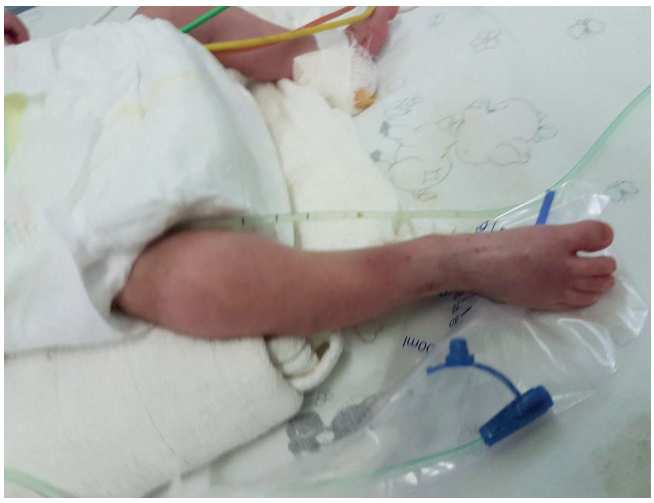

Figure 3. The appearance of the right limb the next day with the full recovery

ing with the evident movements of the auxiliary breathing muscles. Additional examination revealed the respiration frequency of 55 / min (Silverman score 7), thin and livid skin, hemoglobin oxygen saturation of 80%, heart rate of 110 / min with equal beating intervals, clear sound and no pathological murmurs. Primitive reflexes were diminished with generalized hypotonia present.

After detailed observation and microbiological analysis, our patient was placed in the incubator. We intubated infant and continued with prolonged conventional mechanical ventilation. As the primary respiratory distress syndrome ("hyaline membrane disease") was confirmed, we administered poractant alfa, an extract of natural porcine lung surfactant (Curosurf 120mg / 12h) endotracheally. Concomitantly the newborn was rehydrated intravenously with the addition of theophylline preparation. As the risk factors for early sepsis were present, a double antimicrobial therapy (ampicillin + amikacin) was introduced, and corrected on the 6th day of the hospitalization to meropenem + amikacin + fluconazole, considering the deterioration in general condition and the increased levels of inflammation parameters. Convulsions were treated with continuous infusion of phenobarbital and midazolam. On the 7th day of hospitalization, the right leg was without spontaneous motility, easily edematous, mottled and cold with immeasurable pulse and pressure. (Picture 1). In the following days, edema became generalized and the oliguria (diuresis was 0.5mg / kg / h) and abdominal meteorism with bile vomiting occurred.

Blood gas analysis we performed indicated the progression of hypoxia and respiratory acidosis (pH 7.20 - 6.92, pCO2 8.0 - 14.5, pO2 5.1 - 3.5, HCO3 23.5 - 22, 4, BEEcf 4.5 - 10.2), transient hyponatraemia (Na+ 131 - 140), hyperkalemia (K+ 7.3 - 5,3), hypocalcaemia (Ionized Ca^{++} 1,14 - 1,35) and hypoglycaemia (glycaemia 2,7 - 4,5). The results of the complete blood count analysis showed the increase in total number of leukocytes, and the decrease in erytrocites, platelets and hemoglobin (Le 20,5 - 38,9 x 10^9, Er 5,14 - 3,21, Hb 206 - 99, Tr 136 - 40 x 10^9). Biochemical assessment indicated higher level of inflammatory parameters (CRP 3.1 - 129.0 mg/l, PCT 13.210 - > 100.000) and the development of acute renal failure (urea 19.2 mmol/l, creatinine 153 umol/l, urine: proteins +/-, Le 2-3, few epithelial cells, plenty of amorphous urate, some bacteria, urine culture: sterile). Hemoculture at the admission was sterile while we isolated Pseudomonas aeruginosa from the tracheal aspirate (8th day of the hospitalization). An assessment of the coagulation status was performed with the following results PT 23.1 s, INR 2.119, aPTT 42.6 s, fibrinogen 1.882 g/l, d-Dimer 2.60 ug/ml.

Chest X-ray at the admission indicated the existence of a parenchyma with a homogeneous increased density and signs of a hyperventilation (air-bronchogram) on the left lung and a right lower lobe on the right side. On the 3rd day of the hospitalization we performed a second radiograph imaging and noticed reduced transparency of the parenchyma on the right with solitary paracadial darker shadows and suspected partial pneumothorax on the left side.

On the 7th day of hospitalization we asked for the consultancy of the vascular surgeon (Picture 2). His findings were: right leg was without capillary filling, CW Doppler – the flow could be confirmed up to the level of the right iliac artery. The flow distally from the inguinal ligament could not be registered. There were no pathological signs on the left side. Because of artery caliber and elasticity, there were no conditions for the treatment at the Vascular Surgery of the Clinical Center Kragujevac. Vascular surgeon suggested heparin in appropriate doses and warming up of the right leg.

Ductus arteriosus 2-3 mm wide was registered by echocardiographic examination (9th day of the hospitalisaiton). Transfontanellar echosonography (before heparin application) confirmed physiological chambers with small hematoma at the right and moderate hyperechogenicity located periventricular. After heparin administration, we noticed the following: left brain chamber diameter (VPRL) 4.3mm, right brain chamber diameter (VPRD) 4.8mm, larger cavum septum pellucidum (9.4 x 9.1mm) and cavum verge (11.2 x 18.4mm), hematoma on the left sized 14.1 x 5.1 mm, hematoma on the right sized 12.3 x 6.6mm and the diffuse hyperechogenicity with small hypoechogen areas. The same procedure on the 40th day of hospitalization showed: VPRL 5,8mm, VPRD 5,6mm, third ventricle at the physiological level, signs of resorption of hematoma on the left 14 x 6.6mm, hematoma on the right - 12 x 8mm. Moderate hyperechogenicity is still maintained.

After consulting the vascular surgeon and hematologist, we elevated, heated and slightly massaged right limb. Dopamine perfusion dose (2.5mcg / kg / h) and unfractionated heparin were introduced in the therapy. Due to the development of the hemorrhagic syndrome, heparin was switched off after 6 hours and replaced with low molecular weight heparin (enoxaparin-sodium - Clexane 200 IJ / 24h for 5 days) on the 8th day of the hospitalization. The hemorrhagic disease was treated with tranexamic acid, transfusions of concentrated platelets (on the 7th and 8th day of hospitalization) and freshly frozen plasma of corresponding blood group (8th and 10th day of hospitalization). On the 8th day, we noticed signs of recirculation on the right leg with a palpable femoral pulse and the improvement of the skin color (Picture 3). CW Doppler confirmed complete recovery.

Considering severe condition and suspected sepsis, our patient received multiple IgG transfusions and one dose of 20% of human albumin during hospitalization. Anemia has been corrected by transfusion of deplasmatized erythrocytes of the corresponding blood group, on several occasions. During the 4th week of the hospitalization, the general condition of the newborn improved and we extubated the patient. Further, we stimulated spontaneous respiration by applying caffeine citrate (Peyona).

Oxygene therapy was excluded at the end of the 6th week of hospitalization when we also noticed a gaining in body weight. However, in the 12th week, the general condition worsened and the patient became anxious and febrile with

the temperature values of 39.3°C degrees. Lumbar puncture showed sterile liquor with proteinorachia (0.59 g/l) and glycorrhachia (3.00 mmol/l). Hemoculture was sterile while urine culture (taken by urinary catheter) revealed E. coli (10000 cfu/ml) sensitive to meropenem. Performing ultrasound, the child nephrologist noticed the right kidney sized 3.2 x 1.4 cm, preserved parenchyma, without pyelocalics dilatation. Contrary, the dimension of the left kidney was 4.2 x 1.6 cm, with preserved parenchyma but with pyelocalics dilatation (0.6 x 1.3 cm).

At the end of the 13th week, the patient is discharged from the hospital in good general condition and body weight of 2250g with advice for radiology examination of the urinary tract due to suspected vesico-ureteral reflux and to monitor the condition of a central nervous system. The patient was hospitalised for the second time at the Pediatric Clinic, Kragujevac, for repeated crises of consciousness by the type of infantile spasms at the age of 5 months. An electrocortical activity was examined and the patient was diagnosed with the West syndrome. The congenital thrombophilia test showed high levels of protein S, low protein C (34.5%), and the values of Activated Protein C Resistance (Factor V Leiden) (APCR V) and antithrombin were 1.543 and 57.4.

DISCUSSION

Neonatal thromboses may be located in the arterial or in the venous system and may be symptomatic or asymptomatic. Clinical manifestation varies depending on localization (systemic, in lungs or in the central nervous system) and the size of the thrombus. The most common clinical manifestation of the arterial thrombosis includes ischemia of extremities or torso with the onset of pallor or coldness, impaired or absent pulse and reduced or immeasurable blood pressure. Further, signs of the acute renal failure, necrotizing enterocolitis (enteral nutrition intolerance, abdominal meteorism, the gas in the portal vein and intestinal ischemia) or the ischemia of the central nervous system (convulsion, lethargy) are often present. However, lymph flow, pain, cyanosis or hyperemia of the extremities should raise a suspicion of venous thrombosis (5). Long-term complications of arterial thrombosis include hypertension, impaired renal function, deviation in limb growth, claudicacion, paraplegia, epilepsy and cerebral palsy (6).

DIAGNOSIS

Initial laboratory assessment of a newborn with a suspected thrombosis should include complete blood count and coagulation factors, prothrombin time, thrombin time and activated partial thromboplastin time. D-dimers, except for thrombosis may be elevated in the acute phase in all patients with an infection or inflammatory response syndrome. Contrary, negative D-dimers are relatively reliable in excluding thromboses. The platelet count is usually reduced after delivery, but the sudden and significant drop in platelet counts should indicate a possible thrombosis (7).

The ultrasound (Doppler flow measurement in peripheral blood vessels, echocardiography, abdominal ultrasound) demonstrates overall poor performance for the detection of thrombus, but it has certain advantages considering the minimal invasiveness and absence of radiation. Even though a hemodynamically significant duct was registered by an echocardiographic examination, we were unable to include ibuprofen to close it due to thrombocytopenia, acute renal failure and proposed anticoagulant treatment.

Confirmation of thrombosis by performing venography or angiography with contrast could help avoid exposure of the newborn to unnecessary and potentially dangerous treatment. Therefore, they are still considered to be the gold standard in the diagnosis of thrombosis. Angiography is recommended for the diagnosis of ischemic neonatal stroke or neonatal pulmonary embolism. However, the need for transport limits the feasibility of this method in critically ill or extremely immature patients, similar to the newborn we presented here.

TREATMENT

The treatment remains a great challenge for neonatologists because it is necessary to consider the risk/benefit relationship carefully. The anticoagulant therapy should be initiated as soon as possible in order to achieve complete or at least partial recanalization to prevent tissue hypoperfusion, portal hypertension, renal failure, claudication or paraplegia (5). The main potential risk for the application of anticoagulant therapy is a potentially life-threatening hemorrhagic disease (especially intracranial bleeding).

Low molecular weight heparin is a drug of choice since the risk of bleeding is lower, but it is contraindicated in extremely immature children, as well as in cases where intracranial bleeding has already been diagnosed. Relative contraindication for this type of the treatment is thrombocytopenia ($<50 \times 10^9$). However, in most other patients, low molecular weight heparin can be considered appropriate (8).

Over the years unfractionated heparin (UFH) is a standard drug for the prophylaxis and the treatment of thromboembolism (TE). It continues to be used in neonatal medicine for the prevention and treatment of thrombosis. Nevertheless, it is almost completely replaced by a low molecular weight heparin in adult medicine because of its unfavorable pharmacokinetics and pharmacodynamics and a high risk for immediate bleeding. When initiating a low molecular weight heparin, it is necessary to monitor the level of anti-Xa. Preterm infants require a longer time (6 days) to achieve the level of anti-Xa within the target range, comparing to the term infant (2 days) (8). Therefore premature babies require higher doses of fraxiaparin than term newborns.

Indications for systemic thrombolytic therapy are arterial occlusion, massive pulmonary embolism or pulmonary embolism resistant to heparin, as well as the endangerment

of the vital organs or extremities (2). The most commonly used thrombolytic agents in adults are streptokinase and urokinase. However, they express reduced fibrin specificity and high antigen potential comparing to the recombinant plasminogen activator in the tissue (rtPA) and are rarely used in neonatal period (9). Furthermore, rtPA is a favorable choice due to the high fibrin specificity, poor activation of free plasmin, lack of antigen potential and short half-life. The plasminogen level is low in infants compared to adult patients, indicating a potential need for higher doses of rtPA. The use of these agents should be terminated after a few days or when revascularization is achieved. In contrast, low molecular weight heparin (LMVH) is increasingly used in therapeutic concentrations when prolonged thrombolytic therapy (from 6 weeks to 3 months) is necessary.

The invasive method of the treatment involves catheter-directed thrombolysis, mechanical thrombectomy, as well as surgical removal of thrombus and blood vessel recanalisation (9).

Long-term anticoagulant therapy is advised in neonates with symptomatic TE. However, most clinicians do not apply long-term anticoagulation in catheter-induced thrombosis if the patient does not have congenital thrombophilia. The utilization of Varfarin during the neonatal period is uncertain. Many adaptive milk formulas contain a high concentration of vitamin K and therefore could significantly reduce the effects of warfarin.

The platelet function is reduced in newborns compared to older children and adult patients. Anti platelet agents such as aspirin or a novel agent clopidogrel, have been traditionally used in prophylaxis of occlusion in patients with congenital heart disease or shunt-dependent pulmonary or systemic circulation. There is no evidence on the efficacy of the antithrombotic therapy in the treatment of neonatal venous or arterial thrombosis, unlike the use of aspirin as a prophylactic agent in patients with a history of TE events (10,11).

CONCLUSION

Critically ill newborns have a high risk for venous or arterial thrombosis onset, with the presence of catheters being the major exogenous factor. Therefore, in diminishing the incidence of this disorder, heparin application into the catheter (heparinization of the catheter), as well as the prevention of endogenous risk factors development, such as systemic infections and perinatal asphyxia as the leading causes of neonatal morbidity, could be of crucial importance.

We can conclude that the thrombotic therapy should be applied in vital indications irrespective of the potential risk for numerous life-threatening complications. In order to prevent the development of post-thrombotic syndrome or portal hypertension, all newborns with the developed thromboembolic disease shold be tested for congenital thrombophilia. Also, these patients require a long-term follow-up as well.

CONFLICT OF INTEREST

Authors report no conflict of interest.

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PREOPERATIVE ENDOVASCULAR EMBOLISATION OF THE SYMPTOMATIC HEMANGIOMA IN 7TH THORACIC VERTEBRAE: CASE REPORT

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PREOPERATIVNA ENDOVASKULARNA EMBOLIZACIJA SIMPTOMATSKOG HEMANGIOMA SEDMOG TORAKALNOG PRŠLJENA: PRIKAZ SLUČAJA

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ABSTRACT

Although, as asymptomatic, they appear in about 10-12% of the worldwide population, vertebrae hemangiomas are symptomatic in about 0.9-1.2% of all the cases.

We showed the case of the symptomatic hemangioma in the 7th thoracic vertebrae in 67 year old patient, that was successfully preoperative embolised. Magnetic resonance imaging (MRI) detected the tumor in the body of 7th vertebrae with mass effect on the anterior aspect of the spinal cord. Multidetector computed tomography (MDCT) imaging describes this tumor as hemangioma that is in the body of the Th7 vertebrae and in the both pedicules. We performed selective and supraselective spinal angiography which showed pathological vascularisation of the tumor, and then the tumor was embolised. The control angiography detected the reduction of the tumor blood vessels, as a sign of the successful embolisation. Ten days after embolisation, the patient went through corporectomia of the Th7 and the stabilization of the thoracic spine was performed. Intraoperative blood transfusion in our patient was 930 mL, while expected blood transfusion during the surgical intervention without preoperative embolisation is about 1600 mL.

Method of choice in conditions with neurological compressive symptoms caused by vertebral hemangioma is surgery for the decompression of the nerve structures. Embolisation of aggressive vertebral hemangioma is recommended and preoperatively performed for the intraoperative hemorrhage reduction and decreasing of intraoperative complications.

Keywords: *hemangioma, spine, spinal cord compression; angiography, embolisation, therapeutic, hemorrhage*

SAŽETAK

Iako se, kao asimptomatski, sreću u oko 10-12% opšte populacije, hemangiomi kičmenih pršljenova su simptomatski u oko 0,9-1,2% od svih slučajeva.

Prikazali smo slučaj simptomatskog hemangioma sedmog torakalnog (Th7) pršljena kod bolesnice stare 67 godina, koji je preoperativno uspešno embolisan. Pregled magnetnom rezonancom je pokazao tumorsku promenu u telu Th7 pršljena sa "mass" efektom na prednji aspekt kičmene moždine. Na pregledu skenerom promena je okarakterisana kao hemangiom koji zahvata telo Th7 pršljena i oba pedikla. Učinjena je selektivna i supraselektivna spinalna angiografiji na kojoj je prikazana patološka vaskularna mreža tumora, a nakon toga izvršena je embolizacija. Na kontrolnoj angiografiji uočena je redukcija vaskularne mreže tumora, kao znak uspešne embolizacije. Nakon 10 dana od embolizacije pacijentkinji je učinjena korporektomija Th7 i izvršena stabilizacija grudne kičme. Intraoperativna nadohnada krvi kod našeg pacijenta iznosila je 930 ml, dok je očekivana količina krvi za nadohnadu bez preoperativne embolizacije oko 1600 ml.

U stanjima sa neurološkim kompresivnim simptomima, metoda izbora u terapiji vertebralnih hemangioma je hirurška intervencija u cilju dekompresije nervnih struktura. Embolizacija agresivnih vertebralnih hemangioma se preporučuje i izvodi preoperativno u cilju smanjenja intraoperativnog krvarenja i time mogućih intraoperativnih komplikacija.

Ključne reči: *hemangiom; kičma, kompresija kičmene moždine, angiografija, embolizacija, terapijska, hemoragija*

ABBREVIATIONS

Th7- 7th thoracic vertebrae

MRI- Magnetic resonance imaging
MDCT- Multidetector computed tomography

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INTRODUCTION

Agressive vertebral hemangiomas are benign vascular tumours that don't give metastases, instead they go through posterior cortex of the involving vertebrae, spread in epidural space and cause compression on the spinal cord with destabilisation of the spine (1). In cases like this surgery is neccessery for the decompresion of spinal cord and stabilisation of the spine (2). Most vertebral hemangiomas are asymptomatic and are detected in about 10-12% of the worldwide population (3-6). Symptomatic vertebral hemangiomas occur in about 0.9-1.2% of all the cases (7, 8).

Percutaneous embolisation with alcohol in the combination with percutaneous vertebroplastic didn't give satisfying results in motoric deficite elimination (9). Because of the rich vascularisation of hemangioma, hemorrhage is common surgical complication, that sometimes may endanger the life of the patient (1, 10). Spinal hemangioma embolisation with endovascular approach under the control of fluoroscopy can reduce intraoperative hemorrhage (11). Embolic agents such as embolisation fluid, sclerozation fluid, embolisation particles and mechanical occlusion devices are used for the embolisation (11-14).

The purpose of our case report is to present the case of the patient with hemangioma in the Th7 vertebrae that has undergone the surgery after embolisation and corporectomia with spinal stabilization.

CASE REPORT

The sixty-seven year old patient was hospitalised due to the progressive weakness of the lower extremities with the urgent urination, positive Babinski reflex on the right side and an increased sensitivity level at Th7 on both sides. The MRI imaging of thoracic spine with the intravenous application of paramagnetic contrast showed changed signal intesity of Th7 vertebrae, with symmetrical epidural extension, formaninal and paravertebral to the right, which with its MRI characteristics (the increased signal intesity in T2W/STIR sequence, decreased in T1W with significant postcontrast increase of sygnal intesity) can relate to a wider differential diagnosis including aggressive hemangioma. Circular changes with simmilar MRI characteristics described in vertebral bodies of C7, Th2, Th6 and Th9, can be atypical hemangiomas (Figures 1 and 2).

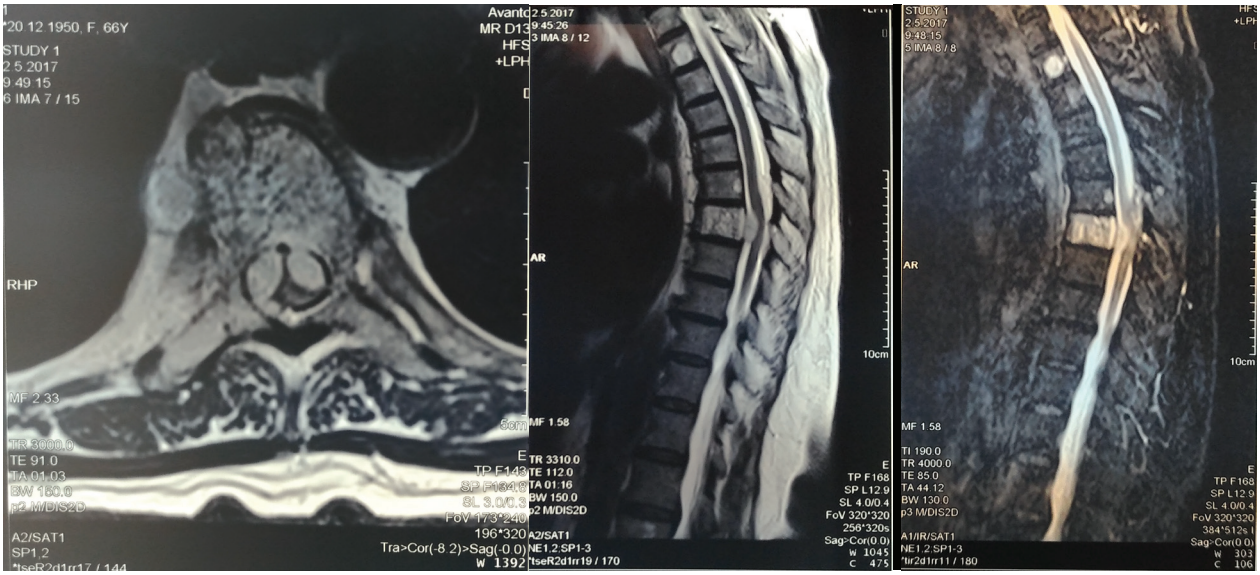

Figure 1. Left and middle: MRI axial and sagittal cross-section in the T2W sequence show an inhomogeneously elevated signal intensity with the capture of the posterior elements to the right and the epidural spreading on both sides and compression of the anterior aspect of the spinal cord. Right: The lesion shows a significant increase in the intensity of the signal postcontrast. Oval changes in similar MRI characteristics in Th2 and Th6 vertebral bodies are also visible.

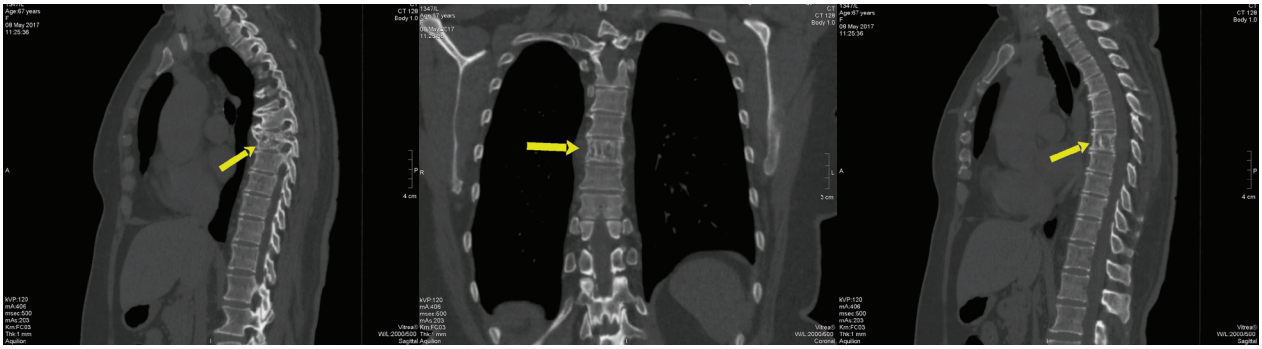

Figure 2. The MDCT examination of the thoracic spine shows rough vertical bone trabecules in the Th7 vertebral bodies.

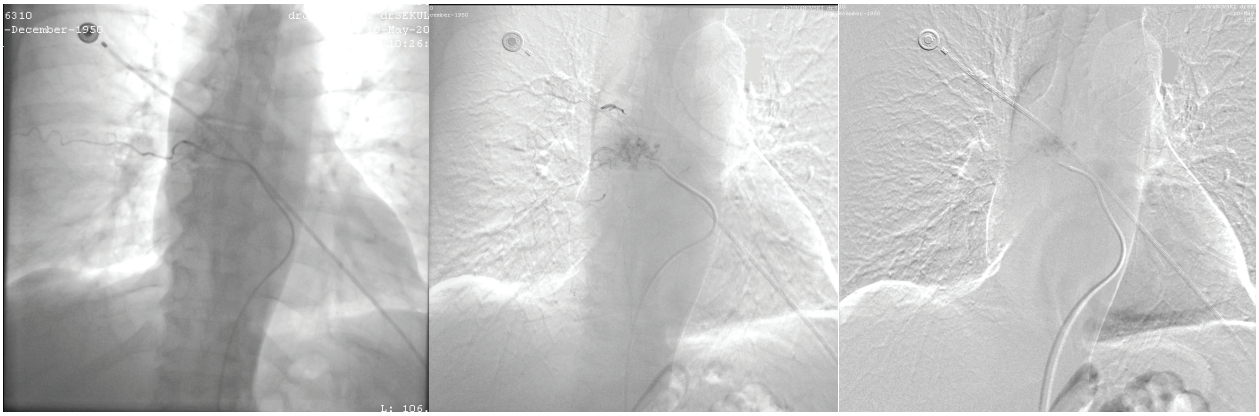

Figure 3. Left - Selective angiography of the VII intercostal artery (a pathological network in the body TH7- hemangioma); Middle - Supraselective angiography with more dinstinctive presentation of the pathological vascular network in the Th7 vertebral bodies; Right - Supraselective angiography after embolisation- significantly reduced pathological network of hemangioma is detected

The MDCT imaging with and without intravenosuosly applied contrast describes changes in Th7 vertebrae as a highly suspected of hemangioma that occupies vertebrae body and both of the pedicules.

Transcatheteric spinal angiography and embolisation are preoperatively carried out. Catheter was selectively placed via right transfemoral approach in the intercostal artery on the level inferior part of Th7 vertebral body that goes under inferior part of the seventh rib on the right side. The contraast was applied and angiography was performed showing pathological vascularisation inside the right aspect of Th7 vertebral body. Then, embolisation particles of Embozene 250 μm and 500 μm were selectively and supraselectively applied in tumour feeding artery. Control angiography showed a reduction of tumour vascularisation, no sign of blush and retention of contrast in tumour and feeding artery, as a sign of succsessfull embolisation (Figure 3).

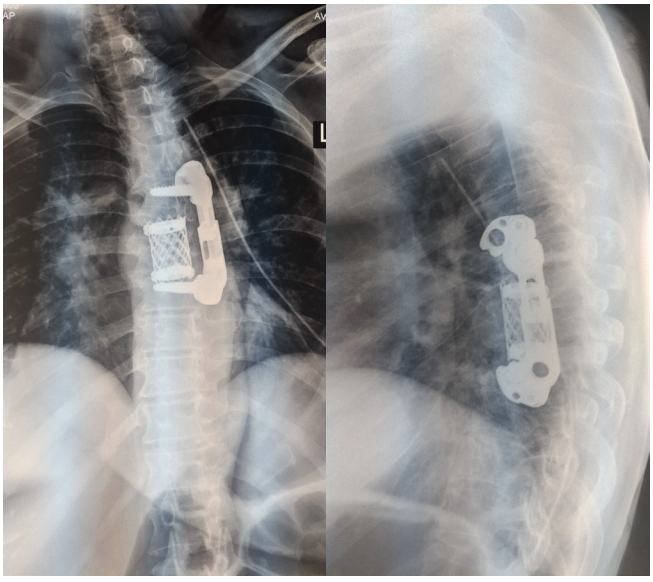

Figure 4. Medical condition after corporectomy Th7 and stabilisation of the thoracic spine by expanding titanium graft and lateral instrumentation

Six days after embolisation in our hospital, the surgery was performed for decompression and stabilisation of the spinal cord. Left thoracotomy and corporectomy of Th7 vertebrae was performed as the stabilisation of thoracic spine with the expanding titanium graft (Figure 4). Patient recived 930 ml of blood transfusion during the surgical intervention.

Pathohystological analyses after resection of the Th7 vertebrae confirmed that it is cavernosus hemangioma.

DISCUSSION

Vertebral hemangiomas are benign lesions made out of venosus structures and capillaries (15). About 1% of vertebral hemangioma give certain clinical symptoms and signs, while the most are asympthomatic (16-18). In 55% patients the sympthom is pain, while in 45% there is a neurological deficit due to the compression on neurological structures - spinal cord, spinal nervs, or because of the vertebral collaps. In pain conditions without neurological compression, preoperative procedures such as embolisation, vertebroplastic, radiotherapy or ethanol injection are performed, while in cases of progressive neurological defficit caused by spreading vertebral hemangioma that makes spinal cord compression, surgical decompression must be performed (2 16, 19-21). However, since these hemangiomas are highly vascular tumours, significant blood loss can occur in resection. Because of that, in order to reduce complications such as preoperative hemorrhagia, many surgeons are for preoperative transcatheteric embolisation of hemangioma, followed by resection and stabilisation of the spine. Because of the high rates of recidivism after partial resection and reconstruction or enlargment of cement mass (2.9-30%), some centers are for total agressive spondilectomia with wide margins for the recidivism reduction (20).

The therapy procedure has not been determined so far, as well as the ideal surgical operation for the patients with sympthomatic vertebral hemangiomas (9, 22, 23).

Therefore, the surgical treatment is required in cases with progressive neurological deficit, for the decompression of the spinal cord (22). Embolisation prior to sympthomtic vertebral hemangioma surgery is recomended by most of the authors as relatively safe procedure with rare complications (11, 18, 24). Postoperative radiotherapy should be performed in the patients with subtotal tumour resection to prevent recurrence (25). However, some authors recommended surgical procedure, embolisation and radiotherapy as the gold standard for the treatment of symptomatic vertebral hemangiomas (15, 26, 27).

Conventional radiography of vertebral hemangiomas can show parallel linear lines or honeycomb apperiance of the vertebrae. The MDCT is also used as an alternative for the MRI for the diagnoses and assessment of the grade and agressivity of the tumour and its contact with anatomical structures around with typical "Polka-dot-sign" due to trabecular aspect of the involved vertebra (15, 18, 26, 28, 29).

Hemangiomas in more than one vertebrae are rare (30, 31). Five hemangiomas in cervical and thoracic vertebrae were detected in our patient, one of which was sympthomatic and embolised, being operated and stabilised afterwards.

Blood loss of about 930 ml during the surgery in our patient was compensated with blood transfusion, and this blood loss is common in treatment of agressive vertebral hemangioma, with preoperativly performed embolisation, which is similar to Robins et al. where the compensation of blood after the tumour embolisation was 981 ml (range of 143-1048 ml), while in patients without embolisation was on an average level was 1629 ml (32). Significant hemorrhage from feeding artery during the surgery and later in epidural space has caused more surgeons to perform preoperative embolisation (15).

In cases with neurological compressive sympthoms, the method of choice for the vertebral hemangiomas therapy is surgery for decompression of nerve structures. Agressive vertebral hemangioma embolisation is recomended and preoperatively performed for the hemorrhage risk reduction (32), but significant factor in intraoperative hemorrhage grading is invasivity of surgical treatment (33, 34). Embolisation is relatively low-risk procedure, and the adverse effects are rare (embolus with cardiovascular problems, stroke, perifery arrterial occlusion and ischemia of the spinal cord, alergic reactions and hemorrhage) (9, 15, 35). Permat et al. showed that combined percutaneous embolisation and vertebroplastic are safe and effective procedures, analysing 26 patients through 19 years with follow-up period of 22-217 months (9).

CONCLUSION

Preoperative embolisation of vertebral hemangiomas significatly reduces intraoperative hemorrhage, typical for operatively treated tumours, which reduces possible complications of surgical treatment.

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THE ASSOCIATION BETWEEN OBESITY AND VISIT-TO-VISIT VARIABILITY IN SYSTOLIC BLOOD PRESSURE: A PROSPECTIVE STUDY

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POVEZANOST GOJAZNOSTI I VARIJABILNOSTI SISTOLNOG KRVNOG PRITISKA PRILIKOM POSETA: PROSPEKTIVNA STUDIJA

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ABSTRACT

With the prevalence of obesity and all accompanying health risks, both prevention and health education, as well as identifying predictors for the development of obesity-related diseases are primary. The pathophysiological relationship between obesity and visit-to-visit variability in systolic blood pressure (SBPV) has not been completely resolved. To investigate the association between obesity and SBPV in hypertensive patients. The prospective study comprised three visits was performed at the hypertension outpatient clinic during the follow up period of 22-months between March 2014 and January 2016. This study included 300 randomly selected hypertensive patients (average 67.76±9.84 years), who were divided in groups of obese/non-obese examinees. SBPV was defined as the standard deviation (SD) from three values of SBP. The values of SBP and SBP-SD were significantly higher in the group of obese hypertensive patients than in the group of non-obese patients (127.06±8.30 vs. 120.37±7.75; 11.29±5.67 vs. 7.37±3.94 mmHg; p<0.01). The highest SBPV was recorded in the 4th quartile in obese patients (43.13±7.50 mmHg). SBPV was strongly correlated with BMI and Waist cirumferences (WC) (ρo=0.425, ρo=0.356, p<0.01). During 22-months follow up there was a significant decrease of SBPV for 8.2 mmHg, BP for 31/5 mmHg, BMI for 3.8 kg/m2, WC for 10 cm and body weight for 8.24 kg. During 22-months follow-up, reduction of body weight was associated with reduction of blood pressure variability in hypertensive patients. Persistently decrease both body weight and long term visit-to-visit variability may explain lower cardiovascular risk in obese-related disease.

Keywords: *Obesity, hypertension, visit-to-visit systolic blood pressure variability.*

SAŽETAK

Sa prevalencijom gojaznosti i svih pratećih zdravstvenih rizika, primarna je preventivna i zdravstvena edukacija, kao i identifikovanje prediktora za razvoj bolesti povezanih sa gojaznošću. Patofiziološki odnos između gojaznosti i varijabilnosti sistolnog krvnog pritiska prilikom poseta pacijenta (SBPV) nije u potpunosti jasan. Istražiti povezanost između gojaznosti i SBPV kod hipertenzivnih pacijenata. Prospektivna studija je obuhvatila tri posete u ambulanti za hipertenziju tokom perioda praćenja od 22 meseca u period od marta 2014 i januara 2016 godine. U ovoj randomiziranoj studiji uključeno je 300 hipertenzivnih pacijenata (prosečne starosti 67,76 ± 9,84 godina), koji su podeljeni u grupu gojaznih i negojaznih ispitanika. SBPV je definisan kao standardna devijacija (SD) tri vrednosti SBP. Vrednosti SBP i SBP-SD bile su znatno veće u grupi gojaznih hipertenzivnih pacijenata nego u grupi negojaznih pacijenata (126.67 ± 8.22 vs. 120.45 ± 7.79 mmHg, 11.00 ± 5.64 vs 7.34 ± 3.96, p <0.01). Najveći SBPV zabeležen je u četvrtom kvartilu kod gojaznih pacijenata (43,13 ± 7,50 mmHg). Dokazana je statistički jaka korelacija između SBPV i BMI / obim struka (OS) (ro = 0,425 / ro = 0,356, p <0,01). Tokom 22-mesečnog praćenja došlo je do značajnog smanjenja SBPV za 8,2 mmHg, BP za 31/8 mmHg, BMI za 3,8 kg / m^2, OS za 10 cm i telesne težine za 8,24 kg. Tokom 22-mesečnog praćenja pacijenata, smanjenje telesne težine bilo je povezano sa smanjenjem varijabiliteta krvnog pritiska kod hipertenzivnih pacijenata. Konstatno smanjenje i telesne težine i dugotrajna varijabilnost sistolnog krvnog pritiska prilikom poseta može se objasniti nižim kardiovaskularnim rizikom kod gojaznih bolesti.

Ključne reči: *gojaznost, hipertenzija, varijabilnost sistolnog krvnog pritiska prilikom poseta.*

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INTRODUCTION

Obesity and hypertension (HT) are health and economic problems that pandemically spread and increase the risk of cardiovascular events. Worldwide, prevalence of arterial hypertension is about 25-40% in the adult population, which is over 1 billion people (1). As per World Health Organization report, more than 1.9 billion adults aged 18 years and older were overweight in 2015. (2) In Serbia 56.3% of people are overweight in 2014. The more and more concerning fact is that younger population in Serbia is obese (3).

Through human evolution adipose tissue have been a number of protective roles: energetic, metabolic and immunological. In modern society, hypercaloric diets, physical inactivity, make adipocytes show their other side, which means they secrete different bioactive molecules. Researchers have documented that the disorder of immunometabolic regulatory mechanisms, altered levels of proinflammatory adipokines, and "state of low-grade inflammation", oxidative stress in adipose tissue, activation of the sympathetic nervous system and renin-angio- tensin-aldosterone system (RAAS) are the pathogenetic link between central fat accumulation, insulin resistance, hyperlipidemia, and hypertension, which precedes the development of metabolic and vascular disorders (4-6). Obesity, especially visceral adiposity is associated with 65–75% increased risk of the primary (essential) hyper- tension in overal population It is well know that blood pressure (BP) increases with the body mass index (BMI) on every kilogram of the body mass increase, the BP in- creases for 2-3 mmHg (7).

On the other hand, recent data indicate that not all obese patients have hypertension and that approximate- ly 10-30% of obese people are not affected by metabolic abnormalities. Recent researchers show that there are 6 different phenotypes of body composition, with differ- ent degree of nutrition and metabolic functions, out of which the fifth phenotype is metabolically 'healthy' obe- sity (MHO). (8,9,10)

Many studies have shown that visit-to-visit variability in systolic blood pressure (SBPV) is independant predictor of clinical events and independant risk factor in hypertensive patients and in overall population (11-13). There are very few studies which examined the correlation between obesity and variability of blood pressure in hypertensive patients (14,15). The questions whether body fat mass, central or peripheral, is harmful for the metabolic status has not been completely resolved, neither has the pathophysiological relationship between blood pressure variability (BPV) and cardiovascular disease.

According to recent reports which indicate that SBPV is independent cardiovascular risk factor and high prevalence of obesity-related diseases, the objective of this study is to investigate the association between obesity and visit-to-visit variability in systolic blood pressure in hypertensive patients.

MATERIALS AND METHODS

Prospective study included 300 randomly selected hypertensive patients (147 men and 153 women, average age 67.76±9.84), who were divided, according to BMI≥30 kg/m², to subgroups of obese (n=153) and non-obese examinees (n=147). The study comprised three visits during the follow up period of 22-months. High and unregulated or inadequately regulated blood pressure was included as criteria in the study. The criteria for non-inclusion were: patients with associated diseases of the digestive and renal systems, acute infections in the past three months, neoplastic diseases, diabetes mellitus, surgery in the previous year, weight changes >5 kg within the previous 6 months. BP values were defined by the arithmetic mean of three measurements each of the study visits.

All participants were included in this study at the Institute for Treatment and Rehabilitation "Niska Banja", Niska Banja, Serbia from March 2014 to February 2016. Ethics Committee of Institute for Treatment and Rehabilitation "Niska Banja" approved the study and fully informed written consent was obtained from each patient prior to the investigation.

All participants signed epidemiological questionnaire which were divaded general information, health information, medication situation, family health information and diet and lifestyle.

Hypertension was defined as BP≥140/90 mmHg and/or antihypertensive drug therapy according to the European Society of Hypertension/European Society of Cardiology (ESH/ESC) guidelines (16). In order to estimate detailed evaluation of distribution SBPV quartiles of SBP-SD were formed. SBPV for each participant was defined using the standard deviation from 3 values of SBP. High SBPV was defined as SBP-SD in the 4th quartile.

Obesity was evaluated through body weight (BW), body mass index BMI≥30 kg/m² and waist cirumferences WC≥94 cm for males and WC≥80 cm for females. BW measurement (kg) was performed using a digital scale with the accuracy of measuring up to 0.1 kg. Body height measurement (cm) was performed using anthropometer (altimeter), with the accuracy of measurement to the nearest 0.5 cm. We used the most recent weight to calculate BMI, which was calculated as weight in kilograms divided by the square of height in meters. The measurement of WC was performed in a standing position, with heels apart, arms relaxed besides the body, and was measured in the middle distance between the rib cage and the iliac bone on the middle axillary line (at the level of the umbilicus). WC was used as an index of central obesity (17).

STATISTICAL ANALYSIS

All analysis were performed using IBM SPSS Statistics 20.0 software using descriptive and analytical methods. All the data were presented as means ± standard deviations (SD), as absolute numbers and percentages, dependent on the statistical method used. The chi-square test was used to analyze differences between categorical data. To analyze continuous data distribution and to compare the means of the two examinee groups was applied the Student T-test. The comparison of mean parameters of obesity, BP and visit-to-visit SBPV was achieved using analysis of variance (ANOVA). The 25th, 50th and 75th percentiles were calculated in order to determine the 4 quartiles of the SBP-SD distribution. Bivariate correlation analysis (Spearman's correlation coefficient) was used to estimate the association between parameters of obesity, BP and SBPV. All statistical analysis were two-tailed, performed for the statistical significance level of $p<0.05$.

RESULTS AND DISCUSSION

Table 1. presents baseline and anthropometric characteristics and visit-to-visit variability in sistolic blood pressure of the study group across visits.

The prevalence of obesity evaluated through BMI in hypertensive subjects at the beginning of the follow-up was 153 (51%) and it was evaluated as WC 192 (64%). During 22-months follow up (across three visits) there was a significant decrease in prevalences of obesity defined according to BMI (51% vs. 32% vs. 17%, $p<0.05$, respectively) and WC (64% vs. 39% vs. 23%, $p<0.01$, respectively). There was significant decrease in the apsolute and relative values of SBPV across the three study visits (16.20±13.34 vs. 9.91±6.80 vs. 8.04±4.43 mmHg, $p<0.01$, respectively) and (10.66% vs. 7.34% vs. 6.64%, $p<0.01$, respectively). Moreover, the values of SBP were significantly lower (152.42±14.97 vs. 135.18±10.13 vs. 121.51±8.17 mmHg, $p<0.01$, respectively).

All parameters of both blood pressure and obesity are in significant, positive and moderate correlations (Table 2). It is also, statistically stronger correlation between SBP- SD and BMI as parameter of total obesity compared to large correlation with WC as parameter of central obesity ($\rho o=0.425$ vs. $\rho o=0.356$, $p<0.01$).

The baseline parameters of blood pressure and obesity after the first and the third visits in non-obese and obese hypertensive patients are shown in Table 3.

All parameters of blood pressure and obesity were significantly lower in obese patients after the third visit compared to baseline values ($p<0.01$), except DBP. The values of SBP and SBP-SD after the third visit were significantly higher in the group of obese hypertensive patients than in the group of non-obese patients (127.06±8.30 vs 120.37±7.75 mmHg, 11.29±5.67 vs 7.37±3.94; $p<0.01$). The difference between DBP was not statistically significant ($p>0.05$).

Characteristics across quartiles of the SBPV in hipertensive obese and non-obese patients after the third visit are presented in Table 4. There was significantly higher value of SBP-SD in hipertensive obese and non-obese patients in the fourth quartile compared to the values recorded in the other three quartiles. The highest SBPV was recorded in the 4th quartile in obese patients compared to SBPV in the 4th quartile of non-obese patients (43.13±7.50 vs. 34.29±5.40 mmHg, $p<0.001$).

Graph 1. presents the change of values of obesity and blood pressure parameters. Comparative analysis of the follow-up data of all hypertensive subjects showed statistically significant average reduction of SBP-SD for 8.2 mmHg, BP for 31/5 mmHg, BW for 8.24 kg, BMI for 3.8 kg/m^2 and WC for 10 cm, at the end of study compared to baseline values.

Table 1. Baseline and anthropometric characteristics of the study group

PARAMETERS	V I S I T E S			P
	1.	2.	3.	value
Age (years)	67.02±9.21	67.73±9.78	68.53±9.97	>0.05
Sex (M/W)	147/153	147/153	147/153	>0.05
BMI≥30 kg/m² (N/%)	153 (51%)	96 (32%)	51 (17%)	**<0.05**
WC≥94(80) cm (N/%)	192 (64%)	117 (39%)	69 (23%)	**<0.01**
Weight (kg)	83.34±10.68	78.14±10.35	75.10±10.77	**<0.01**
Absolute value of SBP (mmHg)	152.42±14.97	135.18±10.13	121.51±8.17	**<0.01**
Relative value of SBP - Cv (%)	9.82%	7.49%	6.76%	**<0.01**
SBPV - SBP-SD (mmHg)	16.20±13.34	9.91±6.80	8.04±4.43	**<0.01**
Relative value of SBP-SD-Cv (%)	10.66 %	7.34%	6.64%	**<0.01**
DBP (mmHg)	85.08±10.68	82.78±10.35	80.14±9.71	**<0.05**

BMI = body mass index, **WC** = Waist circumferences, **BP** = Blood pressure,
SBP = systolic BP, **BPV** = blood pressure variability
SBP-SD = standard deviation of systolic blood pressure,
DBP = diastolic BP. **Cv** - coefficient of variation = SD/mean*100 (%).

Table 2. Correlation between parameters of blood pressure and parameters of obesity (Spearman correlation coefficient)

PARAMETERS	SBPV	Blood pressure		Obesity		
		SBP	DBP	BW	BMI	WC
SBPV	-	0.633**	0.467**	0.428**	0.425**	0.356**
SBP	0.633**	-	0.777**	0.321**	0.359**	0.360**
DBP	0.467**	0.777**	-	0.170	0.262*	0.313**
BW	0.428**	0.321**	0.170	-	0.749**	0.743**
BMI	0.425**	0.359**	0.262*	0.749**	-	0.663**
WC	0.356**	0.360**	0.313**	0.743**	0.663**	-

** P<0.01* P<0.05

SBPV = systolic blood pressure variability, **SBP** = systolic blood pressure, **DBP** = diastolic blood pressure, **BW** = Body weight, **BMI** = body mass index, **WC** = Waist circumferences.

Table 3. Mean values of parameters of blood pressure and obesity after the first and the third visits

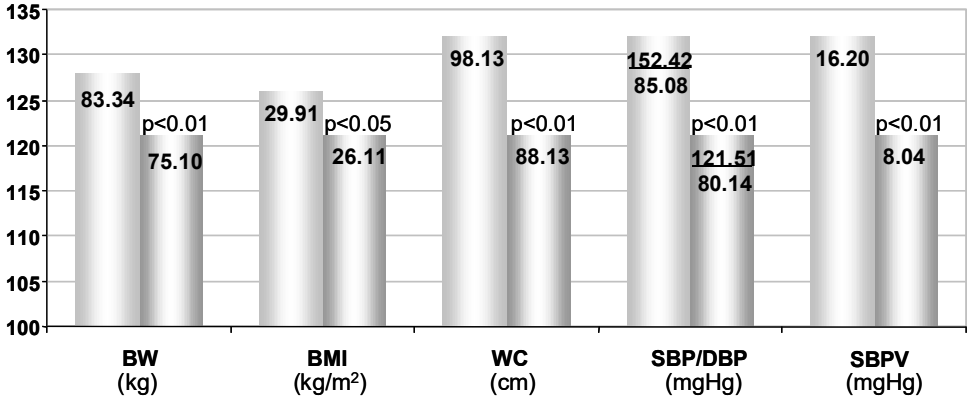
PARAMETERS	**Obese patients** (BMI>30)		**Non-obese patients** (BMI≤30)		P value
	N=153	N=51	N=147	N=249	
	1. visit	3. visit	1. visit	3. visit	
SBPV (mmHg)	16.52±9.41	11.29±5.67	15.87±17.05	7.37±3.94	0.001
SBP (mmHg)	154.21±15.20	127.06±8.30	150.56±14.59	120.37±7.75	0.007
DBP (mmHg)	87.04±9.18	81.18±7.40	83.03±8.75	79.93±5.22	0.058
BW (kg)	91.78±9.45	86.11±8.50	74.56±8.49	72.85±8.24	0.001
BMI (kg/m²)	33.46±2.21	32.05±1.90	26.21±2.45	24.89±2.85	0.001
WC (cm)	106.12±11.17	101.82±11.07	89.81±10.16	85.33±8.86	0.001

SBPV = systolic blood pressure variability, **SBP** = systolic blood pressure, **DBP** = diastolic blood pressure, **BW** = Body weight, **BMI** = body mass index, **WC** = Waist circumferences

Table 4. Characteristics across quartiles of the SBP-SD after the third visit

GROUPS	QUARTILES				P value
	1	2	3	4	
Obese patients	6.67±2.13	11.67±3.56	25.08±4.35	43.13±7.50	p<0.001
Non-Obese patiens	5.52±1.68	8.12±3.25	20.15±4.21	34.29±5.40	p<0.001

Graph 1. Change of values of obesity and blood pressure parameters at the beginninig and the end of the study



Parameters of obesity and SBPV are presented according to the unique SBP scale.

DISCUSSION

With the prevalence of obesity in the young, which should already be considered as a pandemic phenomenon (with all accompanying health risks), both prevention and health education, as well as identifying predictors for the development of obesity-related diseases are primary. Etiopathophysiology of development obesity and hypertension is very complicated including a great variety of factors (socio-economic factors, age, sex, menopause) and biological mechanisms (insulin resistance, chronic proinflamma tory state, stimulation of the sympathetic nervous system as well as the RAAS, renal and heart dysfunction) which lead to abnormal circardial rhythm of blood pressure. On the other hand, the role of fat tissue distribution, adipocyte characteristics and products had been involved in the attempt to explain the occurrence of hypertension in the presence of obesity.

Result of famous The Framingham Heart Study during 44-years, showed that obesity as an independent risk factor for cardiovascular disease and prevalence of obesity (evaluated throught body weihgt, including overweight and obesity) in 5209 hypertensive subjects, was approximately 26% in men and 28% in women (18,19). We have found higher prevalence of obesity, particularly abdominal obesity compared to total obesity in hypertensive patients (64% vs. 51%). During 22-months follow up there was a significant decrease in prevalences of obesity (23% vs. 17%). Our results showed significant differences in prevalence of central obesity in women, probably caused by both mutual up-regulation of protective hormones and fat storage and redistribution. Large adipocytes of visceral fat are dysfunctional due to the increased secretion of proinflammatory factors, reduced secretion of the insulin, estrogen and adiponectin, which increases the presence of chronic subclinical inflammation, the degree of insulin resistance, and metabolic disorders.

Due to frequent spontaneous variation of the blood pressure values during 24 hours, one month or one years, as well as the significance of obesity-related hypertension disease, is important for successful therapy and adequate control BP, without target organ damage. The greatest disadvantages of the classic measurement of BP are the inadequate insight in circardial rhythm and the phenomenon of peripheral resistance. On the other hand, hour 24-hours ambulatory blood pressure monitoring on healthy population has shown technical problems - significant variation on single measurements, compared with the values achived applying classic measurements (20).

The majority of studies (21,22) have analysed sensitivity of many different parameters of visit-to-visit BPV concerning the estimation of prediction of cardiovascular events (CVE): standard deviation, standard deviation independent of the mean (SDIM), coefficient of variation (CV), successive variation (SV), average real variability (ARV), range. We followed the standard deviation of SBP, as it was the simplest and the best indicator of future CVE due to the fact that other parameters of BPV were closely correlated and give similar reflections of BPV.

Just in the past decade, results from research groups (23-25) have documented a relationship between the reduced body weight and abdominal obesity and lower valuae of BP.

Understanding the complex relationship between obesity and hypertension these entities are important in clearing of the increasing prevalence of CVE. According to recent results (14,24,25), we have found, that values of visit-to-visit SBPV were significantly lower in non-obese hypertensive patients compared to obese hypertensive patients (7.37±3.94 vs. 11.29±5.67, $p<0.001$).

This is probably caused by the increase in sympathetic activity, insulin and vascular resistence, and the concentrations of proinflammatory cytokines, which leads to the increased heart pumping activity in hypertension

accompanied with obesity. In non-obese patients, the possible mechanisms in the decreased baroreceptor sensitivity, as the results of dysfunction of neurohumoral regulation, as well as the structural and funtional changes on heart and blood vessels.

Our results showed in consistent with the several study there was significant, positive and moderate correlations between SBPV and all parameters of obesity ($p<0.01$) and there was average decrease SBPV across visits (16.20 vs 9.91 vs 8.04 mmHg, $p<0.01$), and also, the highest SBPV was recorded in the 4th quartile in obese patients (43.13 mmHg). The results of the recent study (14) similarly designed as our research, which included more participants (14988) beloning to general population showed the positive correlation between both total and central obesity with BPV. BPV was 6.89 mmHg across study visits.

In addition, the recent The Dallas Heart Study (26) indicate the significance of the correlation between visceral fat mass distribution measured by dual X-ray absorptiometry (the modern advanced way of measuring abdominal obesity) and short-term and long-term BPV during 5-month period of observing 2595 overweight subjects with mean BP 127/79 mmHg and BMI 29 kg/m^2 Long-term BPV was 9.8 mmHg across overall visits.

Our study included only hypertensive patients with untreated BP values and the registered values of visit-to-visit SBPV was 8.2 mmHg, which was higher compared to general population in the previously mentioned study. Our results indicate the importance of distribution of fat mass, as well as the more significant impact of BMI as indicator total obesity than of WC, as parameter of central obesity on SBPV in hypertensive patients.

The recent study Tadic and all. (15) showed the relationship between parameters of obesity, BPV and remodeling of right ventricle in hypertensive patients with different nutrition degree. The increased body mass leads to the increased metabolic needs of an organism, extracellular fluid volume expansion, faster blood flow through both adipose and non-adipose tissue and organs, especially through heart, kidneys, skeletal muscles, which parallelly leads to the cardial output increase. However, it can later lead to reduction of "reserve" blood flow and damage of flow endothelium-dependent vasodilation. The rapid endothelial dysfunction along with the arterial stiffening and blood-flow variability lead to vascular endothelial dysfunction and the cascade of CVE, which was also proved in the studies (27-29).

Additionally, central fat accumulation contributes to the changes in the serum levels of adipokines, and also contributes to the decrease of insulin sensitivity, as well as the increase of the activity of the sympathetic nervous system. Large adipocytes are dysfunctional due to the increased secretion of proinflammatory factors, reduced secretion of the protective hormones (insulin, estrogen and adiponectin), which increases the presence of chronic subclinical inflammation, and dysregulation of metabolic homeostasis. On the other hand, in hypertensive state, the formed "circulus vitiosus" is easily maintained by the upregulated levels of proinflammatory cytokines and adipokines (30).

Finally, during 22-months follow up, the appropriate diet, the increase in physical activity and antihypertensive therapy, resulted in reduced obesity parameters, particularly body weight, and adequate control of BP, which significantly decreased the values of SBPV.

Similarly to the results presented in a recent study (31) our results showed the long-term effect weight loss on BP, statistically significant average decrease of SBPV for 8.2 mmHg, BP for 31/8 mmHg, BMI for 3.8 kg/m^2 and WC for 10 cm.

Reduction of all parameters of obesity is the most important step in reducing hypertension, and adequate control of BP and metabolically 'healthy' obesity. The results of a recent study showed that during a four- year-follou-up of 181 obese hypertensive patients, a 10 percent weight-loss produced an average of a 4.3/3.8 mmHg decrease in BP. The results of this study showed that the of weight loss for 1 kg produced an average of a 2.1 mmHg reduction in SBP, which could be the results of multiple antihypertensive effects and mechanisms: decrease visceral fat of accumulation, lower simpthatectic nerve activity and improved elasticity of blood vessels. Our results showed that reduction weight for 1 kg produced an average of a 3.76/0.61 mmHg reduction in BP and 1 mmHg in SBPV. Also, reduction weight increased the effectiveness of BP treatment.

Significant factor that contribute to the impact of obesity on BPV in hypertensive patients are distribution of body fat, duration of obesity as well as the degree of target organ injury. The additional important factor is how obesity parameters variability during the long period (several years) influences the BPV, because prolonged obesity leads to development of uncontrolled blood pressures and cardiovascular complications (8,32,33).

CONCLUSION

Results of this study, showed that obesity is strongly correlated with higher variability of systolic blood pres- sure across study visits. During 22-months, reduction of body weight was associated with reduction of blood pres- sure values, and lower value of blood pressure variability. Persistent decrease of both BP and long term visit-to-visit variability may explain lower cardiovascular risk in obese-related diseases.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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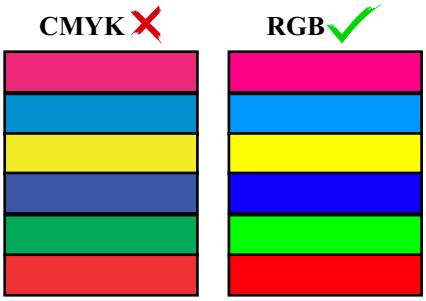

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