World Lung Cancer Day
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According to the American Lung Association, lung cancer is the leading cause of death among cancer patients. When diagnosed early enough, lung cancer survival rates increase five or more years by almost 56%. However, prevention is necessary in order to detect and treat lung cancer before it spreads. Therefore, every year on August 1, World Lung Cancer Day is celebrated with the aim of raising awareness about the disease, its causes, and ways of prevention and treatment.

Prema Američkom udruženju za pluća, karcinom pluća je vodeći uzrok smrti među obolelima od karcinoma. Kada se dijagnostikuje dovoljno rano, stopa preživljavanja od karcinoma pluća se povećava pet ili više godina za gotovo 56%. Međutim, prevencija je neophodna kako bi se karcinom na vreme otkrio i lečio, pre nego što se proširi. Stoga se svake godine, 1. avgusta, obeležava Svetski dan borbe protiv karcinoma pluća sa ciljem podizanja svesti o toj bolesti, njenim uzrocima i načinima prevencije i lečenja.

The influence of personality traits and coping strategies on the quality of life of patients with relapsing-remitting type of multiple sclerosis

Uticaj osobina ličnosti i strategija suočavanja sa stresom na kvalitet života obolelih od multiple skleroze relapsno remitentnog toka

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Abstract

Background/Aim. Multiple sclerosis (MS) is a chronic, progressive autoimmune disease of the central nervous system, that can reduce quality of life. Personality traits and coping strategies are significant factors in interpersonal variance regarding quality of life. The aim of this study was to examine relations between personality traits and coping strategies in patients with relapsing-remitting (RR) type of MS, and to examine relations of personality traits, coping strategies, and quality of life in patients with RR type of MS. **Methods.** This observational cross-sectional study included 66 patients (34 female, 32 male) with clinically definitive MS, based on the McDonald criteria. The following clinical scales were used to examine personality traits, coping strategies, and quality of life: Revised NEO Personality Inventory (NEO-PI-R), Coping Strategies Inventory (CSI), and Multiple Sclerosis Quality of Life (MSQOsL-54). **Results.** The average age of the patients was 41.6 ± 7.1 years. The Pearson's correlation coefficient showed that with regards to coping strategies, the only positive correlation existed with neuroticism (r = 0.502). The correlation was negative between neuroticism and physical (r = -0.284, $p < 0.05$) and mental health (r = -0.289, $p < 0.05$), as well as between passive coping strategies and mental health (r = -0.358, $p < 0.05$), meaning that the patients with a higher level of neuroticism, and who relied on passive coping strategies, had a lower quality of life. **Conclusion:** This research showed the importance of personality traits and coping strategy assessment in patients with RR-type MS. Participation in cognitive-behavioral therapy, with emphasis on active coping strategies, can improve the quality of life in patients with MS.

Key words:
adaptation, physiological; multiple sclerosis; personality; recurrence; quality of life; serbia; stress, psychological; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Multipla skleroza (MS) je hronična, progresivna autoimunska bolest centralnog nervnog sistema, koja može narušiti kvalitet života. Osobine ličnosti i strategije suočavanja sa stresom su bitan faktor za promenljivost između osoba u odnosu na kvalitet života. Cilj istraživanja je bio ispitivanje povezanosti osobina ličnosti i strategija suočavanja kod obolelih od MS relapsno remitentnog (RR) toka, kao i isptitivanje povezanosti osobina ličnosti i strategija suočavanja sa kvalitetom života kod obolelih od MS RR toka. **Metode.** Opservacionom studijom preseka obuhvaćeno je 66 bolesnika (34 osobe ženskog i 32 osobe muškog pola) sa dijagnozom klinički definitivne MS RR toka, postavljene na osnovu Mek Donaldovih kriterijuma. Za procenu osobina ličnosti, strategija suočavanja i kvaliteta života, korišćene su sledeće kliničke skale: Revidirani NEO-Inventar ličnosti (NEO-PI-R), Inventar strategija suočavanja (*Coping Strategies Inventory*) i Kvalitet života MS (*Multiple Sclerosis Quality of Life-54*). **Rezultati.** Prosečna starost ispitanika je bila $41,6 \pm 7,1$ godina. Pirsonov koeficijent korelacije pokazao je da je sa korišćenjem pasivnih strategija suočavanja pozitivno povezan jedino neuroticizam (r = 0,502). Korelacija je bila negativnog smera između neuroticizma i fizičkog (r = -0,284, $p < 0,05$) i mentalnog zdravlja (r = -0,289, $p < 0,05$), kao i između pasivnih strategija suočavanja i mentalnog zdravlja (r = -0,358, $p < 0,05$), što znači da su bolesnici kod kojih je neuroticizam bio više izražen, i koji su koristili pasivne strategije, imali lošiji kvalitet života. **Zaključak.** Rezultati ovog istraživanja ukazuju na značaj

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procene ličnosti i strategija suočavanja kod obolelih od MS. Uključivanje u kognitivno-bihevioralnu terapiju i favorizovanje aktivnih strategija suočavanja može poboljšati kvalitet života obolelih od MS.

Introduction

Multiple sclerosis (MS) can decrease quality of life (QOL) by affecting the patient's ability to work or perform regular functions in everyday life. Patients with MS often experience symptoms involving discomfort and pain. They feel frustrated and isolated, which significantly lowers their QOL [1]. Personality traits and coping strategies are recognized as a notable factor in interpersonal variances regarding QOL [2, 3].

High neuroticism indicates that the individual will experience negative emotions and frustration dealing with everyday stress of living with a chronic neurological disease such as MS. High neuroticism leads to increased pain, somatization, and can be the cause of early death [4]. Individuals with high neuroticism are vulnerable to feelings of helplessness and irrational thoughts, which makes them less capable of impulse control [5].

Adaptive personality traits (extroversion and conscientiousness) are less affected by stress. That means that the patients manage their illness more efficiently, which is important for their psychological well-being and quality of life [6].

Personality traits can predict coping strategies [7]. Relations between personality traits and coping strategies have been the subject of many studies. The results of these studies imply that neuroticism correlates positively with passive coping strategies [8].

Adaptive personality traits correlate positively with active coping strategies (solving problems, seeking support from others, etc). However, some researchers have nevertheless failed to find a significant connection between adaptive personality traits and active coping strategies [8].

For patients with MS, coping strategies play a major role in adapting to psycho-social problems and quality of life. Passive strategies involve withdrawal, avoidance, lower quality of life, and increase the risk of psychological issues such as depression and anxiety [9].

In his study, Strober [2] found that the ''D personality'' type (a combination of a higher neuroticism and a lower extroversion) uses maladaptive coping strategies, and that this personality type is related to lower QOL in patients with MS.

The aim of this study was to examine relations between personality traits and coping strategies in patients with RR-type MS, and to examine relations between personality traits and coping strategies with the QOL in patients with RR-type MS. These relations have been scrutinized in developed countries. So far, no such studies concerning the MS-affected population have been conducted in Serbia.

Methods

Study design, time, and place

The study was designed as an observational cross-sectional study, and it was carried out at the Clinic of Neurology and the Clinic of Psychiatry at the Military Medical Academy (MMA) in Belgrade. The study included patients with RR-type MS treated at the Clinic of Neurology, MMA from March 2014 until March 2015. In accordance with the regulations of Good Clinical Practice (GCP), and with prior approval by the Ethics Committee of the Military Medical Academy Committee (decision issued on December 6, 2013, approval number: 0901-2-50/15) the patients had signed a form declaring consent to participate in the study, having been provided with all necessary information and explanations, including their opportunity to ask relevant questions.

Participants

The study included 66 patients (32 male, 34 female), aged 18–55, diagnosed with clinically definitive MS based on McDonald criteria [10]. The diagnoses were made by a neurologist, who also provided the patients' scores on the Expanded Disability Status Scale (EDSS). Inclusion criteria were: either gender, age 18–55, consent form signed, definitive clinical diagnosis of RR-type MS established according to the revised 2010 McDonald criteria and EDSS score ≤ 6.0.

Exclusion criteria were: central nervous system (CNS) diseases other than MS, major head injury, clinically definitive relapse of the basic illness in the past 3 months, corticosteroid therapy in the past 3 months, history of hepatic/renal insufficiency, diabetes mellitus, arterial hypertension, use of alcohol or psychoactive substances, malignant conditions, impaired hearing, binocular visual acuity $\leq 75\%$, and pregnancy [2].

Instruments

Three clinical scales were used in this study.

Revised NEO Personality Inventory (NEO-PI-R), containing 240 items, measures five major personality traits (neuroticism, extroversion, openness, agreeableness, and conscientiousness). It offers an all-encompassing, detailed assessment of personality of the respondents [11–13]. NEO-PI-R assessment was performed by a clinical psychologist.

Coping Strategies Inventory (CSI) is a self-report scale containing 72 items. It is applied to assess cognitive ability

and behavior relating to specific stressors. Respondents are required to mark the extent to which each coping strategy has been used to cope with the selected stressor. Answers are marked on the five-item Likert scale, with eight primary subscales: problem solving, cognitive restructuring, social support, expression of emotions, problem avoidance, wishful thinking, social withdrawal, and self-criticism. Problem solving, cognitive restructuring, avoidance, and wishful thinking are problem-focused coping strategies, where the first two are active and the other two passive. Social support, expression of emotions, self-criticism, and withdrawal are emotion-focused coping strategies, where the first two are active and the other two passive [14].

Multiple Sclerosis Quality of Life (MSQOL-54) is a self-report scale that measures quality of life. This instrument contains 54 items with 12 subscales. The subscales are: physical functionality, role limitations – physical, role limitations – emotional, pain, emotional well-being, energy, health perception, social function, cognitive function, health distress, overall quality of life, and sexual function [15, 16].

Statistical analysis

The data were analyzed using the SPSS v. 23.0 statistical software (IBM Corp., USA). Sociodemographic clinical data are presented through descriptive statistical methods: absolute and relative numbers, arithmetic mean, standard deviation (SD).

The Pearson's correlation coefficient was used in determining the correlation between personality traits and coping strategies, as well as between coping strategies, personality traits, and quality of life. All *p*-values less than 0.05 were considered significant.

The correlation between personality traits and gender, duration of illness, and the EDSS score was analyzed using MANOVA (multivariant variance analysis). All *p*-values less than 0.05 were considered significant.

Results

The average age of the patients was 41.6 ± 7.1 years. Sociodemographic and clinical characteristics of the patients are presented in Table 1.

Within our patients' groups, there was a statistically significant difference between men and women regarding certain personality traits (MANOVA – Wilks $\lambda = 0.718$, F = 4.326, $p = 0.002$). The personality trait contributing to this difference was neuroticism, which was statistically significantly more present in women than in men (F = 4.015, $p = 0.05$) (Table 2).

The Pearson's coefficient showed that the patients with RR-type MS with a high conscientiousness score had lower score on the EDSS. The correlation was negative and weak,

Table 1

Sociodemographic and clinical characteristics of patients with multiple sclerosis

Variable	Values
Gender, n (%)	
male/female	32 (48.5) / 34 (51.5)
Age (years), mean ± SD	41.6 ± 7.1
Education (years), n (%)	
8–12	4 (6.0)
12–16	46 (70.1)
> 16	16 (23.9)
Marital status, n (%)	
unmarried	7 (10.6)
married	55 (83.3)
divorced / widowed	4 (6.1)
Invalidity, n (%)	
none/yes	34 (51.5) / 32 (48.5)
Psychiatric assistance, n (%)	
yes / no	11 (16.7) / 55 (83.3)
Duration of illness (years), mean ± SD	8.1 ± 5.1
Time since first symptoms (in years), mean ± SD	10.1 ± 4.9
EDSS score, mean ± SD (min–max)	2.4 ± 1.1 (1.0–4.5)

SD – standard deviation; min-max – minimum–maximum;
EDSS – Expanded Disability Status Scale.

Table 2
Personality traits in patients with multiple
sclerosis according to gender

Variable	Male	Female	F	*p*
Neuroticism	141.10	147.68	4.015	**0.050**
Extroversion	151.50	154.84	0.864	0.356
Openness	158.23	165.84	3.755	0.057
Agreeableness	158.00	161.65	1.360	0.248
Conscientiousness	164.27	162.16	0.484	0.489

Results are given as arithmetic mean.

but significant (r = -0.285, *p* < 0.05) (Table 3).

The Pearson's coefficient showed that there was no correlation between personality traits and duration of the illness (Table 3).

The evaluation of correlation between personality traits (NEO-PI-R) and coping strategies (CSI) in patients with RR-type MS is presented in Table 3.

The Pearson's coefficient showed that there was positive correlation only between passive coping strategies and neuroticism (r = 0.502, *p* < 0.01), which means that the patients with higher levels of neuroticism were more likely to use passive coping strategies. All other personality traits were moderately related to active coping strategies.

The assessment of correlation between NEO-PI-R and MSQOL-54 in patients with RR-type MS is presented in Table 3.

The Pearson's correlation coefficient showed that all personality traits, except conscientiousness, correlate positively (at low intensity) with QOL, contentment, mental and physical health. This means that the higher the expression of extroversion, openness and agreeableness, the better QOL. The correlations were significant (*p* < 0.05).

The correlation between neuroticism and physical (r = -0.284) and mental health (r= -0.289) was negative, meaning that the patients with more pronounced neuroticism will report more negative assessment of their contentment, physical and mental health, or the overall QOL.

When it comes to CSI and MSQOL-54, the Pearson's coefficient showed a strong negative correlation between using passive coping strategies and perceived physical and mental well-being (r = -0.358) (Table 4).

The patients who relied less on passive strategies reported a more positive estimation of their contentment and mental health. The correlation was significant (*p* < 0.01).

Discussion

Serbia is one of the countries with a relatively high prevalence of MS. According to the latest figures, there are more than 5,000 registered patients with MS in Serbia [17]. To the best of our knowledge, there was no research on the influence of personality traits and coping strategies on the QOL in patients with MS in Serbia.

The gender balance in our study was almost perfect (34 females and 32 males), even though research indicates that women are three times more likely to develop MS [18]. This can be explained by the fact that the military health care institution is focused on military insurance users and active military personnel, who are predominantly male. Previous research shows that women today make up from 8.7% to 19.3% of the entire military personnel in the Army of Serbia [19].

Personality traits are one of the main factors affecting psychological functionality, as well as the exposure and the ability of individuals to deal with stressful situations in life. A study performed in 2018 on a sample of adult twins (n = 973 pairs), showed the existence of gender differences, based on phenotype rather than genetics, in personality traits, but also that these differences are smaller than a previous study by South et al. [20] implied. Female twins had higher neuroticism compared to male twins [20]. The results of our research also confirmed that female patients with MS had more expressed neuroticism. It would be interesting to investigate whether personality traits at the onset of a chronic disease change over time, since beside physiological changes, such a disease also leads to lifestyle changes (mobility, ability to work, finances, relations with friends and family, life role…), all of which can lead to personality changes in patients [21, 22]. Nevertheless, current studies indicate that personality traits are resistant to effects of

Table 3

Correlation between personality traits and Expanded Disability Status Scale (EDSS) score, illness duration, coping strategies and quality of life in multiple sclerosis patients

Variable	Neuroticism	Extroversion	Openness	Agreeableness	Conscientiousness
EDSS score	0.169	-0.014	-0.107	-0.196	**-0.285***
Illness duration	-0.044	-0.039	-0.001	-0.002	-0.033
Active strategies	0.234	**0.365***	**0.268***	**0.429****	**0.282***
Passive strategies	**0.502****	0.240	0.186	0.236	0.196
Physical health	**-0.284***	**0.254***	**0.257***	**0.261***	0.229
Mental health	**-0.289***	**0.251***	**0.271***	**0.294***	0.193

p < 0.05; ***p* < 0.01.

Table 4

Correlation between coping strategies and quality of life in multiple sclerosis patients

Variable	Active strategies	Passive strategies
Physical health	0.107	0.241
Mental health	-0.002	**-0.358****

***p* < 0.01.

chronic disease [21, 22]. The results of our study show that personality traits are not affected by the duration of the illness, which concurs with the findings of the previous studies mentioned above [21, 22].

Individuals with high neuroticism experience daily frustrations as problematic and are more prone to mood disturbances, self-criticism, hypochondria, and stress vulnerability [23]. Our results indicate that higher neuroticism correlates with negative assessments and physical health (physical functionality, pain, energy, social and sexual function) as well as mental health (emotional problems, cognitive functions, emotional well-being). In other words, patients with RR-type MS with higher neuroticism have decreased quality of life, while RR-type MS patients with more expressed extroversion, openness, and agreeableness have higher quality of life and better physical and mental health. Recent studies in Austria and Italy also suggest that neuroticism correlates with lower QOL in MS [24, 25].

To the best of our knowledge, there is only one study that while examining the association of personality traits and cognitive impairment in MS, also investigated relations between personality traits and EDSS score. Benedict et al. [26] found significant correlation between abnormal personality traits and heightened EDSS score, while the results of our study do not show that. A possible explanation could be lower average EDSS score in our study sample: 2.4 (range 1.0–4.5) compared to the sample used by Benedict et al. [26], where the average EDSS score was 4.1 (range 1.0–8.5), with a significantly smaller sample size of 34 compared to 66 in our study.

Our results showed that RR-type MS patients with higher conscientiousness score had lower score on the EDSS (r = -0.285, $p < 0.05$). It is consistent with the findings that higher conscientiousness implies better organisational skills, deliberation, and healthy behaviour, while lower conscientiousness correlates with impulsiveness and risky behaviour (smoking, alcohol etc.). This is important, considering the results of previous research that showed that smoking had been both a risk factor for developing MS and an accelerator of the illness progression [27, 28].

Personality traits can predict coping strategies [29]. Coping strategies reflect repertoires of responses to stress, both cognitive and behavioural, that the individual will use to manage external and/or internal demands of a stressful event. Passive coping (wishful thinking, withdrawal, avoidance, self-criticism), focuses on ignoring the stressor and involves minimizing, denial, or avoidance in dealing with the stressful situation. Such coping is maladaptive and becomes a new

source of stress. Research so far shows that passive coping correlates with increased pain, invalidity, and depression [30].

In our study, RR-type MS patients with higher neuroticism were shown to be significantly more likely to use passive strategies of coping with stress. Our results also show that other personality traits correlate, albeit weakly, with active strategies, while neuroticism does not correlate with active strategies at all. Research so far also shows that individuals with high neuroticism resort to passive strategies [2, 7, 29, 31]. As for adaptive personality traits, some studies suggest that individuals with high extroversion use active strategies, while other studies do not find any significant correlation between adaptive personality traits and active coping strategies [8, 29]. This discrepancy could be explained by the low intensity of the correlation that our study found.

Patients with RR-type MS who rely on passive strategies estimate their own mental health (psychological well-being) as lower. These results are in accordance with research conducted so far on coping strategies in MS patients. Studies performed on 26 individuals in Mexico [9] and 34 individuals in Lebanon [32], respectively, showed that reliance on active coping strategies leads to more positive assessment of QOL.

Limitations of the study

Our study involved only the patients with RR-type MS; future research on these topics in Serbia should include other clinical types of MS as well. Another significant limitation was that the quality of life questionnaire is MS-specific, making it inapplicable on healthy sample groups.

Conclusion

Our study shows the importance of personality and coping strategy estimation in patients with MS, as they represent major factors affecting their quality of life. Our results show that higher neuroticism and passive coping strategies are connected to lower quality of life in patients with MS.

Therefore, treatment of MS should be multidisciplinary, and take into account individual characteristics in adapting to a chronic disease.

Since personality traits can change during one's life, especially after significant events, inclusion into cognitive-behavioural therapy and adopting active coping strategies can improve MS patients' quality of life.

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The correlation of CBCT analysis derived bone density parameters with primary implant stability – a clinical study

Korelacija vrednosti gustine kosti dobijene pomoću kompjuterizovane tomografije konusnim zrakom i primarne stabilnosti implantata – klinička studija

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Abstract

Background/Aim. There are numerous studies on the usefulness of computed tomography (CT) in the assessment of the bone volume and morphology and on the relationship between CT and primary implant stability. But there is the scarcity of data about the correlation between bone density and the value of primary implant stability. The aim of this study was to examine the correlation of cone beam CT (CBCT) analysis derived bone density with primary stability value. **Methods.** Clinical prospective experimental study was conducted on 38 healthy patients missing one tooth in the lateral region. It was planned to install Bredent Blue Sky Narrow self-taping dental implants with dimensions 3.5 × 10 mm. During preoperative preparation, a CBCT scan was performed on Planmeca apparatus, followed by preimplantation measurements and planning in the CBCT apparatus software (Romexis). The mean value of the average bone density was automatically generated and expressed in Hounsfield units (HU). Upon implant placement, we performed measurements of the primary implant stability using Osstell apparatus. **Results.** Of the 38 patients included in the study, there were 68.4% male patients and 31.6% female patients. The arithmetic mean of the measured bone density of all subjects in the study amounted to 536.2 HU. The arithmetic mean of dental implant primary stability for all subjects in the study was 68.7 ISQ. There was a statistically significant strong positive connection between HU and ISQ ($r = 0.744$, $p < 0.001$). Higher HU values were connected to higher ISQ values. In the multivariate linear regression model, statistically significant predictors of higher ISQ values: males (B = 4.669; $p = 0.047$) and higher HU values (B = 0.032; $p < 0.001$). **Conclusion.** In our clinical study, there was a statistically significant strong positive correlation between the bone density expressed in HU units, measured in the software of the CBCT device and the primary stability of dental implants expressed in ISQ units.

Key words:
bone, density; tomography, computed, cone beam; implants, dental; treatment, outcome.

Apstrakt

Uvod/Cilj. Postoje mnogobrojne studije o korisnosti kompjuterizovane tomografije (KT) u proceni volumena i morfologije kosti, kao i o odnosu između KT i primarne stabilnosti implantata. Međutim, malo je podataka o povezanosti gustine kosti i vrednosti primarne stabilnosti implantata. Cilj studije bio je da se ispita povezanost gustine kosti dobijene putem KT konusnim zrakom i vrednosti primarne stabilnosti. **Metode.** Klinička prospektivna eksperimantalna studija je obavljena kod 38 zdravih pacijenata sa nedostatkom jednog zuba u bočnoj regiji. Planirana je ugradnja samourezujućih *Bredent Blue Sky Narrow* dentalnih implantata dimenzija 3,5 × 10 mm. U preoperativnoj pripremi urađen je snimak KT konusnim zrakom na aparatu Planmeca, a zatim su u softveru aparata za KT konusnim zrakom (Romexis) izvršena preimplantološka merenja i planiranja. Srednja vrednost prosečne gustine kosti je automatski dobijena i izražena u Hounsfield jedinicama (HU). Nakon postavljanja implantata izvršili smo merenja primarne stabilnosti implantata pomoću Osstell aparata. **Rezultati.** Od 38 pacijenata uključenih u studiju, 68,4% je bilo muškog, a 31,6% ženskog pola. Aritmetička sredina izmerene gustine kosti svih ispitanika u istraživanju iznosila je 536,2 HU. Aritmetička sredina primarne stabilnosti dentalnih implantata svih ispitanika u istraživanju iznosila je 68,7 ISQ. Utvrđena je statistički značajna jaka pozitivna povezanost HU

i ISQ (r = 0,744, p < 0,001). Više vrednosti HU bile su povezane sa višim vrednostima ISQ. U modelu multivarijantne linearne regresije statistički značajni prediktori viših vrednosti ISQ bili su: muški pol (B = 4,669; p = 0,047) i više vrednosti HU (B = 0,032; p < 0,001). **Zaključak.** U našoj kliničkoj studiji smo pokazali da postoji statistički značajna jaka pozitivna povezanost između gustine kosti izražene HU jedinicama, izmerene u softveru aparata za KT konusnim zrakom i primarne stabilnosti dentalnih implantata izraženih u ISQ jedinicama.

Ključne reči:
kost, gustina; tomografija, kompjuterizovana, konusna; implantati, stomatološki; lečenje, ishod.

Introduction

Dental implants are used widely and routinely to treat partial or total edentulism [1]. The success of implant therapy depends on a number of factors that may be related to the patient as well as to implant placement procedures. Osseointegration is directly related to implant therapy. Primary implant stability can be used to estimate and predict the success of osseointegration [2–4]. Resonance Frequency Analysis (RFA) method, using Ostell Mentor apparatus, enables clinical measurement of implant stability and the monitoring of biological tissue response and osseointegration over time. The measured resonant frequency amplitude, i.e. the registered vibration generated by the pre-energized implant-mounted smartpeg magnet, is shown numerically and graphically on the analyzer, and its maximum represents the implant stability quantified through implant stability quotient (ISQ) units i.e. implant stability coefficient whose values can range from 0 to 100. The higher the ISQ, the more stable the implant [5].

The most significant factors affecting the primary implant stability are: implant design, surgical technique, bone quality and quantity [6].

Implant macro design plays an essential role in achieving adequate primary stability. Macro design entails implant type and thread design, as well as depth, width, density, angle and thread shape. The difference in primary stability between self-tapping implants, characterized by sharp thread edges and non-self-taping implants, whose threads have a rounded profile [7], as well as implants with parallel walls [8], is particularly noteworthy.

Bone density plays an important role in the success of implant therapy [9]. For this reason, evaluation of bone tissue density stands as an integral part of pre-implant clinical and radiographic examination. The introduction of cone beam computer tomography (CBCT) represents a significant progress in the use of computed tomography [10].

Unlike the classifications based on the subjective assessment of the given criteria according to Misch [11], and Lekholm and Zarb [12], Norton and Gamble [13] proposed bone density classification based on computed tomography (CT) images using interactive software, where bone quality data at the future implant site are obtained based on objective and quantitative result expressed in Hounsfield units (HU). HU unit represents a qualitative radiolucency measure of different tissues at CT. HU scale ranges from – 1,000 (air), 0 (water) to + 1,000 (bone), where the value of this unit depends on the tissue density through which the X-rays pass.

Clinical studies showed higher survival rate for dental implants placed in the mandible [14–16]. The available literature shows studies with a lower survival rate for implants placed in the maxilla [17, 18]. A higher failure rate was recorded in immediate implants placement in maxilla [19]. It is believed that a deviation in survival rates of implants located in the maxilla and mandible results from the bone condition around the implant. It is obvious that, compared to the maxilla, the bone in mandible around the implant has a better volume and quality [20].

Some studies have shown that in lower bone density, type 3 and type 4 according to Lekholm and Zarb bone classification, using self-tapping implants in combination with modified implant bed preparation can achieve superior primary stability compared to classical surgical technique with non-self-tapping implants [20].

In literature, there are numerous studies on the usefulness of CT in terms of the assessment of the bone volume and morphology [21–23] as well as several clinical studies on the relationship between CT values and primary implant stability [24, 25]. However, there have not been a sufficient number of clinical studies attempting to determine the correlation between bone density and the value of primary implant stability [26–29].

The aim of this study was to examine the correlation of CBCT analysis derived bone density with the primary stability value.

Methods

This study was approved by the Institutional Review Board of the Public Health Institution, Clinical Center of Podgorica, Montenegro (No. 0301-4536/1) and written consents were obtained from all subjects. Clinical prospective experimental study was conducted on 38 healthy patients missing one tooth in the lateral region without defects and augmentation, indicated for implant placement under favorable conditions.

The study inclusion criteria were: missing one tooth in the premolar region and/or molar region of the upper and lower jaw, height of the alveolar ridge ≥ 11 mm and width 6 ≤ mm, the remaining teeth repaired together with a signed statement of consent for the procedure as well as completed and signed questionnaire about the patient's health.

The study exclusion criteria were: health conditions contraindicated for the execution of a surgical procedure, pronounced alveolar ridge atrophy, presence of parafunctions and poor oral hygiene.

In all 38 patients missing one tooth, who met the requested criteria, it was planned to install Bredent Blue Sky Narrow self-taping dental implants with dimensions 3.5×10 mm. During the preoperative preparation, a CBCT scan was performed on Planmeca apparatus, followed by pre-implantation measurements and planning in the CBCT apparatus software (Romexis).

The above mentioned CBCT apparatus software enables the volume of bone tissue density to be analyzed at the site of a virtually positioned implant. The bone volume limits for analyzing bone density surrounding the virtually positioned implant are set to include 1 mm of bone around the implant. After setting the bone volume limits for analysis, the mean value of the average bone volume is automatically generated and expressed in HU (Figure 1).

The implants were mechanically placed with a torque of 35 N/cm^2 (Figure 2). Upon implant placement, we performed measurements of the primary implant stability using Osstell apparatus. There was the following procedure with Osstell apparatus: appropriate Smartpeg is placed on the implant, in this case type 49 and tightened manually. The Osstell mentor probe is placed with Smartpeg in 4 positions (buccal, oral, mesial and distal) and the primary stability mean value is calculated (Figure 3).

Statistical analysis

For the analysis of primary data we used descriptive statistical methods, methods for testing statistical hypotheses, methods for examining correlation and methods for examining the relationship between the outcome and potential predictors. Depending on the type of variables, the descriptive data are displayed as n (%) and as mean ± standard deviation (SD) or median (range). The *t*-test was used to test the statistical hypothesis. To test the correlation between two variables, the Pearson linear correlation coefficient was used. The
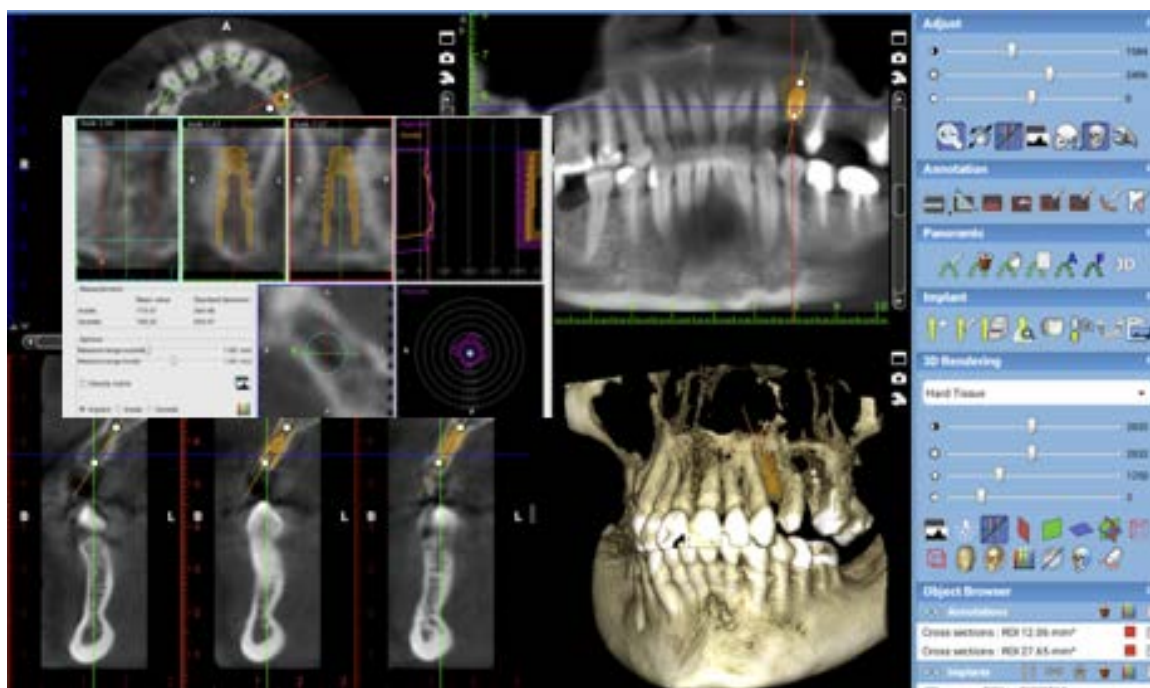
Fig. 1 – Virtual implant placement planning and bone density measurement.

Fig. 2 – Implant site preparation.
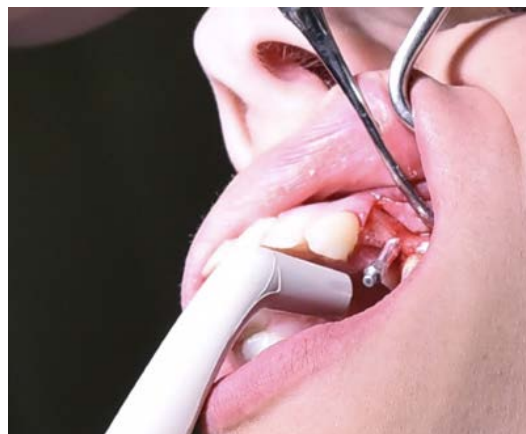
Fig. 3 – Primary stability measurement by Ostell mentor.

linear regression was used to investigate the relationship between ISQ and potential predictors. Statistical hypotheses were tested at a statistical significance level of 0.05. The obtained data were statistically processed to obtain a correlation between the mean value of the bone density and the value of primary stability of the placed implants.

Results

Of 38 patients included in the study, there were 68.4% male patients and 31.6% female patients. A total of 19 implants were placed in the lateral mandible region and 19 in the lateral maxilla region (Table 1).

Table 1

Distribution of subjects by gender and implantation jaw

Gender	Mandible	Maxilla	Total
Male	15 (78.9)	11 (57.9)	26 (68.4)
Female	4 (21.1)	8 (42.1)	12 (31.6)
Total	19 (100.0)	19 (100.0)	38 (100.0)

All values are expressed as numbers (percentages).

The average age of all subjects in the study was 44.6 ± 6.8 years. The youngest subject was 33 years old and the oldest 57 years. A total of 81.6% of subjects were under 50 years of age and 18.4% of patients were over 50 years old (Table 2).

Table 2

Distribution of subjects by age and bone density

Age (years)	n	Bone density (HU)	
≤ 50	31	555.1 ± 159.7	526.3 (350.5–944.7)
> 50	7	452.3 ± 105.4	443.2 (337.6–594.4)

Values are expressed as mean ± standard deviation and median (minimum–maximum).

The arithmetic mean of the measured bone density of all subjects in the study amounted to 536.2 HU [95% confidence interval (CI): 485.1–587.2] (Figure 4).
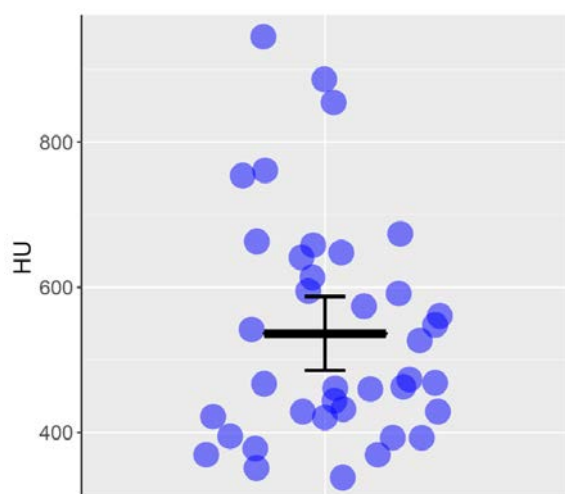
Fig. 4 – Hounsfield unit (HU) values in research subjects.

The arithmetic mean and standard deviation of bone density in the mandible was 642.6 ± 146.8 HU, while in maxilla it was 429.8 ± 64.3 HU, which was a statistically significant difference (t = 5.789; p <0.001). Significantly higher HU values were recorded in the mandible (Table 3).

Table 3

Jaw bone density of research subjects

Implantation jaw	n	Bone density (HU)	
Mandible	19	642.6 ± 146.8	640.7 (420.1–944.7)
Maxilla	19	429.8 ± 64.3	428.4 (337.6–591.0)

Values are expressed as mean ± standard deviation and median (minimum–maximum).
HU – Hounsfield units.

The arithmetic mean of dental implant primary stability for all subjects in the study was 68.7 ISQ (95% CI 65.8–71.5) (Figure 5).
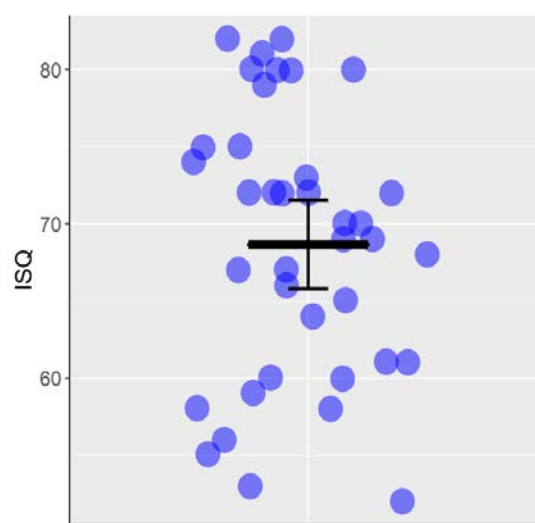
Fig. 5 – Implant stability quotient (ISQ) values.

The arithmetic mean and standard deviation of the dental implants primary stability in the mandible amounted to 73.2 ± 8.1 ISQ, while in maxilla it was 64.2 ± 7.0 ISQ, which ws a statistically significant difference (t = 3.673; p = 0.001). Significantly higher ISQ values were found in the mandible (Table 4).

Table 4

Jaw bone implant stability quotient (ISQ) of research subjects

Parameter	n	Implant stability quotient	
Mandible	19	73.2.6 ± 8.1	73.0 (53.0–82.0)
Maxilla	19	64.2 ± 7.0	64.0 (52.0–75.0)

Values are expressed as mean ± standard deviation and median (minimum–maximum).

There was a statistically significant strong positive connection between HU and ISQ (r = 0.744, p < 0.001). Higher HU values were connected to higher ISQ values (Figure 6).

The model of multivariate linear regression with ISQ as a dependent variable included those predictors that were statistically significant in the model of univariate linear regression at a significance level of 0.05.
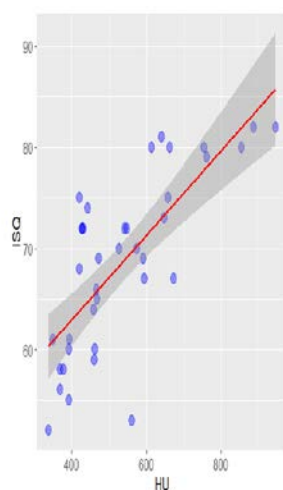
**Fig. 6 – Bone density (HU) and implant
primary stability (ISQ) ratio.**

The model contains 4 predictors listed in Table 5. The entire model (with all predictors) was statistically significant ($p < 0.001$). The model explains 63% of the variance of the dependent variable. There was no multicollinearity between predictors.

Table 5

**Multivariate linear regression with ISQ as a dependent
variable**

Independent variables	Univariate linear regression		Multivariate linear regression	
	B	p	B	p
Jaw (lower *vs* upper)	9.000	0.001	0.138	0.959
Gender (male *vs* female)	10.096	< 0.001	4.669	0.047
Age (up to 50 *vs* over 50)	7.811	0.031	3.782	0.141
Hounsfield units	0.042	< 0.001	0.032	0.001

In the multivariate linear regression model, statistically significant predictors of higher ISQ values were: males (B = 4.669; $p = 0.047$) and higher HU values (B = 0.032; $p < 0.001$) (Figure 7).
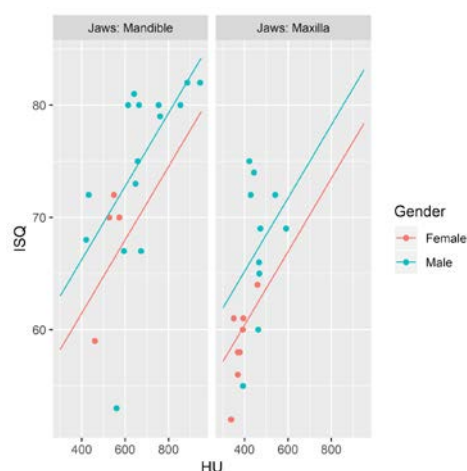
Fig. 7 – Predictors to implant stability quotient (ISQ) ratio.

Discussion

To analyze bone density in this study, we used software analysis based on CBCT imaging.

According to the Norton and Gamble classification, the software-measured bone density at the implant site which is above + 850 HU corresponds to Q1 bone quality according to Lekholm and Zarb and is typical for mandible anterior region. Bone density between +500 and + 850 HU corresponds to Q2 and Q3 bone quality in the posterior mandible and anterior maxilla, while the bone density from 0 to 500 corresponds to Q4 bone quality in the posterior maxilla [30].

Bone density obtained by software analysis in our study of the posterior maxilla was 429.8 HU, which corresponds to Q4 bone quality, and in the posterior mandible region it was 642.6 HU, which corresponds to Q2 and Q3 bone quality which is expected in these regions according to literature.

Several studies have reached similar or slightly higher results of the average bone density in maxilla and mandible compared to our research. Since our research was performed in lateral regions, we believe that this slightly greater bone density found in studies of these authors results from the frontal jaw regions or greater differences in the distribution of subjects' genders [31–35].

The average value of bone density in lateral regions of both jaws, measured in 38 subjects was 536.16 HU. Our results are similar to the results obtained in a clinical study where the arithmetic mean of the average bone density in the posterior regions of maxilla and mandible was 568.5 HU [31].

According to a similar clinical study conducted on a sample of 108 subjects, the mean bone density in the posterior maxilla was 459 HU and in the posterior mandible it was 669 HU. As it was the case in our study, a significant statistical correlation between bone density expressed in HU units and primary implant stability was observed. These authors conclude that bone density values from preoperative CT examination may provide an objective assessment of bone quality, and significant correlations between bone density and implant stability parameters may help clinicians to predict primary stability before implant insertion [27].

In our study, we used a noninvasive resonance frequency analysis (RFA) with Ostell device to measure the primary stability of dental implants. The measurement was performed immediately after the placing of implants, and the average primary implant stability immediately after the implantation was 68.7 ISQ. The average value in the mandible was 73 ISQ, while in the maxilla it was 64 ISQ.

Predictors that were statistically significant in the model of univariate linear regression were included in our research as a model of multivariate linear regression with ISQ as a dependent variable. In the multivariate linear regression model, statistically significant predictors of higher ISQ values were male gender and higher HU values.

In the clinical study, primary stability measured immediately after the implantation in the posterior mandible was 65 ISQ, while in the posterior maxilla it was 60 ISQ [32].

In the clinical study, the average primary stability value in maxilla was 72 ISQ, while in mandible it was 75.8 ISQ [31].

According to a clinical study conducted on 125 patients with type II bone, self-tapping implants showed greater primary stability with 73 ISQ, in type III bone the self-tapping implants recorded ISQ of 74, and for type IV bone, the implant value was 66 ISQ [8].

In the literature, different results of the primary implant stability of numerous clinical studies can be explained by various factors that affect the stability of implants. Different design of implants was used as well as various placement techniques and different toothless regions with different quality and quantity of bone.

The most important factors affecting the primary implant stability are: implant design, surgical technique, bone quality and quantity [6].

Conclusion

We showed that there was a statistically significant strong positive correlation between the bone density expressed in HU units measured in the software of the CBCT device and the primary stability of dental implants expressed in ISQ units. Higher HU values were related to higher ISQ values.

Conflict of interest

None of the authors had any conflict of interest for this study.

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The neurological outcome in the patients with myasthenia gravis who underwent thymectomy via sternotomy and video-assisted thoracoscopic surgery (VATS)

Neurološki ishod lečenja bolesnika sa miastenijom gravis koji su timektomisani sternotomijom i video asistiranom torakoskopskom hirurgijom (VATH)

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Abstract

Background/Aim. Thymectomy is accepted in the surgical treatment of the patients with myasthenia gravis (MG). Earlier thymectomy via sternotomy has recently been replaced by video-assisted thoracoscopic surgery (VATS), which is less invasive. The aim of this study was to determine the effectiveness and reliability of the two methods of surgical removal of the thymus by comparing the neurological outcome in patients with MG. **Methods.** The study included 60 patients with MG who underwent thymectomy at the beginning of their treatment: 30 patients underwent thymectomy via sternotomy, and the remaining 30 patients via VATS. In order to evaluate the effects of these two operation techniques, we compared the data related directly to the operation – the number of postoperative hospital days, the incidence of postoperative complications, as well as the data related to the neurological monitoring of these patients: directly after the operation, one year after the surgery and up to three years after the surgery. **Results.** The data related to the immediate postoperative period indicate a significantly shorter hospitalization after the VATS thymectomy ($p <$ 0.001), but the percentage of postoperative complications in both groups was the same ($p = 0.381$). Clinical deterioration in the first year after the operation showed a uniform distribution, regardless of the type of performed operation ($p = 0.470$). The number of performed rethymectomies in the group that underwent thymectomy via sternotomy vs. VATS was of borderline statistical significance ($p = 0.054$). Complete stable remission, as a confirmation that the thymic tissue was removed in its entirety, was observed in about 11% of the patients who underwent thymectomy. For other patients, clinical remission was maintained with anticholinesterase and immunosuppressive therapy. **Conclusion.** The shorter hospitalization time and faster postoperative recovery, with an equal clinical efficacy and aesthetic component, favors the VATS thymectomy compared to thymectomy via sternotomy as more acceptable in the surgical treatment of the patients with MG.

Key words:
myasthenia gravis; thymectomy; sternotomy; surgery, thoracic, video-assisted; surgery, thoracic, procedures; treatment, outcome.

Apstrakt

Uvod/Cilj. Timektomija je prihvaćena metoda hirurškog lečenja bolesnika sa miastenijom gravis (MG). Ranija timektomija putem sternotomije zamenjena je poslednjih godina video asistiranom torakoskopskom hirurgijom (VATH) kao manje invanzivnom metodom. Cilj rada bio je da se poređenjem neurološkog ishoda utvrde efikasnost i pouzdanost te dve metode hirurškog uklanjanja timusa kod bolesnika sa MG. **Metode.** Studijom je bilo obuhvaćeno 60 bolesnika sa MG koji su timektomisani na početku lečenja: 30 bolesnika je timektomisano sternotomijom, a preostalih 30 putem VATH. U cilju procene efekata te dve operativne tehnike poređeni su podaci koji su se odnosili direktno na operaciju – broj hospitalnih dana posle operacije, incidencija postoperativnih komplikacija, ali i podaci koji se tiču neurološkog praćenja tih bolesnika: neposredno posle operacije, godinu dana posle operacije i do 3 godine posle operacije. **Rezultati.** Podaci koji se odnose na neposredni postoperativni period ukazali su na signifikantno kraću hospitalizaciju posle timektomije putem VATH ($p <$ 0,001), dok je procenat postoperativnih komplikacija u obe grube bio isti ($p =$ 0,381). Utvrđena je uniformna distribucija kliničkog

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pogoršanja prve godine posle operacije, nezavisno od načina operacije (*p* = 0,470). Broj učinjenih retimektomija u grupi bolesnika putem VATH bio je granično statistički značajan (*p* = 0,054). Kompletna stabilna remisija, kao potvrda da je timusno tkivo bilo uklonjeno u potpunosti ostvarena je kod oko 11% timektomisanih bolesnika. Kod ostalih bolesnika klinička remisija je održavana antiholinesteraznom i imunosupresivnom terapijom. **Zaključak.** Kraća hospitalizacija i brži postoperativni oporavak sa podjednakim kliničkim učinkom i estetskom komponentom favorizuju timektomiju VATH u odnosu na timektomiju putem sternotomije kao prihvatljivijom metodom hirurškog lečenja obolelih od MG.

Ključne reči:
miastenija gravis; timektomija; sternotomija; hirurgija, torakalna, video-asistirana; hirurgija, torakalna, procedure; lečenje, ishod.

Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by pathological weakness and fatigue of voluntary muscles. Besides a conservative treatment, thymectomy takes an important role in the treatment of patients with MG [1, 2]. Surgical removal of the thymus gland in the patients with MG allows a stable clinical course, the greater number of clinical remissions, and reduces the dose of a drug required in the conservative treatment of the disease [3].

It is believed that the thymus is responsible for the initiation of the immunological processes, which cause the occurrence of autoantibodies to the acetylcholine receptor (AChR) [4]. Although the association with the anti-MG acetylcholine receptor (AChR) antibodies was first noticed by Lindstrom et al. [5] in 1976, the benefit of thymectomy in patients suffering from MG was observed much earlier, at the beginning of the last century. So, the first thymectomy in patients with MG was performed in 1911 by Ferdinand Sauerbruch, in a transcervical way, after the X-ray recording of the chest showed an increased thymic shadow, and after the thymectomy a clinical recovery was recorded. Since transcervical thymectomy proved insufficient because of the low visibility and since it was accompanied by the large remnants of residual thymic tissue which required rethymectomy, it was quickly replaced by thymectomy via sternotomy [6].

Thymectomy via sternotomy was described for the first time in 1939 by Dr. Alfred Blalock in the case of a 21-year-old patient with MG, following her clinical remission for 3 years [2].

Some time after that, in 1941, thymectomy was performed on 7 MG patients without thymoma, also with a clinical postoperative recovery. Blalock published his results in the series of 20 patients in 1944 [2].

Thymectomy via sternotomy as the optimal method of surgical treatment was long in use, until 1993, when David Sugarbaker for the first time made a video-assisted thoracoscopy (VATS) with laparoscopic approach to the thymus from the auxiliary area, which was far less invasive and accompanied by smaller postoperative scars [7].

The goal of thymectomy is a complete removal of the thymus gland and mediastinal fat tissue that can be a source of ectopic thymic tissue, which is often not available using modern diagnostics.

Since thymectomy via sternotomy approach requires full and open access to the chest cavity, which leaves a big scar (Figure 1), VATS thymectomy has almost entirely taken its place in recent years (Figure 2).

Fig. 1 – The scar after thymectomy via sternotomy.

Fig. 2 – The scar after thymectomy via video-assisted thoracoscopy (VATS).

In our region, VATS thymectomy was first performed in 2012. By the end of 2018, at the Clinic of Thoracic Surgery, Military Medical Academy in Belgrde, 70 VATS thymectomy was performed using at first three, then two, and

now uniportal VATS thymectomy that is described as the standard [8].

This study compared the results of patients with MG who underwent thymectomy via sternotomy and VATS.

Methods

Among 60 patients with clinically, neuropsychologically, immunologically and pharmacologically verified MG treated at the Clinic for Neurology, Military Medical Academy in Belgrade from 2000 to 2018, 30 patients underwent thymectomy via sternotomy and the other 30 via VATS.

VATS thymectomy, as a newer surgical technique has been used at our institution since 2012, and after that all of our patients underwent thymectomy by VATS. In patients operated via VATS method, a unipolar technique by one-sided access to the right or left side was used, which was decided on by a thoracic surgeon with respect to the anatomical localization of the thymus, but the access to the right was more secure because of the relationship with the brachiocephalic vein.

Duo to the pathohistological (PH) findings in the operated thymus, we distinguish atrophic thymus, hyperplasia thymus and the thymoma.

In relation to the way of thymectomy, we compared the following parameters: age, gender, the length of hospitalization, the incidence of complications and lethal outcomes immediately after the thymectomy, the incidence and treatment of postoperative exacerbations, the frequency of rethymectomy, and the length of postoperative recovery.

As a stable and complete remission was the confirmation that the thymic tissue was completely removed, we followed the postoperative outcome immediately after thymectomy, then one year after the operation, as well as for a period longer than one year (up to 3 years). In doing so, the clinical status of the patients was assessed by Oserman and Genkins [9].

In order to evaluate the effect of these two operation techniques, we compared the data related directly to the operation (the number of postoperative hospital days, the incidence of postoperative complications), as well as the data related to the neurological monitoring of these patients in the form of postoperative recurrence and rethymectomies due to residual thymic tissue. The evaluation of the clinical disease achieved by comparing the severity of the disease was performed according to Osserman and Genkins [9] method pre- and postoperatively: immediate postoperatively, on the day of the discharge, one year after the surgery and up to three years after it.

With regard to the recommendation that a thymectomy should be done when the patient is stable, the patients achieved clinical remission with medicaments first, and following that the thymectomy was performed. With medications, the patients were held in a state of clinical remission during the postoperative recovery.

In the postoperative period, the dosages of corticosteroids were carefully reduced because any reduction of the dose of the drug carries a risk of relapse. Non-steroidal im-

munosuppressive drugs were added when a corticosteroid therapy was not enough or in order to avoid its adverse effects. According to the consensus of experts, among non-steroidal immunosuppressive drugs, azathioprine is the first choice for the treatment of MG, which was respected in our group of patients, who were very tolerant and without any side effects from the therapy with azathioprine. The deterioration of their condition most frequently occurred due to the abrupt reduction in therapy, due to an infection or due to residual thymic tissue. Therefore, clinical deterioration was the indication for the control scanner of the chest.

A complete statistical analysis of the data was done with the statistical software package, SPSS Statistics 18.

Most of the variables were presented as the frequency of certain categories, while the statistical significance of differences was tested with the χ^2 test.

In case of continuous data, variables were presented as mean value ± standard deviation (SD), minimal (min) and maximal (max) values. Kolmogorov-Smirnov test was used for the evaluation of normal data distribution. According to the results of this test, statistical significance between the groups was tested by t-test or alternatively by Mann-Whitney or Wilcoxon test.

All the analyses were estimated at $p < 0.05$ level of statistical significance.

Results

As in the total population of our patients (43 women and 17 men), and in both groups of patients operated by different techniques, women significantly dominated: 70.6% in the group with thymectomy via sternotomy and 76.9% in the group operated via VATS ($p < 0.001$); so there were no statistically significant differences in the distribution of the sexes in both groups.

The average age of our patients in the total population was 41.67 ± 13.90 years (min 28, max. 65 years) and there were no statistically significant differences in age among the sexes ($p = 0.236$) or between the groups of patients operated by different techniques ($p = 0.486$).

The average duration of hospitalization for the patients operated via sternotomy was 10.13 ± 2.604 days (the shortest 4 and the longest 16 days), while the average duration of hospitalization for the operated by VATS was 5.04 ± 1.661 (minimum of 2 and maximum of 9 days). Comparing the duration of the hospitalization in relation to the manner of thymectomy performed, statistically significantly longer hospitalization was after the thymectomy via sternotomy ($p < 0.001$).

Among the postoperative complications, all patients reported soft and transient paresthesia in the operated area, which withdrew in the first few postoperative months. Among the more serious complications, there was one death during the first week of operation due to myocardial infarction in the group operated via sternotomy, which proved to be statistically insignificant in terms of postoperative complications among the patients operated with different surgical techniques ($p = 0.381$). Pneumonia and myasthenic crisis,

which are often mentioned as possible postoperative complications, were not seen among our patients.

The average clinical condition score in patients before the operation via sternotomy was 2.65 ± 0.702 (min 2, max 4), and after the operation 1.20 ± 1.08 (min 0, max 3), which indicates a statistically significant recovery ($p = 0.002$). The average preoperative clinical condition score of patients operated via VATS method was 2.0 ± 0.75 (min 1, max 4) and after the operation 1.0 ± 1.0 (min 0, max 3), which indicates a statistically significant postoperative recovery, also ($p \leq 0.001$). In addition, there was no significant difference in the preoperative MG severity between the patients treated with different surgical techniques ($p = 0.062$). To conclude, there was a significant clinical improvement after the thymectomy via sternotomy and VATS method ($p = 0.01$). A comparison of the immediate postoperative outcomes demonstrated that both operational techniques are equally successful when it comes to the immediate postoperative recovery ($p = 0.762$).

Comfort scanner of the chest, performed in 3 of the patients, showed rest of thymic tissue, which was an indication for reoperation (Figure 3). All 3 reoperated patients had been

We observed a recorded patient, reoperated via sternotomy, in whom deterioration was provoked by an infectious syndrome during the first year after surgery.

In patients without the rest of thymic tissue, clinical deterioration was solved by raising the dose of the existing anticholinesterase and corticosteroid therapy (80% operated on via sternotomy and 56.5% operated on by VATS). The introduction of azathioprine (Imuran®) in the therapy was recorded in 20% of the patients operated on via sternotomy and in 34.8% of the patients operated on by VATS); therapeutic plasma exchange (PE) was used in none of the patients operated on via sternotomy and in 8.7% of those operated on by VATS (the PE is useful when other therapies are not sufficient, or when it is necessary to minimize the exacerbations). Using χ^2 test ($p = 0.325$), no statistically significant differences were found in the treatment of the deterioration in the patients operated on by various techniques.

The reliable data about our patients in the next two years were obtained only for 34 patients: for 12 operated on via sternotomy and 22 operated on via VATS. In the observation period of 3 years after the thymectomy, 2 patients
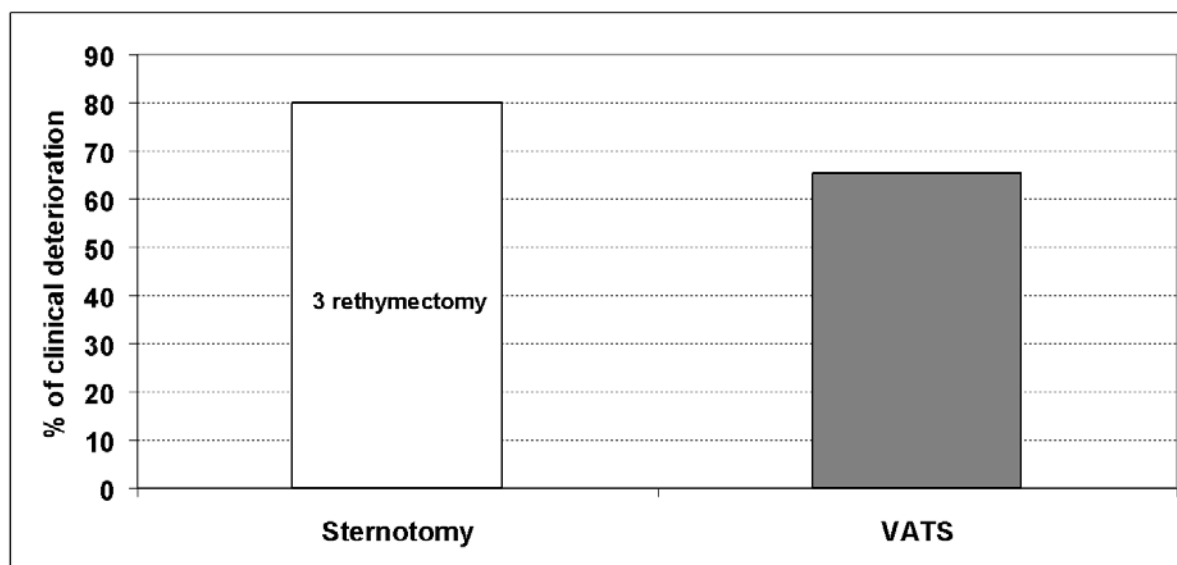
Fig. 3 – The number of rethymectomies during the first year after the thymectomy. VATS – video-assisted thoracoscopic surgery.

operated via sternotomy previously. Among the reoperated patients, two underwent sternotomy again, but one patient underwent VATS. This difference in the number of reoperated patients treated with the different techniques, was of borderline statistical significance ($p = 0.054$). There were no complications in the reoperated patients.

During the first year after the surgery, clinical deterioration occurred in 24 (80%) of the patients who underwent sternotomy and in the group operated on by VATS, deterioration was noted in 15 (65.2%) of the patients (Figure 3). This difference in percentage was not statistically significant, and there was a homogeneous distribution of deterioration regardless of the type of surgery ($p = 0.470$).

from both operated groups were in a complete clinical remission – disease symptom-free and without a treatment for the period of one year.

The second group of patients was only on pyridostigmine (Mestinon®): 3 patients operated on via sternotomy and 1 patient operated on via VATS were included. The third group of patients was on Mestinon® and on corticosteroid therapy: 2 patients underwent sternotomy and 6 patients were operated on by VATS.

The fourth group was treated with Imuran®: 5 patients were operated on via sternotomy and 13 were operated on via VATS. None of the patients underwent PE a year after the thymectomy (Figure 4).
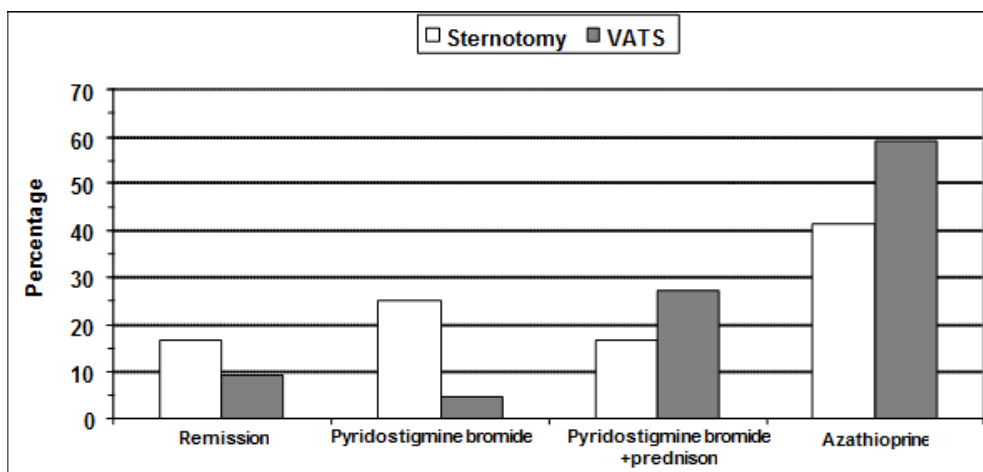
Fig. 4 – Clinical outcome and therapy 3 years after the surgery (% of patients)
VATS – video-assisted thoracoscopic surgery.

The χ^2 test showed that the kind of treatment did not significantly differ in patients operated on by a different techniques more than one year after the operation ($p = 0.358$).

In the group operated on by sternotomy, 2 patients had atrophic thymus, 1 had thymoma and 27 patients had thymic hyperplasia. In the group of patients operated on by VATS, 3 had atrophic thymus, one thymoma and 26 patients thymic hyperplasia.

Among our patients, one was seronegative; he was treated by VATS, and in this patient a thymic hyperplasia was seen.

PH type of thymus was almost uniform in the group of patients operated on by sternotomy and the patients operated on via VATS ($p = 0.896$).

A total of 5 patients were diagnosed with the atrophy of the thymus. The average age of these patients was 67.60 ± 6.986 years and it was a statistically significant difference compared to the patients with thymic hyperplasia and thymoma ($p < 0.001$).

Discussion

Thymectomy is an option which can minimize or even avoid immunosuppressive therapy in seropositive generalized myasthenia. It is suggested in younger than 45 years of age, with the advice to be done as soon as possible, because then the better the results of treatment can be achieved[10].

As MG is a heterogeneous disease, there is no single standard in the treatment of this disease that has been accepted as the best for everyone with MG [11]. Initial therapy dose of pyridostigmine should be adjusted to the symptoms of the disease. Corticosteroids and immunosuppressive therapy are introduced when pyridostigmine is not enough to achieve therapeutic goals [11, 13].

Younger patients, with a less severe and shorter duration of the disease, more likely have a complete remission after thymectomy [3].

Clinical remission refers to the situation in which a patient is disease-free and may have some weakness in the closing of the eyes, but without any other weakness of the muscles [11].

Also, thymectomy is done in seronegative generalized MG patients, when there is weak and uncertain response to immunosuppressive therapy [11].

If a patient does not respond or has insecure course to immunosuppressive therapy or if there are side-effects after its initiation, thymectomy is the method of choice in elderly patients, also [10]. As we followed this recommendation, the average age of our patients was 41.67 ± 13.902 years.

All MG patients with thymoma should be operated on [12].

Women were dominant in our group of the operated patients, corresponding to the data from the literature, where there is a higher incidence of women with the early-onset MG (before the age of 50), in which thymectomy is primarily indicated [1, 13].

Publications of sporadic positive effects of thymectomy on the clinical course of patients with MG have recently been replaced by the publications which compare the clinical course of patients treated surgically and conservatively [3, 8, 14].

One of them has shown a significantly more likely achieved clinical remission after thymectomy than without the operation (20% vs. 10%) [6].

In our group of a total of 60 patients, a significant improvement in the clinical picture of patients directly after thymectomy ($p = 0.01$) has been noted.

The effect of thymectomy in different decades in the period from 1940 to 2000 was followed by another large study [6], which observed that in all the decades the number of patients in complete clinical remission is about 10%. It corresponds to our results that about 11% of our patients were in complete clinical remission and without a treatment for a period longer than one year [6]. The number of patients who had postoperative recovery gradually grew from 30% to almost 60% at the closing decade of the year 2000. Clinical remission of our patients was achieved by combining differently anticholinesterase and immunosuppressive drugs but no patient required PE in the period after one year after thymectomy.

The percentage of subjects [3, 10] whose condition continued to deteriorate and those who died decreased from decade to decade, which perhaps explains the modern concepts of pharmacological treatment of this disease.

It is known that MG with early or late beginning has a different HLA gene and various autoimmune targets, but the same mechanism of development. The existence of different targets of autoimmunity confirms the differences in thymic pathology: follicular hyperplasia of the thymus is often seen in the early onset of a seropositive MG, whereas the atrophy of the thymus is the most common finding in late onset of MG (15–20%) [6, 13]. There is an equal distribution of different PH findings of thymus: thymoma, thymic atrophy and hyperplasia ($p = 0.896$) among our patients operated on by different techniques.

Atrophic thymus was found in five of our patients (16.7%). They were significantly older (67.60 ± 6.97 years) compared to the patients with hyperplasia and thymoma ($p < 0.001$) in the corresponding data from the literature.

Thymoma is seen in 10% to 15% of cases [15], usually between 45 and 55 years of age, which corresponds to our results because thymoma was found in one patient in both groups.

Following parameters directly related to the operation, such as the duration of the operation, intraoperative blood loss, the perception of pain, duration of hospitalization, the need for artificial respiration, postoperative complications resulting in death have been most commonly used in comparison effects of thymectomy via sternotomy and via VATS [15, 16]: It has been shown a certain advantage of VATS thymectomy in relation to thymectomy via sternotomy due to a shorter hospitalization, minor loss of blood and better cosmetic effect. A meta-analysis of 12 studies that dealt with this problem also gives the advantage to VATS thymectomy because fewer postoperative complications and low frequency of myasthenic crisis [17, 18]. Similar data were obtained in our group of patients: significantly longer hospitalization after sternotomy ($p < 0.001$), and one death in the group operated via sternotomy, which did not come out statistically significant, while myasthenic crisis and pneumonia, often referred to as postoperative complications in the literature, were not observed among our patients [17].

The incomplete removal of the thymus leads to an unstable postoperative course, while the criteria for a complete stable remission is that the thymic tissue was removed in its entirety [18]. Therefore, apart from the results related directly to the operation, we followed the clinical status of our patients immediately after the surgery, then one and 3 years after the operation.

Immediate postoperative recovery was statistically significant ($p = 0.002$), and both operational techniques were equally effective ($p = 0.762$).

The number of clinical deteriorations in the first year after the operation showed that there was a homogeneous distribution of deterioration, regardless of the type of surgery ($p = 0.470$). In 3 patients the cause of deterioration was the remnant of thymic tissue, which was an indication for rethymectomy.

All 3 patients who had a need for rethymectomy had previously been operated by sternotomy. This difference in the number of rethymectomies between patients treated by various techniques was of borderline statistical significance ($p = 0.054$).

Other exacerbations were solved by correcting anticholinesterase therapy and corticosteroids, the introduction of azathioprine and a series of PE.

It was concluded that there were no statistically significant differences ($p = 0.325$) in the treatment of deterioration in the patients operated on by various techniques.

Three years after the surgery, two (around 11%) of the patients in both operated groups were in complete clinical remission: symptom-free and without a treatment for a period longer than one year [8]. Other patients were on the combination of anticholinesterase therapy and immunosuppressives, and none of them had a need for PE. Types of treatment, 3 years after the operation, were not significantly different in patients operated on by different techniques ($p = 0.358$).

Conclusion

The sternotomy and VATS thymectomy achieve equal clinical results, but shorter hospitalizations and better cosmetic effects favor VATS, and it is now a more acceptable technique for thymectomy in the patients with MG.

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The effect of hyperbaric oxygenation on cardiodynamics and oxidative stress in rats with sepsis

Efekti hiperbarične oksigenacije na kardiodinamiku i oksidacioni stres kod pacova sa sepsom

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Abstract

Background/Aim. Dysfunctions at the cellular, tissue, and organ level, which can result in death, are caused by metabolic changes and affection on the regulation of gene transcription and micro- and macrocirculation. The aim of the present study was to assess the impact of hyperbaric oxygenation (HBO) on isolated heart as well as on the oxidative status of rats with sepsis. **Methods.** The investigation included male Wistar albino rats classified into three groups: the first group was a control group (CTRL); the second group included animals exposed only to the induction of sepsis without HBO treatment (the Sepsis group), while the third group included animals treated with HBO after the induction of sepsis (the Sepsis + HBO group). For the induction of sepsis, fecal peritonitis model was used (3 mL/kg of fecal suspension administered intraperitoneally). After the induction of sepsis, the rats were exposed twice a day (on 12 hours) to HBO treatment at 2.8 atmospheres absolute (ATA) for 90 minutes over a period of 3 days. 72 h after the confirmation of sepsis, the animals were sacrificed and the hearts were retrogradely perfused on the Langendorff apparatus at a gradually increased coronary perfusion pressure (CPP = 40–120 cm H$_2$O). The

following parameters of heart function were continuously recorded: maximum and minimum rate of left ventricular pressure development (dp/dt max, dp/dt min); systolic and diastolic left ventricular pressure (SLVP and DLVP); heart rate (HR). Coronary flow (CF) was measured flowmetrically. Following oxidative stress markers were measured: nitrites (NO$_2^-$), superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), index of lipid peroxidation (TBARS), activity of superoxide dismutase (SOD) and catalase (CAT) and the level of reduced glutathione (GSH). **Results.** There were no significant differences in dp/dt max, dp/dt min, SLVP and HR between the groups. CF was statistically significantly higher (p < 0.01) in the sepsis group. The values of all cardiac oxidative markers were lower in the sepsis + HBO group (p < 0.05), while systemic pro-oxidative and antioxidative parameters were unchanged. **Conclusion.** Our results showed that HBO treatment was not associated with improved cardiac function and coronary perfusion, while expressed promising beneficial effects on cardiac oxidative stress.

Key words:
hyperbaric oxygenation; oxidative stress; sepsis; heart; rats.

Apstrakt

Uvod/Cilj. Disfunkcija na nivou ćelije, tkiva ili organa, koja može imati za posledicu smrtni ishod, javlja se usled metaboličkih promena i poremećaja regulacije transkripcije gena i mikro- i makro cirkulacije. Cilj ove studije bio je da se proceni uticaj hiperbarične oksigenacije (HBO) na izolovano srce, kao i na oksidacioni status pacova sa sepsom. **Metode.** Istraživanjem su obuhvaćeni mužjaci Wistar albino pacova klasifikovani u tri grupe: prva grupa je bila kontrolna grupa (CTRL), drugu grupu su činile životinje

izložene samo sepsi bez HBO tretmana (grupa Sepsa), dok su u trećoj grupi životinje tretirane HBO nakon indukcije sepse (grupa Sepsa + HBO). Za indukciju sepse korišćen je model fekalnog peritonitisa (3 mL/kg fekalne suspenzije, intraperitonealno). Posle indukcije sepse, pacovi su bili izloženi dva puta dnevno (tokom 12 sati) HBO tretmanu sa 2,8 apsolutnih atmosfera (ATA) tokom 90 minuta u periodu od 3 dana. 72 h nakon potvrđivanja sepse, životinje su žrtvovane, a srca su retrogradno perfundovana na Langendorfovom aparatu, pri postepenom povećanju koronarnog perfuzionog pritiska (CPP = 40–120 cm H$_2$O). Sledeći par

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ametri srčane funkcije su kontinuirano mereni: maksimalna i minimalna stopa promene pritiska u levoj komori (dp/dt max, dp/dt min); sistolni i dijastolni pritisak leve komore (SLVP i DLVP) i srčana frekvenca (HR). Koronarni protok (CF) je meren floumetrijski. Određivani su sledeći markeri oksidacionog stresa: nitriti (NO_2^-), superoksid anjon radikal (O_2^-), vodonik peroksid (H_2O_2), indeks lipidne peroksidacije (TBARS), aktivnost superoksid dismutaze (SOD) i katalaze (CAT) i nivo redukovanog glutationa (GSH). **Rezultati.** Nije bilo značajne razlike u dp/dt max, dp/dt min, SLVP i HR između grupa. CF je bio statistički značajno veći ($p <$ 0,01) u grupi sa sepsom. Vrednosti svih srčanih oksidacionih markera bile su niže u grupi sepsa + HBO ($p <$ 0,05), dok su sistemski pro-oksidacioni i antioksidacioni parametri bili nepromenjeni. **Zaključak.** Naši rezultati su pokazali da HBO tretman nije bio povezan sa poboljšanom funkcijom srca i koronarnom perfuzijom, dok je ostvario obećavajući korisne efekte na oksidacioni status u srcu pacova.

Ključne reči:
hiperbarička oksigenacija; stres, oksidativni; sepsa; srce; pacovi.

Introduction

A life-threatening organ dysfunction caused by a disrupted response of the host organism to infection is sepsis, a condition whose global epidemiological significance is difficult to assess, and which places a heavy financial burden on health systems. Dysfunctions at the cellular, tissue, and organ level, which can result in death, are caused by metabolic changes and affection on the regulation of gene transcription and micro- and macrocirculation [1–3]. Much progress has been made in the knowledge of the pathogenetic mechanisms mentioned above in the last few decades, but extensive trials are still needed to implement newer modalities of therapy into sepsis treatment.

In addition to immune and metabolic disorders, cardiovascular disorders develop. The two manifestations of cardiac dysfunction are the hyperdynamic (warm shock) and the hypodynamic (cold shock) phase [4, 5]. The hyperdynamic phase is characterized by the elevated vascular tone and low cardiac output, while in the hypodynamic phase we have a reverse situation – decreased vascular tone and increased cardiac output, and a difference in the form of manifestation in adults and the pediatric population was observed [5]. Tests on the isolated cardiomyocyte revealed that with the passage of time from the onset of sepsis, an increase in heart rate was observed, as well as a decrease in the maximum and minimum rate of left ventricular pressure development and a decrease in diastolic pressure in the left ventricle [5–7]. The basis of these changes is the production of cytokines, whose indirect influence is reflected by the increased production of vasoactive mediators [5]. The association of Ca^{2+} receptors, which control Na^+/K^+-ATPase function in cardiomyocytes, with the onset of cardiac dysfunction in sepsis in an animal model has also been demonstrated [4].

Hyperbaric oxygenation (HBO) is the process of exposing the whole organism to 100% oxygen at elevated pressure [greater than 1 atmosphere absolute – ATA (760 mmHg)] at different time intervals. The effect of this treatment, whose effects can be divided into primary and secondary, is based on the principles of Henry and Dalton's law. The primary effect is the percentage increase in dissolved oxygen in the circulation, while the secondary effects are vasoconstriction, neovascularization, and a decrease in gas volume [8–10]. Vasoconstriction leads to an increase in vascular resistance and arterial pressure, and an increase in pressure in the left ven-

tricle, an increase in the maximum and minimum rate of left ventricular pressure development, bradycardia and a decrease in cardiac output [11, 12]. The mechanisms underlying this cardiovascular response are linked to baroreceptor-mediated regulation [11].

HBO is a potential intervention for the prevention of septic shock and can also be used in the treatment of severe pancreatitis, diabetic foot ulcer, carbon monoxide poisoning and other conditions [13]. Studies addressing the use of HBO in the treatment of sepsis have primarily addressed the benefits of HBO in the domain of cytokine production, especially about the increased expression of interleukin (IL)-10 and the decreased levels of IL-6 [13]. More importantly, due to its mechanism of action, HBO can affect the production of oxidative stress biomarkers and thus change redox homeostasis in sepsis, which could be responsible for the potential positive impact of this procedure [14].

Having in mind that there are almost no studies that investigate the effects of hyperbaric oxygenation on cardiac function and coronary circulation during sepsis, we aimed to assess the potential impact of this therapeutic approach on isolated heart as well as on cardiac and systemic oxidative status of rats with sepsis.

Methods

Animals and study design

The study was carried out on eighteen male Wistar albino rats, 8 weeks old, body weight 200 ± 30 g. The animals were kept in an artificial 12-h light–dark cycle (8:00 a.m–8:00 p.m.) at room temperature ($22 \pm 1°C$). Water and food were available *ad libitum*. The animals were housed in their respective groups in a collective cage and received water and standard laboratory chow. The animals were classified into three groups.

The first group (n = 6) was the control group (CTRL). The second group (n = 6) included animals exposed only to the induction of sepsis without HBO treatment (the Sepsis group), while the third group (n = 6) included animals treated with HBO after the induction of sepsis (the Sepsis + HBO group).

Ethical standards

This research was carried out in the Laboratory for Cardiovascular Physiology of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The study protocol was ap-

proved by the Ethical Committee for the welfare of experimental animals of the Faculty of Medical Sciences, University of Kragujevac, Serbia. All experiments were performed according to EU Directive for the welfare of laboratory animals (86/609/EEC) and the principles of Good Laboratory Practice.

Sepsis induction protocol

Firstly, fresh feces were collected from a heterogeneous group of rats. The feces were then dissolved in saline and this mixture was homogenized and filtered through gauze to obtain fecal suspension. Animals were anesthetized by intraperitoneal administration of ketamine and xylazine (10 mg/kg, 5 mg/kg, respectively) and 3 mL/kg of fecal suspension was administered intraperitoneally. For the confirmation of sepsis, the previously established clinical rat scoring system (Table 1) was used for each animal as well as rectal body temperature and biochemical indicators of sepsis [C reactive protein (CRP) and procalcitonin (PCT) values]. Animals from both groups were monitored for the next 72 hours before sacrifice [15].

Table 1

Clinical rat scoring system for the confirmation of sepsis and the severity of sepsis

Characteristic	Scoring range
Hunched	0–1
Bloated	0–1
Conjunctival injection/mucky eyes	0–1
Piloerection	0–1
Lack of movement	0–2
Lack of alertness	0–2

Legend for scoring: absence (0), presence (1) or marked presence (2).
The total score of 0 to 3 denotes mild sepsis and ≥ 4 severe sepsis.

HBO treatment protocol

HBO treatment was carried out in a specially constructed hyperbaric chamber for rats (HYB-C 300). After the induction of sepsis, the rats were exposed twice a day (on 12 hours) to 100% O_2 at 2.8 ATA for 90 minutes for 3 days. To avoid the effects of diurnal rhythm variation, the HBO session started at the same time, each day [16].

Ex vivo assessment of heart function

72h after the confirmation of sepsis, animals from all groups were anesthetized with short-term narcosis induced by intraperitoneal application of ketamine (10 mg/kg) and

xylazine (5 mg/kg) and sacrificed by decapitation. The hearts were then rapidly isolated and retrogradely perfused on Langendorff apparatus (Langendorff apparatus, Experimetria Ltd, 1062 Budapest, Hungary) through the ascending aorta at a gradually increased coronary perfusion pressure (CPP = 40–120 cm H_2O). The hearts were perfused with Krebs–Henseleit solution, while a transducer was inserted in the left ventricle to continuously record the following parameters of myocardial function: maximum and minimum rate of left ventricular pressure development (dp/dt max, dp/dt min), systolic and diastolic left ventricular pressure (SLVP and DLVP), and heart rate (HR). Coronary flow (CF) was measured flowmetrically. The perfusion started at CPP = 70 cm H_2O and hearts were allowed to equilibrate until HR and contractility reached steady-state. After stabilization (approximately 30 min), the CPP was gradually increased from 40, 60, 80, 100 and 120 cm H_2O to estimate coronary autoregulation. At each value of CPP, the coronary venous effluent was collected for the determination of oxidative stress parameters.

Evaluation of systemic oxidative stress

At the moment of sacrificing animals, blood samples were collected from jugular vein in order to estimate following pro-oxidants in plasma: nitrites (NO_2^-), superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), index of lipid peroxidation (thiobarbituric acid reactive substances, TBARS) and parameters of antioxidative defense system in erythrocytes samples: activity of superoxide dismutase (SOD and catalase (CAT) and the level of reduced glutathione (GSH). All parameters were measured on the spectrophotometer apparatus (Shimadzu UV 1800, Japan).

Evaluation of cardiac oxidative stress

The coronary venous effluent from each value of CPP (40–120 cm H_2O) was collected for the determination of oxidative stress parameters from the isolated rat heart. The following oxidative stress parameters were determined spectrophotometrically: NO_2^-, O_2^-, H_2O_2 and TBARS.

Markers of oxidative stress

Nitric oxide decomposes rapidly to form stable metabolite nitrite/nitrate products. NO_2^- level was measured spectrophotometrically at a wavelength of 543 nm and used as an index of nitric oxide (NO) production using the Griess reagent as previously described by Green et al. [17]. The measurement of H_2O_2 is based on oxidation of Phenol Red by hydrogen peroxide, in a reaction catalyzed by horseradish peroxidase (HRPO) at 610 nm, as previously described by Pick and Keisari [18]. The index of lipid peroxidation was estimated by measuring of TBARS using 1% thiobarbituric acid (TBA) in 0.05 NaOH incubated with the plasma as previously described [19]. The concentration of O_2^- was measured by Nitro Blue Tetrazolium (NBT) reaction in hydroxymethylaminomethane (TRIS) buffer with a plasma sample at 530 nm as previously described by Auclair and Voisin [20]. The whole analysis was determined using the spectrophotometrical

method (UV-1800 UV – Vis Spectrophotometer by Shimadzu Scientific Instruments Inc).

Antioxidative enzymes

For the determination of antioxidant parameters, isolated erythrocytes were prepared according to McCord and Fridovich [21]. SOD activity was determined by the epinephrine method described by Misra and Fridovich [22]. A 100 μL lysate and 1 mL carbonate buffer were mixed, and then 100 μL of epinephrine was added. Detection was performed at 470 nm. CAT activity was determined according to Beutler [23]. Lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove hemoglobin and then, 50 μL CAT buffer, 100 μL sample, and 1 mL 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. The level of reduced glutathione (GSH) was determined based on GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid, as previously described by Beutler [24]. Measuring was performed at 420 nm.

Determination of CRP and PCT values

Serum CRP and PCT were detected using specific enzyme-linked immunoassay kits according to the manufacturer's instructions [25].

The measurement of rectal temperature

The rectum temperature was continuously monitored using a digital thermometer (PIC solutions, Artsana S.p.A., Grandate, Italy).

Drugs

All kits, reagents and substances used in the study were purchased from Sigma-Aldrich Chemie GmbH Eschenstrasse 5, 82024 Taufkirchen, Germany.

Statistical analysis

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

Results

Confirmation of sepsis and scoring of sepsis severity

The distribution of the septic score was presented in Table 2. Of the septic animals surviving to sacrifice (n = 12), 22% were scored mild and 78% severe sepsis (Table 2). In-

addition, a whole group of animal's biochemical parameters of sepsis, as well as rectal temperature confirmed the state of sepsis (Table 3).

Table 2

Average clinical rat sepsis score for the whole group of animals (n = 12)

Animals with sepsis (%)	Score
22	3 (mild sepsis)
78	4–6 (severe sepsis)

Table 3

Average rectal temperature and values of biochemical indicators of sepsis for the whole group of animals before the induction of sepsis and at the moment of sacrificing (n = 12)

Parameter	Before sepsis mean ± SD	At the moment of sacrificing mean ± SD
Rectal temperature (°C)	36.9 ± 0.4	38.6 ± 0.8
CRP (ng/mL)	325.74 ± 23.46	527.86 ± 20.67
PCT (pg/mL)	76.35 ± 10.84	200.54 ± 38.21

CRP – C-reactive protein; PCT – procalcitonin.

Cardiac function ex vivo

Cardiodynamic parameters for the assessment of cardiac function in *ex vivo* model are presented in Figure 1. As it can be seen, there was no significant difference in dp/dt max during all CPPs among the groups. Dp/dt min was higher in the control group (CPPs = 60 – 100 cm H_2O) than in the Sepsis and Sepsis + HBO group but without statistical significance. The SLVP and HR values were almost similar in all groups, while the DLVP was insignificantly higher in the control group. On the other hand, CF was statistically significantly higher ($p < 0.05$) in the Sepsis group than in the Sepsis + HBO group and also in the control group in comparison to other two groups at almost all CPPs (60–120 cm H_2O).

Cardiac oxidative stress

Biomarkers from coronary venous effluent as indicators of cardiac oxidative stress are presented in Figure 2. The values of O_2^- were significantly lower in the Sepsis + HBO group compared to the Sepsis group (at CPPs = 40, 60 and 80 cm H_2O, $p < 0.05$), while between the control and the Sepsis + HBO group there were no statistical differences. Similarly, the H_2O_2 values were lower in the Sepsis + HBO group compared to the Sepsis group with strong
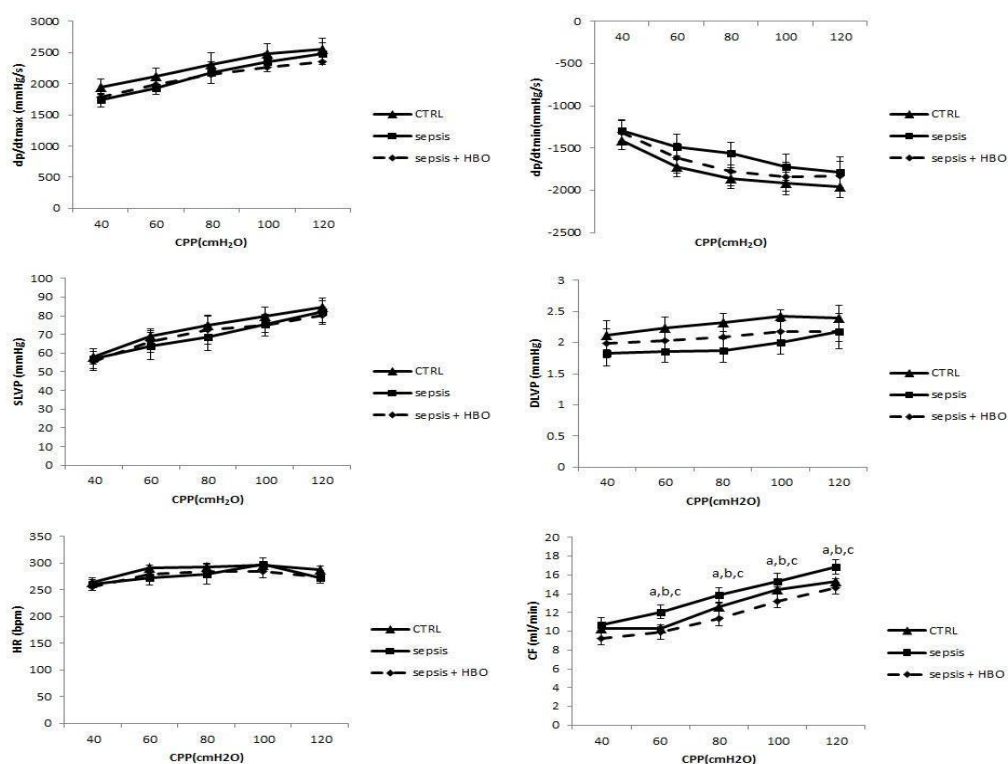
Fig. 1 – Parameters of cardiac function *ex vivo* (data are presented as mean values ± standard deviation)

dp/dtmax – maximum rate of pressure development; dp/dtmin – minimum rate of pressure development;
SLVP – systolic left ventricular pressure; DLVP – diastolic left ventricular pressure; HR – heart rate; CF – coronary flow;
CPP – coronary perfusion pressure.
Statistically significant differences ($p < 0.05$) among the groups at the same coronary perfusion pressure (CPP) are marked
as follows: a – comparison between the control group (CTRL) and the group of rats with sepsis (the Sepsis group);
b – comparison between the control group (CTRL) and the group of rats with sepsis treated with HBO (the Sepsis + HBO
group); c – comparison between the group of rats with sepsis (the Sepsis group) and the group of rats with sepsis treated
with HBO (the Sepsis + HBO group).
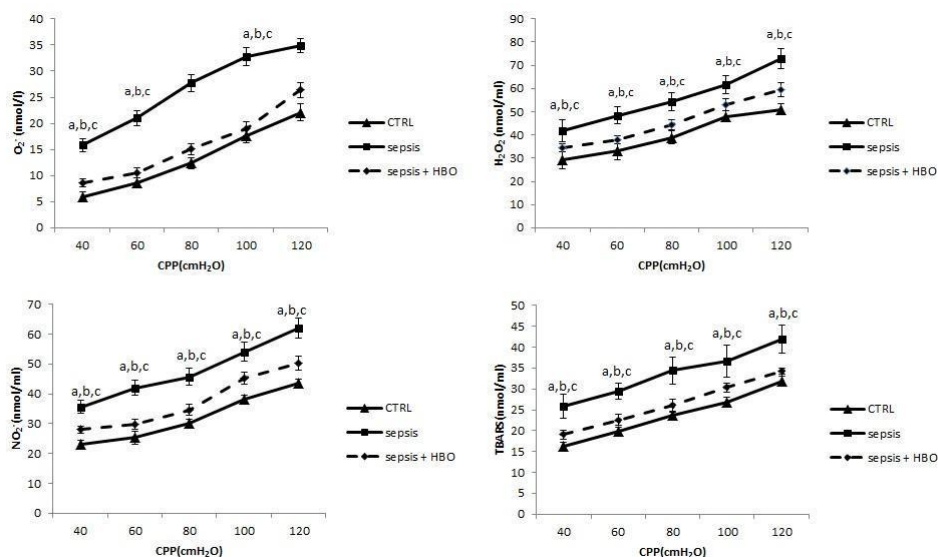
Fig. 2 – Parameters of cardiac oxidative stress (data are presented as mean values ± standard deviation).

O_2^- – superoxide anion radical; H_2O_2 – hydrogen peroxide; NO_2^- – nitrites; TBARS – thiobarbituric acid reactive substances
(index of lipid peroxidation).

Statistically significant differences ($p < 0.05$) between the groups at the same coronary perfusion pressure (CPP) are marked as
follows: a – comparison between the control group (CTRL) and the group of rats with sepsis (the Sepsis group);
b – comparison between the control group (CTRL) and the group of rats with sepsis treated with HBO (the Sepsis + HBO
group); c – comparison between the group of rats with sepsis (the Sepsis group) and the group of rats with sepsis treated with
HBO (the Sepsis + HBO group.

statistical significance at all CPPs ($p < 0.01$), without significant differences between the control and the Sepsis + HBO group. The same trend in the Sepsis + HBO group had values of NO_2^- with statistical significance at all CPPs ($p < 0.05$). As in previous cases, TBARS values were also significantly lower in the Sepsis + HBO group (CPPs = 40–100 cm H_2O ($p < 0.05$)) compared to the Sepsis group without significant differences between the control and the Sepsis + HBO group.

Systemic oxidative stress

The values of pro-oxidants from plasma of both groups of rats are presented in Figure 3. Unlike cardiac oxidative stress markers, in the values of all measured systemic pro-oxidants, a significant difference between the Sepsis group and the Sepsis + HBO group was not observed. Namely, the values of NO_2^- and H_2O_2 were insignificantly lower in the Sepsis group, while the values of O_2^- and TBARS were insignificantly lower in the Sepsis + HBO group. Compared to the control group, the values of pro-oxidant were significantly higher in both, the Sepsis and Sepsis + HBO groups. In addition, the values of antioxidant enzymes from erythrocyte lysate are presented in Figure 4. Although it can be seen that SOD and GSH values were higher in the Sepsis + HBO group, there was no statistical confirmation. The CAT values were also without significant difference between the Sepsis and the Sepsis + HBO group, but compared to the control group, the values of this antioxidant enzyme were significantly lower in the Sepsis + HBO group.
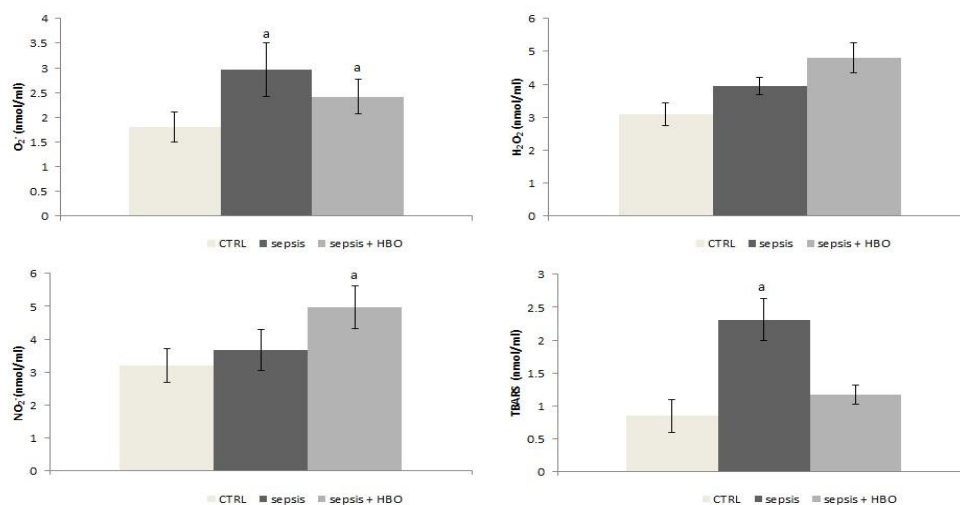
Fig. 3 – The values of pro-oxidants in plasma samples (data are presented as mean values ± standard deviation).
O_2^- – superoxide anion radical; H_2O_2 – hydrogen peroxide; NO_2^- – nitrites; TBARS – thiobarbituric acid reactive substances (index of lipid peroxidation).
Statistically significant differences ($p < 0.05$) are marked as follows:
a – statistical significance difference compared to the control group (CTRL).
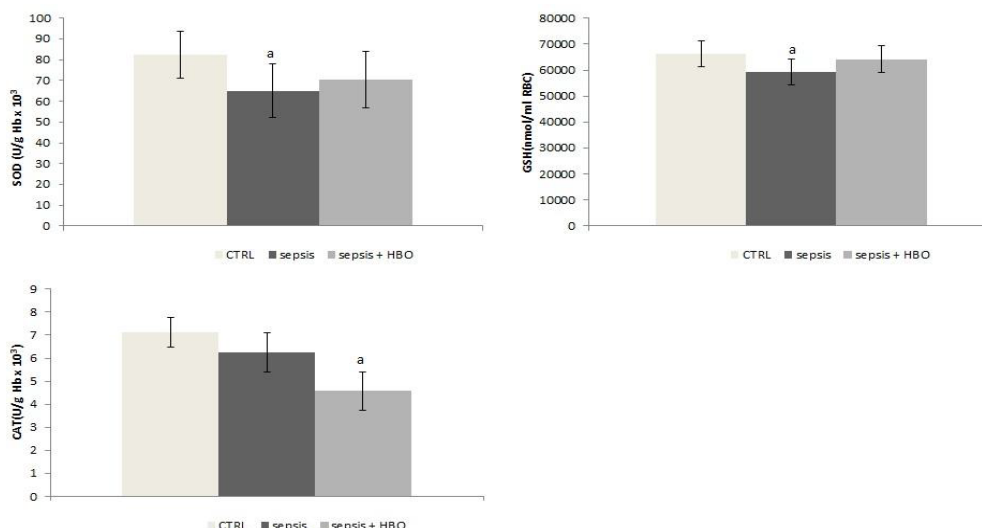
Fig. 4 – The values of antioxidant enzymes in erythrocyte lysate samples (data are presented as mean values ± standard deviation).
SOD – superoxide dismutase; CAT – catalase; GSH – glutathione.
Statistically significant differences ($p < 0.05$) are marked as follows:
a – statistical significance difference compared to the control group (CTRL).

Discussion

The present study aimed to assess the effects of HBO on cardiac function as well as cardiac and systemic oxidative state in rats with sepsis. The estimation of pro-oxidative and antioxidative markers was chosen to estimate a potential role of oxidative stress in the changes of cardiac function during sepsis and after HBO pretreatment. The function of the heart from aspect of cardiodynamics in sepsis and a possible impact of HBO were almost uninvestigated, with poor and heterogeneous data in the literature.

Cardiac dysfunction is a consequence of severe sepsis and is characterized by impaired contractility, diastolic dysfunction, as well as reduced cardiac index and ejection fraction (EF) [26]. The mechanisms involved in adverse effects of sepsis on myocardial function are little-known. Inflammatory mediators through impaired calcium turnover can lead to the alteration of cardiomyocyte contraction [26]. Other factors imply diminished β-adrenergic stimulation or reduced ATP production, which both cause cardiomyocyte dysfunction [27, 28].

On the other hand, HBO was introduced in recent years as a promising tool in the treatment of sepsis. The beneficial influence of HBO on sepsis is based on the enhancement of killing capacity of leukocytes, which substantially depends on the amount of oxygen [29]. Although some studies highlighted the positive effects of HBO pretreatment on kidney and liver injury in a model of rat sepsis [30], there are no data regarding the heart tissue.

In this study we evaluate the function of the isolated rat heart through the whole set of different cardiac parameters. In that manner, our results showed that dp/dt max, as an indirect indicator of the inotropic properties of the heart, was changed neither after sepsis nor after the HBO therapy. Similarly, dp/dt min, as an indirect indicator of lusitropic properties of the heart, was also unchanged in sepsis with a slight improvement after HBO, but still not enough for statistical confirmation. The same results were noticed with all other parameters, except CF. Namely, coronary endothelial response in healthy animals and after HBO treatment was weaker (Figure 1). However, coronary perfusion was in all groups in physiological range for this kind of protocol. These findings pointed out that hyperbaric oxygenation limited the effect on coronary endothelium and not on cardiomyocytes. In addition, it can be assumed that the longer time of exposure or different HBO protocols may achieve other effects.

The possible explanation for these results could be the fact that septic animals often develop tachycardia that progressed, especially in non-survivors. This includes mechanisms such as sympathetic overstimulation, despite the use of continuous opioid analgesia to manage pain [31]. It was previously reported that stroke volume and heart rate could be good predictors of the early phase of sepsis in a 3-day rat model of fecal peritonitis, which is the same design we used [31].

In the other part of the study we evaluated the possible impact of HBO on cardiac and systemic redox state during sepsis and estimated the role of oxidative stress in achieved effects. As expected, cardiac oxidative markers were the lowest in healthy animals indicating correlation between sepsis and increased oxidative stress within the heart. In terms of septic conditions, our findings showed the evident and strong depressed release of all investigated pro-oxidants in isolated rat hearts (Figure 2) after HBO treatment, indicating powerful protective influence of this procedure. When considering these results in light of rat cardiodynamics, it can be assumed that beneficial effects of HBO are firstly seen on molecular levels and that for functional improvement, longer time of HBO exposure is needed. The mechanisms of positive HBO impact on the cardiac oxidative status are not easy to explain. It seems that in the heart, exogenously derived hyperbaric oxygen is some which succeeded in suppressing the production of endogenous reactive oxygen species, but this claim is difficult to prove.

Unlike this, we did not find any changes in systemic oxidative status (Figures 3 and 4). However, although insignificant, noticed trend of increased activity of SOD and GSH level as well as a drop in the production of O_2^- and TBARS, pointed out that perhaps for more prominent results longer duration of exposure to HBO and/or higher ATA could be applied.

Most of the literature data covering the impact of HBO on oxidative damages during sepsis are gathered from animal models focused on liver and kidney. It is previously documented that hyperbaric oxygen applied at 2 ATA for 60 min and 1, 4, 9, and 24 h after the induction of sepsis, reduces synthesis of free radicals and mortality in rats [32]. The difference in comparison with results of our study could be the fact that authors used a lipopolysaccharide model of sepsis and a different protocol of HBO therapy. Moreover, we followed the animals for 72 hours after the induction of sepsis with intense and frequent HBO sessions.

Others investigated the effects of HBO on rat renal damage and oxidative stress markers after the induction of sepsis with an intraperitoneal injection of *Escherichia coli* cells (2.1×10^9) while HBO treatment was conducted through five sessions of 2 ATA at intervals of 6 h [33]. It was found that hyperbaric oxygen increased SOD and CAT activity and consequently reduced oxidative damages of the kidney induced by sepsis [33].

Oter et al. [34] assessed the effects of HBO on liver function and morphology as well as oxidative status in rats with sepsis caused by intraperitoneal application of *Escherichia coli* cells (2.1×10^9). In that study, HBO (which applied as six sessions at 2 ATA for 90 min at 6h intervals) in combination with cefepime reverses sepsis-induced both histopathological and functional changes of liver potentially through improved antioxidant activity.

Interestingly, the newest researches support the approach that for the best antioxidative results in sepsis, HBO should be used along with antibiotic therapy [35]. All of these studies partially correlate with the present investigation along with the fence that studies differ in sepsis model and HBO protocols.

Conclusion

To the best of our knowledge, this is one of only few studies that estimate the influence of hyperbaric oxygenation on cardiac function and coronary circulation during septic conditions. In that sense, the findings of the present research may be an important basis for designing future experiments, as well as clinical investigations. In the present study, we showed that HBO treatment was not associated with improved cardiac function and coronary perfusion, while it expressed promising beneficial effects on cardiac oxidative stress. A deeper assessment of this topic including underlying molecular mechanisms requires further investigations.

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Therapeutic potential of agmatine in the experimental autoimmune encephalomyelitis

Terapijski potencijal agmantina u eksperimentalnom autoimunskom encefalomijelitisu

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Abstract

Background/Aim. Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis (MS), in which we investigated the neuroprotective effect of agmatine (AGM), known as a primary amine produced via the decarboxylation of L-arginine. **Methods.** Dark Agouti rats were divided into groups: control (C), Complete Freund`s Adjuvant (CFA), EAE rats decapitated on the 13th day *post* immunization (dpi) (EAE13) and on the 20th dpi (EAE20), EAE animals given three (EAE+AGM13) and 10 (EAE+AGM20) doses of AGM, and healthy animals administered three/10 doses of AGM (AGM). Thiobarbituric acid-reacting substances (TBARS), SH groups (SH), total glutathione (GSH), glutathione peroxidase activity (GPx), superoxide dismutase activities (tSOD, MnSOD, CuZnSOD) and nitrite/nitrate concentration (NO_2+NO_3) were assessed in plasma and brain structures [whole encephalitic mass (WEM) and brainstem (BS)]. **Results.** The obtained results showed that AGM treatment successfully attenuated severe clinical deficits in EAE. Applications of AGM in EAE rats induced normalized TBARS, SH, GSH, GPx and

NO in WEM. In BS, AGM expressed less prominent effects, inducing normalized TBARS, GPx and NO, but no effect on SH and GSH. In both brain structures, tSOD activity lowered and normalized at the peak and in the remission phase of the disease, post-AGM treatment. The effect of AGM on the MnSOD in EAE was expressed in WEM/BS only in the remission phase as a reduced activity. **Conclusion.** Milder clinical form of developed EAE in rats indicates promising therapeutic effect of AGM in MS. The activated antioxidant system and suppressed oxidative/nitrosative stress development may denote a successful blockade of neuroinflammation initiated by EAE immunization. The study implies the capability of AGM to attenuate oxidative/nitrosative damage at the peak of EAE by modulating antioxidative defense capacity during the time-course of the disease. Thus, AGM may be considered as an agent with a beneficial effect on neuroinflammation in EAE.

Key words:
agmatine; antioxidants; encephalomyelitis; multiple sclerosis; neuroprotective agents; oxidative stress; rats; treatment.

Apstrakt

Uvod/Cilj. Eksperimentalni autoimunski encefalomijelitis (EAE) je model multiple skleroze (MS) u kome su ispitivani neuroprotektivni efekti agmatina (AGM), poznatog primarnog amina koji se dobija dekarboksilacijom L-arginina. **Metode.** Dark Aguti pacovi su podeljeni u grupe: kontrola (C), kompletni Frojdov adjuvans (CFA), EAE pacovi dekapitovani 13 dana (EAE13) i 20 dana (EAE20) nakon imunizacije, EAE životinje sa tri (EAE+AGM13) i 10 (EAE+AGM20) doza AGM i zdrave životinje sa tri/10 doza AGM (AGM). Reaktivne supstance koje reaguju sa tiobarbiturnom kiselinom (TBARS), SH grupe (SH), koncentracija ukupnog glutationa (GSH), aktivnost glutation pero-

ksidaze (GPx), aktivnost superoksid dizmutaza (tSOD, MnSOD, CuZnSOD) i koncentracija nitrita/nitrata (NO_2+NO_3) su određivani u plazmi i moždanim strukturama [kompletna encefalitična masa (WEM) i produžena moždina (BS)]. **Rezultati.** Dobijeni rezultati su pokazali da tretman sa AGM uspešno smanjuje teški klinički deficit u EAE. Aplikacija AGM kod EAE pacova je normalizovala TBARS, SH, GSH, GPx i NO u WEM. U BS, AGM je doveo do manje izraženih efekata normalizacijom TBARS, GPx i NO, ali je bio bez efekata na SH grupe i GSH. U obe moždane strukture, tSOD je bila smanjena i normalizovana u piku i remisiji bolesti nakon tretmana sa AGM. Efekat AGM na MnSOD u EAE je bio izražen u WEM/BS samo u toku remisije bolesti i manifestovao se kao redukcija

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aktivnosti enzima. **Zaključak.** Blaža forma razvijenog EAE pokazuje izraženi terapijski efekat AGM kod MS. Aktivirani antioksidativni sistem i supresija razvoja oksidativnog/ nitrozativnog stresa mogu predstavljati uspešnu blokadu neuroinflamacije indukovanu EAE imunizacijom. Studija pokazuje sposobnost AGM da ublaži oksidativno/ nitrozativno oštećenje u piku EAE modulacijom kapaciteta antioksidativne odbrane u toku trajanja bolesti. U tom smislu, AGM se može smatrati agensom za antioksidativno lečenje i prevenciju neuroinflamacije u EAE.

Ključne reči:
agmatin; antioksidansi; encefalomijelitis; multipla skleroza; neuroprotekstivi; stres, oksidativni; pacovi; lečenje.

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune neuroinflammatory disease, concerning the animal model used to study human multiple sclerosis (MS). The autoimmune molecular target(s), which have been identified and used in biological models, seem to be proteins expressed by myelin-producing oligodendrocytes [1].

Neuroinflammation in EAE targets the spinal cord/ cerebellum, triggering so-called flaccid paralysis characterized by reduced muscle tone that progress from the tail upward along the body [2]. Dark Agouti (DA) rats are genetically susceptible to EAE [3]. The disease follows a predictable clinical course, characterized by a prodromal period of 10–15 days followed by ascending paralysis beginning in the tail and hind limbs and progressing to the fore-limbs concurrent with weight loss. Progressive hind-limb paralysis during EAE is the consequence of demyelination and damaged axonal conduction followed by inflammation [4].

The control of reactive oxygen and nitrogen species (ROS, RNS) and their cytotoxic bioproducts in affected cells are performed through scavenging enzymes or thiols, which participate in tissue repair and survival during EAE. Oxidative and nitrosative stress play an important role in myelin loss and degeneration of nerve tissue in MS [5]. It is one of the critical steps in the progression of neurodegenerative diseases and conditions where oxidative stress causes damage to mitochondria, with consequent energy failure, through the production of ROS/RNS [6, 7].

Nitric oxide (NO$^\bullet$) seems to be a target for new therapies in human demyelinating disease [8]. In physiological conditions, NO$^\bullet$ was created from the oxidation of the terminal guanidine nitrogen of arginine by the enzyme NO$^\bullet$ synthase (NOS) presented in three isoforms (neuronal-nNOS, endothelial-eNOS, and inducible-iNOS) [9]. Besides its role as a transsynaptic retrograde messenger in the brain, in EAE and MS, NO$^\bullet$ promotes neuronal injury, inducing mitochondrial dysfunction, lipid peroxidation (LPO), as well as nitration of key protein and ion channel disability [10]. Suppressed NO$^\bullet$ metabolism seems to express beneficial effects during the course of experimental MS [11].

Malondialdehyde (MDA) is an end-product of polyunsaturated fatty acids peroxidation and represents the severity of oxidative stress-induced brain injury [12]. An indicator of LPO was determined by measurable parameter thiobarbituric acid reactive substances (TBARS) [13]. Antioxidant defense against free radical-induced toxicity include enzymes that scavenge ROS/RNS, such as catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), but also non-enzymatic biomolecules: glutathione (GSH), vitamins C and E, uric acid [14]. Their synergistic action regulates oxido-reductive balance and thus suppresses oxidative injury [15].

The present study examined potentially beneficial effect of agmatine – AGM (4-aminobutyl) guanidine, on oxidative and/or nitrosative stress development in the EAE – the model of MS. AGM is an amine that is formed by decarboxylation of L-arginine with the enzyme arginine decarboxylase and hydrolyzed through the enzyme agmatinase to putrescine [16]. It exerts neuromodulation properties, particularly assuring the control and modulation of NO pathway, influencing glutamate N-methyl-D-aspartate (NMDA) receptors and limiting oxidative/ nitrosative stress development. By its antioxidant activities, AGM reveals neuroprotective outcomes [17]. We proposed that treatment with AGM administered during the acute phase of EAE would attenuate disease severity, both clinically and biochemically. Based on the presented data, the aim of this study was to examine the role of ROS/RNS and the effectiveness of AGM treatment on rats' whole encephalitic mass (WEM) as well as on brainstem (BS) in EAE.

Methods

Animals

A permission of the Ethics Committee for the welfare of experimental animal's No. 119-01-5/14/2017-09 was obtained from the Ministry of Agriculture and Environmental Protection – the Veterinary Directorate of the Republic of Serbia. Inbred two-month-old female DA rats were kept in cages under standardized housing conditions (ambient temperature: 23 ± 2 °C, relative humidity: 55% ± 3% and a light/dark cycle: 13/11 h) and had free access to standard laboratory pellet food and tap water. All the experiments were performed after 7 days of adaptation to laboratory conditions and were carried out between 9 a.m. and 1 p.m. For the principle of welfare, during the period of paralysis, water and food were given manually.

Experimental procedure

Experimental autoimmune encephalomyelitis was induced by the subcutaneous (sc.) injection of 0.1 mL solution of rat spinal cord tissue homogenate (50% w/v in saline) dissolved in Complete Freund`s Adjuvant (CFA; Sigma, St. Louis, MO, USA) in the right hind footpads. The dose of

AGM (75 mg/kg bw.; dissolved in water) was selected on the data based on our previous studies with other models, which showed that a dose of 75 mg/kg bw. was not toxic (no change in body weight or food intake in rats, and no visible morphological changes) [18]. EAE-untreated rats received an equal volume of saline or CFA. From the day *post* immunization (dpi), the rats were daily monitored for the clinical score. Applied protocols with AGM in all animals were performed starting from the 10th day; by default, every day intraperitoneal application. The time point decapitation was performed on the 13th dpi (EAE13; the peak of the disease; animals received three doses of AGM) and on the 20th dpi (remission of the disease; animals received 10 doses of AGM). Before the immunization, as well as before the decapitation, all animals were intraperitoneally anesthetized (sodium-pentobarbital 45 mg/kg bw.). Clinical evaluation of EAE was undertaken daily in a double-blind manner 20 dpi [19].

The animals were randomly divided into experimental groups: control group (C, n = 8); group treated with Complete Freund`s Adjuvant (CFA, n = 8); EAE group that was decapitated on the 13th dpi (EAE13, n = 8); EAE group that was decapitated on the 20th dpi (EAE20, n = 8); EAE group treated with three doses of AGM (EAE+AGM13, n = 8); EAE group treated with 10 doses of AGM (EAE+AGM20, n = 8), as well as healthy animals treated with three (n = 8) and 10 (n = 8) doses of AGM integrated into a common group (AGM, n = 16).

The decapitation was performed 24 hours after the last AGM application or at the appropriate time point. The animals' brains were immediately put on liquid nitrogen and stored at -20 °C until analysis.

Clinical evaluation

All animals were scored daily according to the clinical signs on a scale of 0–5. EAE clinical expression was considered as 0 = no abnormalities; 0.5 = partial loss/ reduced tail tone and inability to rotate the back end of the tail; 1 = tail atony; 1.5 = slightly/ moderately unsteady gait and reduced straightening up ability or combination; 2 = hind limb weakness; 2.5 = partial hind limb paralysis; 3 = complete hind limb paralysis; 3.5 = complete paralysis of hind limbs and forelimb weakness; 4 = quadriplegic with breathing difficulties; 5 = moribund state or death [19]. Numerous parameters of the disease were observed to estimate the severity of EAE: mean clinical score (average clinical scores for all rats within a group on a specified day); mean maximal severity score (the mean of the maximal clinical score that each animal in a group extended over the course of the experiment); duration of paralysis (the mean number of days for which the rats had a score of 2 or more).

Measurement of oxidative/nitrosative status indicators in plasma and brain homogenates

Blood samples for determining oxidative/nitrosative status parameters were collected from the external iliac vein into vials containing heparin and EDTA.

The brain structures (WEM and BS) were dissected on ice, and 0.1 g of each tissue slice was transferred into 0.9% sodium chloride (normal saline). Homogenization was performed on ice by a homogenizer (Tehtnica, Zelezniki, Slovenia) at 800 rotations/ min. The homogenates were centrifuged ($1,000 \times g$, 15 min, 4 °C), the precipitates were redispersed in sodium chloride, centrifuged ($2,500 \times g$, 30 min, 4 °C) and the obtained precipitates were dissolved in 1.5 mL of deionized water. The samples were centrifuged again ($2,000 \times g$, 15 min, 4 °C) and the supernatants (crude mitochondrial fractions) were stored at -70 °C [20]. The total protein concentration was determinate by Lowry et al. [21] in WEM and BS.

Lipid peroxidation analysis in the plasma and WEM/BS was measured as thiobarbituric acid reactive substances (TBARS) production using the method described by Girotti et al. [22]. The results are expressed as µM/L in plasma and µM/mg proteins in brain homogenates.

The determination of total SH groups in plasma and WEM/BS was carried out according to the method of Elman [23]. The results are expressed as mM/L in plasma and nM/mg proteins in brain homogenates.

The total glutathione (GSH+1/2GSSG, in GSH equivalents) content was established by the DTNB-GSSG reductase recycling assay, spectrophotometrically at 412 nm [24]. The results were expressed as nM/mg proteins.

Glutathione peroxidase analysis (Randox Laboratories, USA) was performed spectrophotometrically at 340 nm [25]. The unit of enzyme activity of GPx is defined as the number of micromoles of NADPH oxidized per min (µM NADPH/min). The results were expressed as U/mg proteins.

Superoxide dismutase (EC 1.15.1.1.; SOD) activity was measured spectrophotometrically, as inhibition of epinephrine spontaneous auto-oxidation for 10 min at 480 nm [26]. Cytosolic SOD (CuZnSOD) was calculated as the difference of total (tSOD) and mitochondrial (MnSOD) enzyme activity. All three isoforms of SOD were expressed as U/mg proteins.

Nitrite and nitrate ($NO_2 + NO_3$) accumulation, as an indirect measure of NO release, was determined in WEM/BS, using the colorimetric method of Griess at 492 nm [27]. The results were expressed as nM/mg proteins.

Statistical analysis

One-way ANOVA and Tukey`s *post hoc* tests were used (software GraphPad Prism, version 5.03) for statistical data analysis. Values are presented as means ± standard deviation (SD). The linear regression analysis was performed to determine the relation between the obtained values of parameters, using the statistical program GraphPad Prism. Differences were considered statistically significant for $p < 0.05$.

Results

All immunized animals developed clinical signs of the disease (100% incidence) (Table 1).

Most AGM treated animals developed a milder form of EAE in comparison to EAE rats and completely recovered at the end of the observing interval (Figure 1). The mean

Table 1

The influence of agmatine (AGM) on the extent of induced experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13) and in the remission of EAE (EAE20), as well as after three (EAE+AGM13) and 10 (EAE+AGM20) doses of AGM in EAE animals

Parameter	EAE13	EAE+AGM13	EAE20	EAE+AGM20
Animals, n	8	8	8	8
Incidence, n	8/8	8/8	8/8	8/8
Mean maximum severity score	2.79 ± 0.57	1.50 ± 0.71†	2.50 ± 1.12	1.00 ± 0.50‡
Duration of paralysis in days	2.13 ± 0.35	1.38 ± 0.52†	4.00 ± 0.45	1.43 ± 0.79‡
Mortality rate	1/8	0/8	3/8	1/8

Data are presented as mean ± standard deviation (SD).
†Indicates a statistically significant difference from the EAE group at the peak of the disease (EAE13); ‡Indicates a statistically significant difference from the EAE group in the remission of the disease (EAE20).
p – values were obtained by one-way ANOVA followed by Tukey`s test ($p < 0.05$).
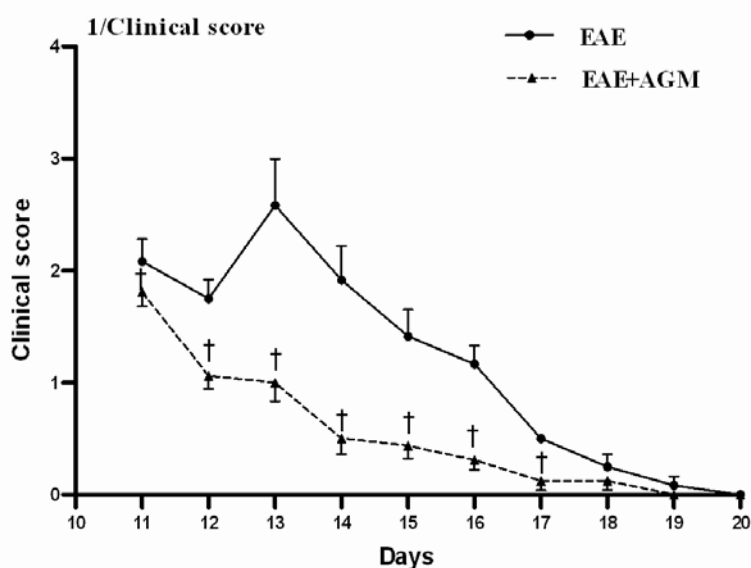
Fig. 1 – The effect of agmatine (AGM) treatment on the experimental autoimmune encephalomyelitis (EAE) clinical signs from 11th – 20th day post immunization (dpi).
Results are given as mean ± standard deviation (SD).
†$p < 0.05$ statistically significant difference compared to the EAE group.

maximal severity score was significantly lower in the AGM treated EAE group at the peak of the disease (1.50 ± 0.71 vs. 2.79 ± 0.57, $p < 0.05$) and in the remission of EAE (1.00 ± 0.50 vs. 2.50 ± 1.12, $p < 0.05$) compared to the appropriate EAE group. In addition, the duration of paralysis was significantly shorter in the EAE+AGM13 (1.38 ± 0.52 vs. 2.13 ± 0.35, $p < 0.05$) and EAE+AGM20 (1.43 ± 0.79 vs. 4.00 ± 0.45, $p < 0.05$) groups in comparison to the EAE rats. Additionally, mortality rate was lower in the EAE+AGM13 group compared to the EAE13 group (12.5%) and in the EAE+AGM20 group (12.5%) compared to the EAE20 group (37.5%).

Concentrations of TBARS in plasma increased in the the EAE13 and EAE+AGM13 groups compared to the control group. In the remission of the disease, TBARS concentration lowered compared to the EAE13 (Figure 2A). In tissue, compared to controls, TBARS increased in WEM of the EAE13 and EAE20 groups, while in BS, TBARS increased at the peak of the disease (EAE13) (Figure 2B). Compared to the appropriate EAE group (EAE13/EAE20), the administration of AGM reduced TBARS in WEM (EAE+AGM13/20), while in BS, AGM lowered TBARS at the peak of the disease (EAE+AGM13).

Among all groups, total SH groups content in plasma was decreased only at the peak of EAE (EAE13) compared to the control (Figure 3A). A similar trend of results was registered in tissue: decreased SH in WEM and BS at the peak of EAE (EAE13) and normalized SH in WEM in the remission of EAE, as well as post AGM application. Assuming higher control values in BS, SH depleted in both investigated time points of the disease (EAE13 an EAE20) and after AGM administration at the peak of the disease (EAE+AGM13) (Figure 3B).

The results showed a decreased GSH content in WEM of the EAE13 group, while in the remission of the disease, GSH values normalized to controls in the EAE20 group and
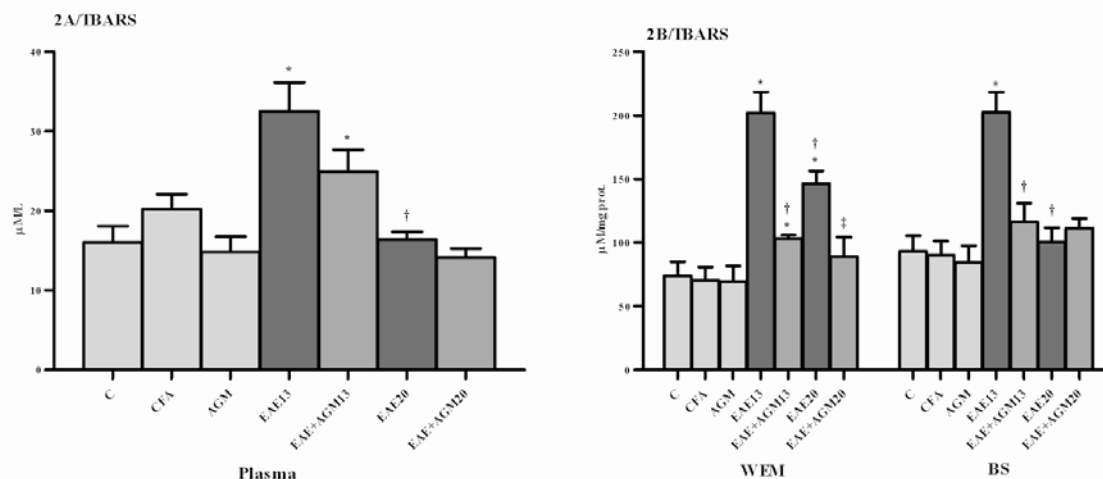
A)   B)

Fig. 2 –Thiobarbituric acid reactive substances (TBARS) in (A) plasma (μM/L), and (B) brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; μM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are expressed as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

A)   B)

Fig. 3 – Total SH groups (SH) in (A) plasma (mM/L), and (B) brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; nM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), EAE at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

both the EAE+AGM13 and EAE+AGM20 groups (Figure 4). In BS, similar to SH groups, GSH content was higher in the control groups (C, CFA, AGM) and markedly reduced at the peak and the remission of EAE (EAE13 and EAE20), but also after 10 administered doses of AGM in EAE (EAE+AGM20). Three doses of AGM at the peak of EAE induced GSH repair in BS.

The activity of GPx in both WEM and BS decreased at the peak of the disease (EAE13), while in all other investigated groups, it restored to controls (Figure 5).
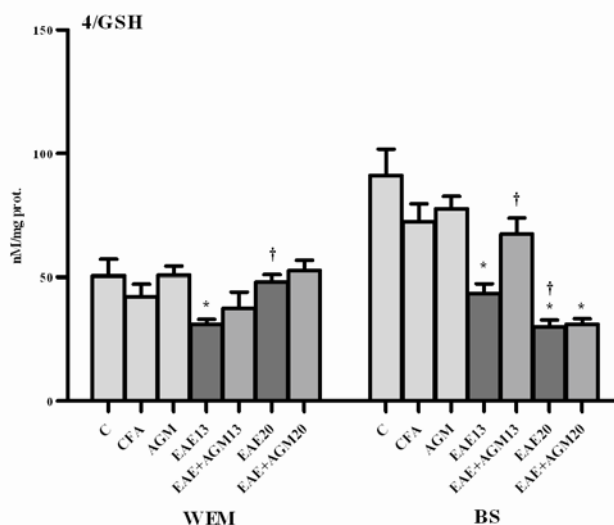
Fig. 4 – The total glutathione content (GSH) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; nM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to [*]the control group (C), compared to [†]the EAE group at the peak of the disease (EAE13) and compared to [‡]the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

Fig. 5 – The glutathione peroxidase activity (GPx) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; mU/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to [*]the control group (C), compared to [†]the EAE group at the peak of the disease (EAE13) and compared to [‡]the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

The activity of tSOD in WEM increased in the EAE13 and EAE20 groups compared to the control, while a reduced tSOD was registered in the EAE+AGM13 and AGM groups (Table 2). In BS, we noted the increased tSOD activity at the peak (EAE13) and at the remission (EAE20) of the disease compared to the control, and a significantly decreased enzyme activity after AGM treatment at both intervals of the disease (EAE+AGM13, EAE+AGM20) compared to the appropriate EAE group.

Mitochondrial SOD activity increased in both WEM and BS at the peak of EAE (EAE13 and EAE+AGM13) as well as in the remission of EAE (EAE20 and EAE+AGM20) compared to the control (Table 2).

Cytosolic SOD activity in WEM increased at the peak of EAE (EAE13) and significantly decreased after AGM treat-ment (EAE+AGM13) compared to the control. In BS, we noted the elevated CuZnSOD activity at the peak (EAE13) and at the remission (EAE20) of the disease compared to control values, however, AGM treatment significantly decreased enzyme activity at the peak of EAE (EAE+AGM13) compared to the EAE13 group of animals (Table 2).

The results showed the increased NO_2+NO_3 concentrations in both WEM and BS of the EAE13 group and normal-ized $NO^\bullet$ level after AGM treatment at the peak of EAE (EAE+AGM13). In the remission phase of the disease (EAE20), NO_2+NO_3 lowered in both WEM and BS compared to the peak of the disease (EAE13). Ten-day application of AGM induced additional reduction of NO_2+NO_3 in WEM (EAE+AGM20) compared to EAE20 and a significant elevation of NO_2+NO_3 in BS compared to EAE20 (Figure 6).

Table 2

Activities of superoxide dismutases [total superoxide dismutase (tSOD), manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD)] in whole encephalitic brain (WEM) and brainstem (BS) at the peak of the disease (experimental autoimmune encephalomyelitis – EAE13) and in the remission of EAE (EAE20), as well as after three (EAE+AGM13) and 10 (EAE+AGM20) doses of agmatine (AGM) in EAE animals

Superoxide dismutases	Brain structure	Groups						
		C	CFA	AGM	EAE13	EAE+AGM13	EAE20	EAE+AGM20
tSOD	WEM	727 ± 121	850 ± 112	$514 \pm 115^*$	$1,743 \pm 343^*$	$574 \pm 101^{*,\dagger}$	$909 \pm 105^{*,\dagger}$	756 ± 178
	BS	1052 ± 85	1152 ± 114	$1,038 \pm 217$	$2,189 \pm 402^*$	$1,190 \pm 349^\dagger$	$1,716 \pm 464^*$	$1,193 \pm 287^\ddagger$
MnSOD	WEM	71 ± 12	102 ± 23	73 ± 9	$210 \pm 42^*$	$169 \pm 37^{*,\dagger}$	$145 \pm 28^{*,\dagger}$	$99 \pm 4^{*,\ddagger}$
	BS	102 ± 23	115 ± 25	$125 \pm 16^*$	$324 \pm 10^*$	$228 \pm 37^{*,\dagger}$	$232 \pm 72^{*,\dagger}$	$153 \pm 41^*$
CuZnSOD	WEM	656 ± 122	812 ± 122	$438 \pm 130^*$	$1,533 \pm 348^*$	$406 \pm 106^{*,\dagger}$	$764 \pm 109^\dagger$	628 ± 108
	BS	950 ± 91	1115 ± 115	913 ± 212	$1,864 \pm 404^*$	$879 \pm 212^\dagger$	$1,484 \pm 404$	$1,039 \pm 287$

Data are presented as mean ± standard deviation (SD).
C – control (saline); CFA – Complete Freund`s Adjuvant.
*Indicates a statistically significant difference from the control group of animals; †Indicates a statistically significant difference from the EAE group at the peak of the disease (EAE13); ‡Indicates a statistically significant difference from the EAE group in the remission of the disease (EAE20).
p – values were obtained by one-way ANOVA followed by Tukey`s test ($p < 0.05$).
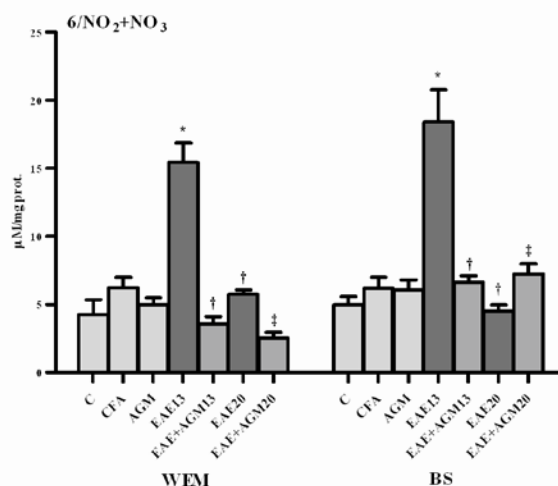
Fig. 6 – The nitrite and nitrate concentration (NO_2+NO_3) in brain homogenates [whole encephalitic mass (WEM) and brainstem (BS)]; µM/mg proteins in following groups of animals: control (C), Complete Freund`s Adjuvant (CFA), healthy animals treated with three and 10 doses of agmatine (AGM), experimental autoimmune encephalomyelitis (EAE) at the peak of the disease (EAE13), EAE and AGM at the peak of the disease (EAE+AGM13), EAE in the remission of the disease (EAE20) and EAE and AGM in the remission of the disease (EAE+AGM20).
Values are given as mean ± standard deviation (SD).
The labels of significance: compared to *the control group (C), compared to †the EAE group at the peak of the disease (EAE13) and compared to ‡the EAE group in the remission of the disease (EAE20).
Statistical significance was considered at $p < 0.05$ (One Way ANOVA, Tukey`s tests).

Discussion

The obtained results showed that AGM treatment successfully attenuated severe clinical deficit and suppressed oxidative/ nitrosative stress in EAE. The most severe clinical score was revealed around 13 dpi, followed by the signs of a recovery (Figure 1). This biological model of EAE is complementary with the previously published results in mice EAE, where maximum clinical signs appeared around 20 dpi [18]. During the disease expansion, AGM expressed a strong protective effect, reducing the clinical score in rats with EAE (EAE+AGM) at the peak of the disease compared to the EAE group (Table 1). Also, in the remission of the disease, EAE neurological signs in EAE animals were significantly more severe when compared to the EAE+AGM rats, suggesting the importance of NO metabolism in EAE pathogenesis. The better clinical score in the EAE+AGM group compared to the EAE animals suggests that AGM suppressed inflammation in EAE, which is in accordance with the previously published results [28, 29].

Oxidative stress in MS is a toxic condition in which the excessive production of ROS overcomes the intrinsic antioxidant capacities [30]. Many studies revealed the existence of oxidized phospholipids and MDA in the myelin membranes of apoptotic oligodendrocytes, together with oxidized DNA in oligodendrocyte nuclei [31]. An increase of TBARS in the plasma of EAE rats indicates the increased oxidative damage of lipids with systemic generalized expression (Figure 2A). Following the immunization, we showed the increase in TBARS in brain homogenates (WEM, BS) of EAE rats at the peak of the disease (Figure 2B) which is in accordance with the previous studies [32]. Agmatine treatment, which is known for its immunomodulatory and antioxidative effects, is followed by a significant decrease in TBARS (EAE+AGM20) compared to EAE13 in plasma and in WEM/BS (EAE+AGM13/20) (Figure 2) [33].

Although protein SH groups is determined by the structure and function of proteins, non-protein thiols are predominant in the cellular defense against oxidative stress. The key antioxidant reserve corresponds to several thiol groups, but additionally they can be the major targets for ROS and RNS. Membrane SH groups increase membrane permeability to calcium ions (Ca^{2+}) following excitotoxicity, as well as the production of ROS/RNS that leads to the increase in lipid peroxidation and the decrease in SH groups in plasma of EAE animals at the peak of the disease (Figures 2A, 3A). Plasma SH is modified in MS patients, indicating that SH content is a useful biochemical marker of *in vivo* redox reactions [34]. The results of decreased total SH groups in plasma and WEM/BS homogenates in the EAE13 group (Figures 3A, B) denote the excessive redox-dependent changes in EAE, which, additionally, affect the activity of the mitochondrial respiratory chain complex [35]. The demyelinating condition is associated with the increased protein SH groups nitrosylation, leading to the total SH content depletion [36]. In contrast, during AGM therapy, which led to repairing the resulting oxidative damage, total SH in plasma and tissue homogenates (WEM, BS) were restored to control values (Figures 3A, B).

Depleted GSH in WEM/BS is in accordance with the decreased SH groups at the peak of the disease (EAE13), while in the remission phase of EAE (EAE20), SH groups and GSH restore in WEM (Figure 4). Assuming that GSH is the major thiol present in brain tissue as redox buffer and the fact that MDA is considered as a good marker of LPO, inverse relationship between GSH level and MDA concentration in the EAE13 group indicate the current state of oxidative stress [37]. In EAE20, together with normalized TBARS in BS, SH and GSH were not restored, meaning the prolonged pro-oxidative loads. The treatment by AGM induced the repair of GSH in BS at the peak of the disease, sustaining its antioxidant profile [18]. Normalized TBARS at the peak of EAE (EAE13) and on the 20th dpi indicate the better antioxidant outcome of AGM on lipid components than on proteins.

The result of toxic oxygen metabolites in the brain, generated by neurons and glial cells is the production of superoxide anion ($O_2^{\bullet-}$), which is dismutated to hydrogen peroxide (H_2O_2) as a precursor of high destructive hydroxyl radical ($^{\bullet}OH$). They are all able to react with membrane lipids and cause LPO [38]. The destructive effects of these ROS are interrupted with GPx/GSH enzyme system, which is one of the crucial cell redox pathways [39]. Lower GPx activity in WEM of EAE13 rats revealed reduced antioxidative capacity against oxidative stress (Figure 5), which may result from its inhibition and inactivation by ROS in the presence of $O_2^{\bullet-}$ [40]. As a result of the increased oxidative stress in the EAE13 group, we registered a significantly reduced GPx, followed with reduced GSH in WEM and BS (Figures 4, 5). It could mean that tissue oxidative stress during EAE is predominantly induced by the suppressed non-enzymatic role of GSH rather than by its cofactor function in GPx. The treatment with AGM after three doses in EAE animals (EAE+AGM13) led to restored GPx in WEM (Figure 5). The AGM pathway not only seems to promote neuronal health through its transformation to polyamines, but additionally suppresses inflammation and excitotoxicity, affecting the glutamatergic transmission, reducing glutamate release and inhibiting NOS activity [33]. These multifaceted aspects of the AGM pathway can clarify the restored GPx activity in EAE groups with AGM, as well as their possible therapeutic potential and drug discovery.

The elevated MnSOD and CuZnSOD, as well as total SOD activity in WEM and BS of EAE13 and EAE20, denoted the compromised oxidative balance (Table 2). The application of AGM in EAE13 induced a decreased total SOD and cytosolic SOD in WEM, contrary to significantly increased MnSOD compared to controls, meaning that AGM in WEM influenced predominantly cytosolic and extracellular SOD. At the peak of EAE, the AGM application induced normalized total SOD and CuZnSOD in BS, while MnSOD stayed elevated (same as in EAE). The remission of EAE in both structures (WEM, BS) is characterized by depressed SOD isoforms (tSOD, MnSOD, CuZnSOD) compared to the EAE13 group. AGM induced normalized activity of tSOD and CuZnSOD in the remission of the disease in WEM and BS, with a mild elevation of MnSOD compared to controls.

Such results indicate AGM-induced predominant influence on mitochondrial SOD in BS, throughout EAE, which could be promising, assuming that the mitochondria respiratory chain is an important source of ROS [41].

Besides its beneficial neuro/immunomodulatory effects, NO• participates in the disruption of the blood-brain barrier (BBB), promoting inflammatory, and cytotoxic effects, and inducing oligodendrocyte injury and demyelination, axonal degeneration, and impairment of axonal conduction [42, 43]. Elevated NO_2+NO_3 in EAE13 are followed by AGM-induced normalized NO in both WEM and BS (Figure 6). Astrocytes in MS plaques express high levels of constitutive NOS, producing NO, which interact with $O_2^{•-}$ and produce highly reactive peroxynitrite ($ONOO^-$). The reaction rate between NO and $O_2^{•-}$ is three times higher than superoxide dismutation by SOD, which may harm oligodendrocytes and axons [44]. The mechanisms underlying AGM inhibitory action on astrocytes might, therefore, include the inhibition of NO• production at the peak of the disease and subsequent pathological effects of NO• hyperproduction [45]. The significant positive correlation between MnSOD activity and NO_2+NO_3 (r = 0.8095, $p < 0.05$) in WEM of the EAE+AGM13 rats at the peak of the disease may suggest mitochondria as a crucial generator of ROS/RNS in EAE and as a place of the beneficial effects of AGM.

Depleted NO in both WEM and BS after the AGM treatment may suggest that NOS interconnects its effect with AGM, accomplishing physiological effects in the brain [46]. Being structurally similar to L-arginine, AGM is a competitive NOS inhibitor [47]. It also protects neurons against glutamate toxicity and this effect was mediated by NMDA receptor blockade, with AGM interacting at a site located within the NMDA channel pore [48]. The increased NO_2+NO_3 level in the inverse proportion to the GSH content in EAE rats can be explained by the oxidation of GSH within free radical neutralization, as well as its lower synthesis, resulting from a decreased production of cysteine as the limiting GSH precur-

sor. The beneficial effect of AGM could be related to its ability to inhibit iNOS or to block NMDA receptors and/or voltage-dependent Ca^{2+} channels [49]. A possible explanation for the decrease NO and TBARS concentrations might be that NO acts as ROS scavenger, protects cells from LPO and, consequently, prevents the progressive increase of TBARS level.

Conclusion

These results indicate that LPO might have a more important role in protection after AGM administration in EAE animals. Additionally, the AGM treatment in EAE rats which caused a significant decrease in TBARS concentration, suggested the activation of the antioxidant system, resulting in an aggressive oxidative mechanism blockade initiated by EAE immunization. The neuroprotective roles of AGM were acknowledged through the oxidative stress development indicators, such as decreased TBARS and increased GSH concentration.

The study denotes that AGM can attenuate oxidative/ nitrosative stress at the peak of EAE by modulating antioxidative defense capacity during the time-course of the disease. Several studies that have investigated the neuroprotective effects of AGM, through its ability to reduce oxidative stress, suggested the beneficial effect of AGM in the treatment of neuroinflammation in MS experimental models. The precise mechanisms of AGM action remain to be elucidated.

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The effect of cardiovascular rehabilitation on physical strain tolerance – does gender really matter?

Uticaj kardiovaskularne rehabilitacije na toleranciju fizičkog napora – da li je pol zaista bitan?

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Abstract

Background/Aim. Gender as a risk factor for cardiovascular diseases has been the subject of research in numerous studies. All of them warn of shortcomings in the diagnosis and treatment of women with a coronary artery disease. The aim of this study is to determine whether there is a difference in the effects of cardiovascular rehabilitation (CVR) on the tolerance of physical strain related to gender in examinees with the coronary artery disease. **Methods.** The study involved 684 patients, 506 (74.0%) men and 178 (26.0%) women. All respondents were referred to the CVR program after surviving a heart attack, percutaneous coronary intervention or surgical myocardial revascularization. During a three-week program of CVR, patients were subjected to the dosed and personalized physical training. At the beginning and at the end of rehabilitation, all patients were tested for physical strain. **Results.** The average strain level in men was significantly higher in the second test ($t = 4.368$; $p < 0.001$). Also, the duration of the test was significantly longer in the second test ($Z = 11.836$; $p < 0.001$). In women, the average strain level was significantly higher ($t = 5.352$; $p < 0.001$), and the duration of the test was significantly longer in the second test ($Z = 7.471$; $p < 0.001$). **Conclusion.** A three-week program of CVR led to an improvement in the tolerance of physical strain in both men and women. Our research once again proved that women have an equal benefit as men from the implementation of CVR. Nevertheless, women rarely participate in the CVR programs. It is necessary to make additional efforts in order to further educate physicians and other medical staff about the importance of sending women to the CVR program.

Key words:
cardiovascular diseases; risk factors; sex, factor; rehabilitation; exertion, physical; coronary artery disease.

Apstrakt

Uvod/Cilj. Pol, kao faktor rizika od kardiovaskularnih bolesti (KVB), je bio predmet ispitivanja u brojnim studijama. Sve one upozoravaju na nedostatke u dijagnostičkom pristupu i načinu lečenja žena obolelih od koronarne bolesti srca. Cilj ovog rada bio je da se utvrdi da li postoji razlika u efektima kardiovaskularne rehabilitacije (KVR) na toleranciju fizičkog napora u odnosu na pol kod ispitanika sa koronarnom bolešću srca. **Metode.** U ispitivanje je bilo uključeno 684 bolesnika, od toga 506 (74,0%) muškaraca i 178 (26,0%) žena. Svi ispitanici su upućeni na program KVR u Institut za lečenje i rehabilitaciju Niška Banja nakon preživelog srčanog udara, perkutane koronarne intervencije i/ili hirurške revaskularizacije miokarda. Tokom tronedeljnog programa stacinonarne rehabilitacije, bolesnici su bili podvrgnuti doziranom i personalizovanom fizičkom treningu. Na početku i kraju rehabilitacije svim bolesnicima je urađen test fizičkog opterećenja. **Rezultati.** Kod muškaraca prosečan nivo opterećenja je bio značajno veći na drugom testu ($t = 4,368$; $p < 0,001$). Trajanje testa je bilo takođe znatno duže na drugom testu ($Z = 11,836$; $p < 0,001$). I kod žena prosečan nivo opterećenja je bio značajno veći ($t = 5,352$; $p < 0,001$), a trajanje testa značajno duže ($Z = 7,471$; $p < 0,001$) na drugom testu. **Zaključak.** Tronedeljni program KVR doveo je do poboljšanja tolerancije fizičkog napora i kod muškaraca i kod žena. Naše istraživanje je još jednom pokazalo da žene imaju podjednaku korist od sprovođenja KVR. I pored toga žene u značajno manjoj meri participiraju u programima KVR. Neophodno je učiniti dodatni napor kako bi se medicinsko osoblje dodatno edukovalo o značaju upućivanja žena na program KVR.

Ključne reči:
kardiovaskularne bolesti; faktori rizika; pol, faktor; rehabilitacija; napor, fizički; koronarna bolest.

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Introduction

Cardiovascular diseases (CVD) are the leading cause of death in the world. According to the latest data from the World Health Organization (WHO), about 17.9 million people die annually of CVD, which is about 31% of the total mortality [1]. The most lethal CVD are the coronary heart disease (CAD) and stroke. Every year, more than seven million people die of CAD and it is the main cause of death and disability in developed countries [2]. Fibrinolytic therapy, coronarography and, in particular, percutaneous coronary intervention (PCI) have led to a decline in the mortality of CAD [3].

Gender as a risk factor for CVD has been the subject of research in numerous studies. It is believed that almost 1/2 of women over the age of 20 and about 1/3 of men suffer from some cardiovascular diseases [4, 5]. The Framingham study has shown that CAD is more common in men, especially in relation to premenopausal women [6]. On the other hand, recent studies warn of shortcomings in the diagnosis and treatment of women with CAD [7]. A large meta-analysis done by Kim et al. [8] indicated a higher incidence of complications and higher early mortality in women after percutaneous coronary intervention or surgical myocardial revascularization.

Cardiovascular rehabilitation (CVR) is of paramount importance in the secondary prevention of cardiovascular events [9, 10]. This relates primarily to those patients who have suffered a heart attack, have had a surgical revascularization of the myocardium and/or PCI. Cardiovascular rehabilitation does not only include a dosed physical activity, but also an adequate patient education, the struggle against modifying risk factors, psychosocial adaptation, and adequate cardioprotective medication therapy [11]. As such, CVR has been shown to have a great effect on the quality and duration of life [12, 13].

The aim of this study was to determine whether there is a difference in the effects of CVR on the tolerance of physical strain related to gender in examinees with ischemic heart disease.

Methods

The study involved 684 patients, 506 (74.0%) men and 178 (26.0%) women. The average age of the subjects was 60.84 ± 9.81 years. All respondents were referred to the CVR program at the Institute for Treatment and Rehabilitation Niška Banja after surviving a heart attack, PCI and/or surgical myocardial revascularization. During a three-week program of CVR, patients were subjected to the dosed and personalized physical training that involved bicycle riding, walking and cardiovascular exercise programs. At the beginning and at the end of rehabilitation, all the patients were tested for physical strain. The tests were done on the treadmill track using the Bruce protocol. Tests were limited by submaximal heart rate (calculated as 85% from 220-age equation); symptoms and signs like chest pain, lack of air, dizziness, etc., complex heart rhythm disorders, and/or electropathological changes on the electrocardiogram. Electropathological changes involved the oc-

currence of horizontal and/or down-sloping ST depression ≥ 01 mm. Complex heart rhythm disorders included long-term episodes of bigeminy of ventricular premature complex (VPC) (long term meaning at least four consecutive second-to-normal VPC), couplets of VPC, and ventricular tachycardia.

Over six thousand patients were considered to participate in the study but it involved only 684 of them, as we included only those patients who finished the entire three-week rehabilitation program and had at least two tests during their stay at the Institute. Patients who did not finish the entire program and/or did not have two tests during their stay at the Institute were not included in our study.

Data on the ejection fraction of the left ventricle and other echocardiographic parameters were obtained from the accompanying medical documentation of the patients, while the data on risk factors for CVD were taken with a detailed anamnesis and basic clinical examination.

Statistics

Data were analyzed using SPSS software-version 20. Qualitative data were expressed as frequencies and percentages, while quantitative data were presented as mean $\pm$ standard deviations (SD). Data distribution was tested using the Kolmogorov-Smirnov test. Normally distributed data were compared by the Student t-test, while the Mann-Whitney test, the Wilcoxon signed Ranks test and the Mc Nemar test were used for abnormally distributed data. For the comparison of frequencies, the chi-square test was used. Statistical significance was accepted for $p < 0.05$.

Results

The age structure of patients did not significantly differ between genders (men: 60.6 ± 9.81 versus women: 61.54 ± 9.83, $t = 1.102$, $p = 0.271$). Myocardial infarction (MI) was more common in women than in men ($\chi^2 = 6.283$; $p = 0.012$). Men were more often subjected to coronary artery bypass grafting (CABG) ($\chi^2 = 12,100$; $p = 0.001$). On the other hand, there was no difference in the incidence of PCI in relation to the gender (Table 1).

Table 1

Distribution of MI, PCI and CABG among genders

Parameters	Male	Female	χ^2/t	p
MI				
no	109 (21.5)	23 (12.9)	6.283	0.012
yes	397 (78.5)	155 (87.1)		
CABG				
no	286 (56.5)	127 (71.3)	12,100	0.001
yes	229 (43.5)	51 (28.7)		
PCI				
no	230 (45.5)	73 (41.0)	1.449	0.485
yes	276 (54.5)	105 (59.0)		
No. of stents	0.88 ± 1.12	0.97 ± 1.14	0.868	0.386

Results are given as mean ± standard deviation or n (%).
MI – myocardial infarction;
CABG – coronary artery bypass grafting;
PCI – percutaneous coronary intervention.

Distribution of smoking status differed significantly by gender ($\chi^2 = 7.871$; $p = 0.020$). A significantly higher number of non-smokers were among women, while among men there were significantly more former smokers. The incidence of other risk factors for CAD (hypertension, hyperlipidemia, diabetes mellitus, heredity) did not differ between the groups (Table 2).

Also, there was no significant difference in the values of the ejection fraction between men and women (men: 49.9 ± 9.3% versus women: 50.9 ± 9.2%; $p = 0.254$). At the beginning of the CVR program, the first exercise stress tests (EST) were performed. The tolerance of strain on the first test between men and women is shown in Table 3. The test lasted longer in men (Z = 2.621; $p = 0.009$) and men achieved a higher level of strain compared to women ($t = 4.758$; $p < 0.001$). There was no significant difference in the

double product before the test, double product standing for systolic blood pressure x heart rate. The double product after the test was higher in men (Z = 2.293; $p = 0.022$). The incidence of complex heart rhythm disorders or ST depression did not differ between the genders.

After a three-week rehabilitation, the patients were again subjected to exercise stress test. The results obtained were almost identical to the first test. The average strain level was significantly higher among men ($t = 5.123$; $p < 0.001$), as well as the duration of the test ($t = 2.264$; $p < 0.024$). There was no significant difference in the duration of the test and the double product before the test. The double product after the test was higher in men (Z = 2.549; $p = 0.011$). The incidence of complex arrhythmias or ST depression did not differ between genders (Table 4).

Table 2

Risk factors for coronary artery diseases

Risk factors	Male	Female	χ^2	p
HLP				
no	68 (13.4)	16 (9.0)	2.421	0.120
yes	438 (86.6)	162 (91.0)		
DM				
no	396 (78.3)	134 (75.3)	0.670	0.413
yes	110 (21.7)	44 (24.7)		
AH				
no	89 (17.6)	21 (11.8)	3.272	0.070
yes	417 (82.4)	157 (88.2)		
Smoking				
no	228 (45.1)	99 (55.6)		
yes	101 (20.0)	36 (20.2)	7.871	0.020
former	177 (35.0)	43 (24.2)		
Heredity				
no	296 (58.5)	105 (59.0)	0.013	0.909

Results are given as n (%).
HLP – hyperlipidemia; AH – arterial hypertension;
DM – diabetes mellitus.

Table 3

The first exercise stress test (EST)

Parameters	Male	Female	t/Z*/χ^2	p
EST level	2.37 ± 0.95	1.97 ± 0.98	4.758	< 0.001
EST duration (min)	5.31 ± 2.63	4.72 ± 2.78	2.621*	0.009
Double product before	9,814.05 ± 3,969.42	10,045.42 ± 2,190.65	1.916*	0.055
Double product after	23,308.71 ± 12,184.88	20,809.19 ± 3,784.94	2.293*	0.022
ST depression	62 (12.3)	17 (9.6)	0.941	0.332
Arrhythmia		5 (2.8)	3.180	0.075

Results are given as mean ± standard deviation or n (%).

Table 4

The second exercise stress test (EST)

Parameters	Male	Female	t/Z*/χ^2	p
EST level	2.69 ± 0.94	2.28 ± 0.95	5.123	< 0.001
EST duration (min)	6.23 ± 2.71	5.62 ± 2.85	2.264*	0.024
Double product before	9,641.18 ± 2,235.39	9,813.15 ± 2,002.67	1.916*	0.055
Double product after	21,962.51 ± 3,622.89	21,071.10 ± 3,467.63	2.549*	0.011
ST depression	81 (16.0)	25 (14.0)	0.387	0.534
Arrhythmia	39 (7.7)	10 (5.6)	0.864	0.352

Results are given as mean ± standard deviation or n (%).

At the end of the rehabilitation, we compared the results of the first and the second test in order to determine whether there were differences in the effects of CRV on the tolerance of physical strain in relation to the gender of the patients.

The average strain level in men was significantly higher in the second test ($t = 4.368$; $p < 0.001$). The duration of the test was significantly longer in the second test ($Z = 11.836$; $p < 0.001$). A double product before the test did not show a significant difference, but the double product after the test was significantly lower in the second test ($Z = 2,311$; $p = 0.021$). ST depression was more frequent in the second test ($\chi^2 = 5.891$; $p = 0.015$) (Table 5).

In women, the average strain level was significantly higher in the second test ($t = 5.352$; $p < 0.001$). The duration of the test was also significantly longer in the second test (Z = 7.471; $p < 0.001$). A double product before and after the test did not differ significantly. The frequency of ST depression in the second test was significantly higher ($\chi^2 = 6.369$; $p = 0.021$); (Table 6).

Thus, a three-week program of CVR has led to the improvement in the tolerance of physical strain in both men and women. Namely, in both groups of patients, the second test lasted significantly longer than the first one, and the patients achieved a higher level of loading in the second test (Tables 5 and 6).

Tables 7 and 8 show the effect of CVR on physical strain rate tolerance in patients with different types of revascularization. In patients with PCI the average strain level was significantly higher in the second test for both men and women ($t = 8.321$; $p < 0.001$ for men; $t = 4.333$; $p < 0.001$

Table 5

Comparison between the first (EST 1) and the second exercise stress test (EST 2) in men

Parameters	EST 1	EST 2	$t/Z^*/\chi^2$	p
EST level	2.37 ± 0.95	2.69 ± 0.94	4.368	< 0.001
EST duration (min)	5.31 ± 2.64	6.23 ± 2.72	11.836*	< 0.001
Double product before	$9,814.05 \pm 3,696.42$	$9,641.18 \pm 2,235.39$	0.493*	0.622
Double product after	$23,308.71 \pm 12,184.88$	$21,962.51 \pm 3,622.89$	2.311*	0.021
ST depression	62 (12.3)	81 (16.0)	5.891	0.015
Arrhythmia	32 (6.3)	39 (7.7)	1.029	0.310

Results are given as mean ± standard deviation or n (%).

Table 6

Comparison between the first (EST 1) and the second exercise stress test (EST 2) in women

Parameters	EST 1	EST 2	$t/Z^*/\chi^2$	p
EST level	1.97 ± 0.98	2.28 ± 0.95	5.352	<0.001
EST duration (min)	4.71 ± 2.78	5.62 ± 2.85	7.471*	<0.001
Double product before	$10,045.42 \pm 2,190.65$	$9,813.15 \pm 2,002.66$	1.266*	0.205
Double product after	$20,809.19 \pm 3,784.94$	$21,071.10 \pm 3,467.63$	0.485*	0.628
ST depression	17 (9.6)	25 (14.0)	6.369	0.021
Arrhythmia	5 (2.8)	10 (5.7)	1.029	0.227

Results are given as mean ± standard deviation or n (%).

Table 7

Comparison between the first (EST 1) and the second exercise test (EST 2) in patients with percutaneous coronary intervention

Parameters	EST 1	EST 2	$t/Z^*/\chi^2$	p
Male, n = 276				
EST level	2.51 ± 0.98	2.88 ± 0.97	8.231	< 0.001
EST duration (min)	5.56 ± 2.70	6.47 ± 2.75	7.519*	< 0.001
double product before	$9,317.40 \pm 1,840.18$	$9,350.82 \pm 2,026.95$	0.013*	0.989
double product after	$23,155.51 \pm 16,066.83$	$22,182.48 \pm 3,767.56$	1.294*	0.196
ST depression	38 (13.8)	49 (17.8)	69.637	< 0.001
submaximal HR	161 (58.3)	198 (71.7)	87.515	< 0.001
Female, n = 105				
EST level	2.08 ± 1.05	2.40 ± 0.98	4.333	< 0.001
EST duration (min)	4.99 ± 3.07	5.91 ± 2.99	4.068	< 0.001
double product before	$9,778.29 \pm 1,959.29$	$9,841.90 \pm 1,938.57$	0.430*	0.667
double product after	$20,719.62 \pm 3,641.52$	$21,261.67 \pm 3,391.25$	1.375*	0.169
ST depression	10 (9.5)	13 (12.4)	61.383	< 0.001
submaximal HR	56 (53.3)	67 (63.8)	33.726	< 0.001

Results are given as mean ± standard deviation or n (%).
HR – heart rate.

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for women). Also, the duration of test in these patients was significantly longer in the second test for both men and women (Z = 17.519; $p < 0.001$ for men; Z = 4.068; $p < 0.001$ for women). Moreover, a significantly higher percentage of patients reached submaximal heart rate during the second exercise test for both genders with PCI ($\chi^2 = 87,515$, $p < 0.001$ for men; $\chi^2 = 33.726$; $p < 0.001$ for women) (Table 7).

In patients with CABG the average strain level was significantly higher in the second test for both men and women ($t = 5.406$; $p < 0.001$ for men; $t = 1.926$; $p = 0.060$ for women). Also, the duration of test was significantly longer in the second test for both men and women (Z = 10.025; $p < 0.001$ for men; Z = 2.953; $p = 0.003$ for women). Significantly, a higher percentage of patients reached submaximal heart rate during the second exercise test for both genders with CABG ($\chi^2 = 71.136$; $p < 0.001$ for men; $\chi^2 = 24.874$; $p < 0.001$ for women) (Table 8).

A three-week program of CVR has led to an improvement in the tolerance of physical strain in both men and women regardless of the type of revascularization.

Table 9 shows the distribution of drugs used among men and women. It was found that women were significantly

more likely to use clopidogrel ($\chi^2 = 5.713$; $p = 0.007$). The use of other drugs did not differ significantly by gender. The most commonly used drugs in both groups of patients were beta-blockers, acetylsalicylic acid and statins.

Discussion

Cardiovascular rehabilitation is an essential part of the secondary prevention of cardiovascular events in patients with CAD [14]. It has been proven that CVR significantly improves the quality of life, reduces mortality and significantly reduces the possibility of a re-coronary event [15–17]. Also, CVR leads to a better lipid profile, weight loss, blood pressure reduction and reduction of cigarette consumption, anxiety and depression [18–19]. However, less than half of the patients are involved in cardiovascular rehabilitation programs [20, 21]. The reason for such low participation lies in the financial and psychosocial reasons, the lack of motivation and age, and the inadequate education on the benefits of CVR [22, 23]. On the other hand, patients are often not referred to the CVR by an authorized physician [24, 25]. The reason lies in inadequate education and not-well-informed medical staff on the importance of CVR.

Table 8

Comparison between the first (EST 1) and the second exercise test (EST 2) in patients with coronary artery bypass grafting (CABG)

Parameters	EST 1	EST 2	$t/Z^*/\chi^2$	p
Male, n = 220				
EST level	2.11 ± 0.84	2.36 ± 0.82	5.406	< 0.001
EST duration (min)	4.93 ± 2.47	5.83 ± 2.48	10.025*	< 0.001
double product before	10,775.00 ± 9,681.32	10,101.95 ± 2,479.19	0.855*	0.393
double product after	21,970.32 ± 11,944.42	21,713.59 ± 3,504.81	2.256*	0.024
ST depression	25 (11.4)	33 (15.0)	93.464	< 0.001
submaximal HR	122 (55.5)	147 (66.8)	72.136	< 0.001
Female, n = 51				
Level	1.76 ± 0.73	1.94 ± 0.79	1.926	0.060
Duration	4.59 ± 2.40	5.33 ± 2.58	2.953*	0.003
Double product before	10,172.06 ± 2,189.75	10,031.18 ± 2,283.83	0.338*	0.753
Double product after	20,075.49 ± 4,187.71	20,237.35 ± 3,835.98	0.174*	0.862
ST depression	4 (7.8)	8 (15.7)	23.330	< 0.001
Submaximal HR	19 (37.3)	28 (54.9)	24.874	< 0.001

Results are given as mean ± standard deviation or n (%).
HR – heart rate.

Table 9

Therapy

Drugs	Male	Female	χ^2	p
Beta blockers	484 (95.7)	172 (96.6)	0.320	0.572
ASA	483 (95.5)	172 (96.6)	0.448	0.504
Clopidogrel	163 (32.2)	75 (42.1)	5.713	0.017
Ticagrelor	152 (30.0)	50 (28.1)	0.241	0.624
Statins	477 (94.3)	170 (95.5)	0.394	0.530
Sartans	46 (9.1)	14 (7.9)	0.247	0.619
Ca antagonist	101 (20.0)	39 (21.9)	0.307	0.579
ACE inhibitors	380 (75.1)	146 (82.0)	3.553	0.059
Trimetazidine	98 (19.4)	44 (24.7)	2.292	0.130
Nitrate	45 (8.9)	18 (10.1)	0.234	0.629
Tiazide	52 (10.3)	27 (15.2)	3.053	0.081

Results are given as n (%).
ASA – acetylsalicylic acid; ACE – angiotensin converting enzyme.

Most studies on the CVR emphasized that women participate in CVR programs in a significantly lower percentage than man. The reasons are numerous, looking after family members, transport problems and the existence of comorbidity [26–28]. Also, it should be noted that women were significantly less directed to CVR by physicians [29–32]. This again emphasizes a different attitude of the medical profession towards women in terms of adequate diagnostics, timely therapy and appropriate rehabilitation. Namely, numerous studies that compared the importance of gender differences in relation to the frequency of CVD, timely diagnosis and adequate therapy have come to the conclusion that women are not so rarely subdiagnosed and inadequately treated compared to men [33–35]. Lavie and Milani [36] pointed out the effects that CVR has on the quality of life of women with CAD, while Balady et al. [37] proved that CVR improves the tolerance of physical strain for both men and women. A recent research suggests a significant effect that CVR has on female patients. Thus, Colbret et al. [38] proved that CVR resulted in a significant reduction in mortality in women compared to men. Nevertheless, women participate in CVR programs in a significantly lower percentage than men.

The percentage of men and women who participated in the CVR program during the course of our research speaks in favor of the previous claim. Only 26% of the respondents were female patients. During the involvement of the respondents in the study, it was noticed that female patients, more often than men, left rehabilitation earlier. This points to another problem in the implementation of the rehabilitation program in women, which is a low adherence. Female patients have a significantly less adherence during the implementation of rehabilitation programs than men and have previously abandoned rehabilitation [39]. Women examinees who did not complete the three-week program of CVR at the Institute for Treatment and Rehabilitation Niška Banja were not included in our research.

In our study, as well as in most previous ones, the presence of risk factors for CVD was the same in both genders, which again emphasizes that a cardiovascular disease is no longer a "disease of men" as previously stated [40, 41]. It is known that CVR has a positive impact on the reduction of modifying risk factors. Thus, it was proved that patients after the completion of rehabilitation had a better lipid profile, greater weight loss, better tolerance of physical effort and significantly better psychological profile [42–44]. This again emphasizes the importance of rehabilitation in cardiovascular patients, regardless of the gender.

Comparing the first and the second test, we came to a conclusion that both men and women had better tolerance to physical strain after the completion of rehabilitation. It should be emphasized that female examinees, as well as men, in the outcome test achieved a significantly higher level of strain and that the outcome test was significantly longer than the test at the beginning of the rehabilitation. This again emphasizes the positive impact of CVR on improving the tolerance of physical strain for both men and women. Cardioprotective medication therapy did not differ between male and female gender examinees. The most commonly used drugs were beta-blockers, antithrombotic drugs, and statins. A somewhat more frequent use of clopidogrel among female patients supports previous studies that the new P2Y12 inhibitors are slightly less effective than clopidogrel in women, although this does not have a greater impact on the risk reduction of a new CV event [45].

Conclusion

A three-week program of CVR led to the improvement in the tolerance of physical strain in both men and women. Our research has once again proved that women have an equal benefit from the implementation of the CVR programs. Nevertheless, women rarely participate in the CVR programs. It is necessary to make additional efforts in order to further educate physicians and other medical staff about the importance of sending women to CVR.

Acknowladgment

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Evaluation of dentists' knowledge about the use of radiology in pediatric dentistry in Serbia

Procena znanja doktora stomatologije u Srbiji o primeni rendgenologije u dečjoj stomatologiji

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Abstract

Background/Aim. Radiology is a crucial part of dentists' everyday practice. It plays a vital role in diagnosis, treatment planning and the follow-up care of patients. The aim of this study was to evaluate the knowledge of dentists in Serbia concerning the use of radiology in pediatric dentistry. **Methods.** In total, 252 dentists from across Serbia participated in the study. A questionnaire was created in order to examine dentists' knowledge about the use of radiology in pediatric dentistry. The questionnaire consisted of 39 questions. All questions were analyzed in SPSS 24 statistical software. **Results.** According to the results, 42.1% of dentists worked in private practice, 17.8% at University clinics and 40.1% in National Health Services. Out of total 252 respondents, only 82 (32.5%) had an x-ray unit. A total of 199 (79%) dentists replied they would diagnose early caries lesions only by inspection, but 30 (11.9%) dentists said they would make a diagnosis by doing both the inspection and taking a radiograph. The results show that 187 (74.2%) respondents said that they did not make an OPG for every patient. When asked about the disposal of radiological waste, 84.3% of dentists did not answer the question. **Conclusion.** Attempts should be made in order to upgrade and update Serbian dentists overall knowledge about the use of radiology in pediatric dentistry.

Key words:
dentists; knowledge; dentistry, pediatric; radiography; surveys and questionnaires; serbia.

Apstrakt

Uvod/Cilj. Rendgenologija predstavlja važan deo stomatološke prakse. Njena primena je veoma značajna u dijagnozi, planiranju i kasnijem praćenju zdravstvenog stanja pacijenta. Cilj istraživanja bio je da se proceni znanje i stavovi doktora stomatologije u Srbiji o primeni rendgenologije u dečjoj stomatologiji. **Metode.** U studiji su učestvovala 252 stomatologa iz Republike Srbije. Konstruisan je upitnik za ispitivanje stavova stomatologa o primeni rendgenologije u dečjoj stomatologiji. Upitnik se sastojao od 39 pitanja. Sva pitanja su obrađena u SPSS 24 statističkom program. **Rezultati.** Na osnovu dobijenih rezultata, 42,1% stomatologa radilo je u privatnoj praksi, 17,8% na univerzitetskim klinikama i 40,1% u domovima zdravlja. Ukupno, 199 (79%) stomatologa odgovorilo je da bi ranu karijesnu leziju dijagnostikovali samo inspekcijom, dok je 30 (11,9%) stomatologa odgovorilo da bi postavili dijagnozu na osnovu inspekcije i radiografisanja. Od ukupno 252 ispitanika, samo 82 (32,5%) imalo je rendgen aparat. Ukupno 187 (74,2%) ispitanika odgovorilo je da ne prave ortopantomografski snimak za svakog pacijenta. Na pitanje o odlaganju radiografskog otpada, 84,3% stomatologa nije imalo odgovor. **Zaključak.** Postoji potreba da se znanja stomatologa u Srbiji o primeni rendgenologije u dečjoj stomatologiji nadogradi i unapredi.

Ključne reči:
stomatolozi; znanje; stomatologija, dečija; radiografija; ankete i upitnici; srbija.

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Introduction

The discovery of x-rays changed the medical world. Dentistry was no less affected by this finding. The use of x-rays in pediatric dentistry is not only vital for the diagnosis of a disease and treatment planning, but also for the follow-up care of patients. The negative side of using x-rays is the inevitable exposure to radiation, of both patients and medical staff. This downside cannot be eliminated, but using adequate protective equipment and defining indications for the use of radiographs, exposure to radiation can be reduced significantly [1].

The radiation dose should be kept as low as it can reasonably be achieved both for a patient and an operator. The younger the individual, the higher the vulnerability to radiation is because of the large number of cell divisions occurring in small children [2].

Protective equipment such as lead aprons, protective thyroid collar and radiation protective shields are one of the basic ways to prevent high exposure to x-rays. Evidence shows that this protective equipment is efficient in reducing unnecessary x-ray radiation [3].

Having in mind the side effects of x-rays, every radiograph has to be justified. The most effective way to reduce the radiation dose in dental radiography is to avoid unnecessary x-ray examinations [1]. Knowing how important radiology is in dental medicine, avoiding x-ray examination altogether is impossible. This is why establishing guidelines for intraoral and extraoral radiography is a top priority. Indications for pediatric dental radiography are numerous, but most commonly they include diagnostics of dental caries, periapical dental disease, dental trauma, development abnormalities, periodontal disease and many more [4].

Methods

In total, 252 dentists from across Serbia participated in the study. A questionnaire was created in order to examine dentists' knowledge towards radiology in pediatric dentistry. The questionnaire consists of 39 questions (Table 1). There were five segments in which all questions were divided: demographic

Table 1

Questionnaire

1. How old are you? _____
2. How long have you been practicing dentistry? _____
3. Title: a) General practitioner; b) Dental specialist; c) Assistant professor; d) Professor
4. Workplace: a) Private practice; b) University clinic; c) National Health Service
5. The city and district of workplace: _____
6. Approximately, how many periapical radiographs are taken at your clinic every week? a) 0-10; b) 11-20; c) 21 and more
7. Do you take an orthopantomograph (OPG) for every patient? a) Yes; b) No
8. Do you use a negatoscope while analyzing radiographs? a) Yes; b) No
9. The risk of excessive x-ray exposure is efficiently reduced by: a) Protective equipment; b) Establishing adequate indications; c) Reducing the exposure time; d) Collimator is used
10. What method is the most suitable for diagnosing a fractured jaw? a) Cone beam computed tomography (CBCT); b) Computed tomography (CT); c) I do not know
11. What method is the most suitable for diagnosing orthodontic anomalies? a) CBCT; b) OPG
12. Can pregnant women, with the use of protective equipment, be exposed to: a) Intraoral radiography; b) OPG; c) Both; d) I would not make radiographs of pregnant women
13. What are the indications for radiographs in your everyday use? _____
14. When do you use radiology in traumatology? a) Fractures of deciduous teeth; b) Fractures of permanent teeth; c) Both
15. What radiological method is the most suitable for diagnosing an early caries lesion? a) Periapical radiograph; b) Bitewing x-ray
16. Do you use a reduced x-ray field in pediatric dentistry? a) Yes; b) No
17. What are the indications for 3D radiographs in pediatric dentistry? _____
18. How do you measure working length of teeth in children? a) Radiographs; b) Electronic apex locator
19. What method is the most suitable for diagnosing an early caries lesion? a) Inspection; b) Radiography; c) Both
20. Do you have an x-ray unit in your clinic? a) Yes b) No
21. What type of x-ray unit do you have? a) Digital x-ray machine; b) Standard x-ray machine c) Both d) None of the above
22. Age of the x-ray equipment: a) _____b) I do not know
23. The kilovolt (kV) and milliampere (mA) of your x-ray equipment: a) _____b) I do not know
24. The cone type of your x-ray: a) Short cone; b) Long cone; c) Pointed; d) I do not know; e) Two or more
25. Which type of collimator do you use? a) Rectangular; b) Rounded; c) I do not know; d) I do not use one
26. Do you adjust the exposure time according to the location of the tooth where the radiograph will be taken? a) Yes b) No
27. Do you use a film holder while taking radiographs? a) Yes; b) No
28. Do you or your assistant hold the x-ray film with a finger while taking radiographs? a) Yes; b) No
29. Who manages the radiological equipment at your clinic? a) Dentist; b) X-ray technician; c) Nurse
30. Do you use a dosimeter to measure the radiation dose? If you do use it, please indicate the type: a) Yes _____; b) No
31. The brand of a processing solution that you use: a)_____; b) I do not know
32. Do you have a license for your x-ray equipment? a) Yes; b) No
33. Is your x-ray equipment inspected regularly? a) Yes; b) No
34. Do you have an OPG machine at your clinic? a) Yes; b) No
35. How do you dispose your radiological waste? _____
36. Are the walls of the x-ray room covered with lead? a) Yes; b) No
37. Do you have a protective barrier in your practice? a) Yes; b) No
38. Do your patients wear a lead apron while being exposed to x-rays? a) Yes; b) No
39. Do your patients wear a thyroid collar while being exposed to x-rays? a) Yes; b) No

characteristics of dentists, indications for radiography, radiographic equipment, radiographic techniques, and radiation protection.

The statistical analysis was performed in the SPSS 24 statistical software. χ^2-test was used to determine the significance of differences between two independent groups. In the process of examining the relationship between variables ANOVA test was used. The level of significance was set at 5% ($p < 0.05$).

Results

Profile of respondents

The average age of pediatric dentists who participated in the study was 40.6 ± 9 years (range 23–65 years). The average duration of their practice was 14 ± 8.8 years (range 1–37 years). Out of 252 participants, 208 (82.2%) practiced in primary [private practice and National Health Services (NHS)] and 44 (17.8%) in tertiary health care (University clinics). The data obtained showed that 130 (51.6%) participants defined themselves as general practitioners who work with pediatric patients, 78 (31%) as pediatric dental specialists, 20 (7.9%) as assistant professors and 24 (9.5%) as professors of pediatric dentistry. According to the results, 42.1% dentists worked in private practice, 17.8% at University clinics and 40.1% in NHS.

Indications for radiography

The most commonly mentioned reason for taking radiographs was the diagnosis of dental caries (46.8%). A total of 199 (79%) dentists replied they would diagnose early caries lesions only by inspection, but 30 (11.9%) dentists said they would make a diagnosis by doing both the inspection and taking a radiograph. The results showed that the respondents were divided when choosing a suitable method for diagnosing an early caries lesion with 109 (43.3%) dentists answering periapical and the same number answering bitewing

radiographs. The rest of the respondents (13.5%) did not know the answer to this question. A majority of respondents (61.5%) responded that they would take radiographs of both deciduous and permanent teeth in case of dental trauma.

There was a significant difference between the workplace of dentists and method of measuring the working length of teeth in children ($p < 0.000$) during endodontic procedures. Only 2.3% of dentists working in a hospital used radiography as a preferred method of measuring the working length, whereas 42.6% of dentists working in public health care used the same method.

Only 6.3% of the respondents replied they would expose pregnant women to both intraoral radiography and orthopantomography.

Indications for 3D radiographs in pediatric dentistry were various. Dentists working at University clinics (31.2%) reported to use 3D radiographs mostly in orthodontics, whereas the majority of dentists working both in private practice (36.8%) and NHS (32.7%) did not have an answer to this question (Table 2). A large percent of dentists (77%) agreed that they would rather use an orthopantomogram (OPG) rather than cone beam computed tomography (CBCT) for diagnosing orthodontic anomalies. Only 31 (12.3%) dentists replied that they would use computed tomography (CT) instead of CBCT for diagnosing a fractured jaw.

Radiographic equipment

Out of total 252 respondents, only 82 (32.5%) have x-ray units. Dentists who practice at University clinics were better equipped with the results showing 54.6% of them have an x-ray unit. The results showed a significant difference between the dentists' workplace and the use of a panoramic unit ($p < 0.000$). Out of all dentists who practiced at University clinics, 61.4% had an access to a panoramic unit, whereas 80.4% of dentists working in private practice did not have a panoramic unit in their workplace.

All data about digital x-ray units are shown in (Table 3).

Table 2

Indications for 3D radiographs in pediatric dentistry

Indications	Private practice	NHS	University clinic	Total
Endodontics	2.1	12.6	10	7.35
Orthodontics	32.6	20.1	31.2	26.35
Periodontology	0	0	0	0
Oral surgery	18.1	10.7	12.5	14.4
Diagnostics of cavities	0	1.9	0	0.95
Dental trauma	10.4	22	22.5	16.2
No answer	36.8	32.7	23.8	34.75
Total	100	100	100	100

All values are expressed as percentages.
NAS – National Health Service.

Table 3

Type of the x-ray unit

X-ray unit type	Private practice	NHS	University clinic	Total
Digital x-ray	25.2	1.3	52.3	25.4
Standard x-ray	5.6	15.8	2.3	9.1
Both	2.8	13.9	11.4	8.7
None of the above	66.4	56.4	34.1	56.7
Total	100	100	100	100

All values are expressed as percentages.
NHS – National Health Service.

A noticeably large percent of dentists (81%) reported they do not know the age of the x-ray equipment used for radiographs. Similar results were obtained concerning the question about the number of kilovolt and milliampere of the x-ray unit. Only 9 (3.6%) of the dentists knew the answer to this question. Just 76 (30.2%) respondents confirmed having a license for their x-ray equipment.

Different cone types (short cone, long cone, short pointed cone and two or more cones) varied from one institution to another. Long cone was the most preferred cone type among the dentists (9.5%), but a greater number of the dentists responded that they did not know which cone type is used for radiographs (79%).

Only 3 (1.2%) respondents answered having rectangular collimators and 212 (84.1%) reported that they did not know the type of collimator they used. There were 65 (25.8%) dentists who replied that they use a dosimeter to measure the radiation dose. Examining the knowledge of the respondents, results showed that 158 (62.7%) dentists use a negatoscope while analyzing radiographs.

A significant percent of the dentists (94.5%) did not know the type of chemicals they use for their radiographs, and only 2.7% reported that they use automatic processor. The results showed that the majority of the dentists (57.9%) did not answer if they check their x-ray equipment regularly and a smaller percentage (35.7%) check their equipment on a regular basis. When asked about the disposal of radiological waste, 84.3% of the dentists did not answer the question.

Radiographic techniques

The largest amount of periapical radiographs per week are taken at University clinics (34.1%) and NHS (34.2%) with the most common answer of 21 and more taken radiographs per week. The results show that 187 (74.2%) respondents said that they do not make an OPG for every patient. A notably high percent of dentists answered that they use a reduced x-ray field in pediatric dentistry (72.2%), but 18.7% did not have an answer to this question. There were 136 (54%) dentists who did not answer if they set the exposure time according to the location of tooth while taking a periapical radiograph. Only 35.7% of dentists adjust the exposure time depending on the tooth location. As a preferable method, 20 (7.9%) respondents use the method of holding the film with their finger while taking radiographs. The results show that film holders are not in common use among the dentists practicing in Serbia. Only 17.5% of the dentists taking part in the study use a film holder.

According to the results, the percentage of personnel in charge of taking radiographs according to the place of employment showed that 51.4% of pediatric dentists did not know the answer to this question, and the nurses were least common to work with the x-ray equipment (2.8%) (Table 4).

Radiation protection

Interesting results were obtained concerning the use of radiation protective equipment (lead walls of the x-ray room, lead aprons, protective barriers and thyroid collars). There was a statistical difference found between the place of employment and the use of protective equipment ($p < 0.000$). Dentists who worked in private practice mostly answered that they do not know if the walls should be covered with lead (62.6%), whereas the majority of pediatric dentists who worked at University clinics reported positively to this question (63.6%). According to the results, protective barriers are mostly used at University clinic with 70.5% of dentists working at University clinics replying they use protective barriers. Only 28% of pediatric dentists working in private practice had the same answer. There were 102 (40.5%) participants who replied they use thyroid collars regularly and 124 (49.2%) participants did not answer this question. Data about the knowledge regarding the use of lead aprons are shown in Table 5.

Table 4
Personnel in charge of taking radiographs according to the place of employment

Personns	Private practice	NHS	University clinic	Total
Dentist	17	1	6.8	8.8
X-ray technician	15.1	50.5	59.1	37.1
Nurse	3.8	2	2.3	2.8
I do not know	64.2	46.5	31.8	51.4
Total	100	100	100	100

All values are expressed as percentages.
NHS – National Health Service.

Table 5
Use of lead apron according to workplace

Answers	Private practice	NHS	University clinic	Total
Yes	29.9	55.4	65.9	46.4
No	9.4	1	9.1	6
No answer	60.7	43.6	25	47.6
Total	100	100	100	100

All values are expressed as percentages.
NHS – National Health Service.

Discussion

Indications for radiography

The purpose of radiographic examination for dental caries is to examine tooth surfaces at risk, which cannot be readily visualized by direct methods. Periapical or bitewing views may also be used to augment clinical examinations in cases in which the presence or extent of carious involvement is in question. An official report of the American Academy of Oral and Maxillofacial Radiology [5] says the best way to detect dental caries in the posterior quadrants is by bitewing radiographic technique and periapical projections are made for the anterior teeth due to the buccolingual inclination of the teeth. These methods for diagnosing dental caries are commonly used among pediatric dentists in Serbia as well. Though most of them would diagnose dental caries only by oral examination, the rest were divided between periapical and buccolingual radiographs.

The use of x-ray units in dentistry is a complex question when considering pregnant women as patients. One of the studies on this topic says radiographic examination in pregnant women must be performed only in the second trimester in order to reduce the chances of deleterious effects [6]. We found that a majority of pediatric dentists in Serbia would not expose women to any kind of radiography. A research of North Queensland dentists [7] had an opposite result. The most preferred procedural change for pregnant patients was to take only intraoral radiographs if urgent for diagnosis (95.2%). Kelaranta et al.[8] conducted a study of radiation exposure of the fetus and breasts during dental x-ray examinations and the effect of lead shields. The information gathered based on the results of intraoral, panoramic, cephalometric and CBCT dental modalities shows the upper estimates of fetal doses varied from 0.009 to 6.9 µGy without lead shielding and from 0.005 to 2.1 µGy with lead shielding. Most importantly, this study explains that pregnancy is never a reason to avoid or to postpone a clinically justified dental radiographic examination.

CT imaging is the most significant technological advance in maxillofacial imaging since the introduction of panoramic radiography. All CT scanners consist of an x-ray source and a detector mounted on a rotating gantry. Although providing similar images, using CBCT we can adjust exposure factors such as the field size, selected for each patient based on individual needs and therefore reduce the exposure to x-rays [4]. Majority (34.75%) of dentists participating in our study answered that they did not know the indications for the use of CBCT. Second and third highest scored were orthodontics (26.35%) and dental trauma (16.2%). In a Scandinavian study [9], it was determined that the two most common reasons for a CBCT are assessment of an ectopic canine and localization of an impacted second premolar. A study published in 2019 about digital radiography and CBCT [10] states that dental trauma was cited as the reason for using CBCT by 70 (61.3%) subjects, dental development issues by 71% and pathology in the jaws by 75.8%. In our study, CBCT was shown to be the most preferable method for diagnosing jaw fractures. A cross-sectional analysis of the National Hospital Ambulatory Care [11] states that the number of CT neuroimagings among children with head injuries did not decrease from 2007 to 2015. This result is supported by the American Academy of Oral and Maxillofacial Radiology [5], which says that one of the indications for the use of CT is when cases involve bone grafts or complex orofacial trauma.

Radiologic examination is essential for evaluating trauma to the teeth and jaws. Although a panoramic image may be useful for localizing injuries to the teeth and supporting structures, it may not have the image resolution to reveal injuries involving the anterior mandible or maxillae or the teeth. Dentoalveolar trauma always requires intraoral images to obtain adequate anatomic details [4]. This rule is applied by the majority of pediatric dentists in Serbia. The European DIMITRA project (Dentomaxillofacial pediatric imaging: an investigation toward low-dose radiation induced risks) published a review about the use of CBCT in pediatric dentistry, in which it is stated that CBCT is more useful in the diagnostics of periapical pathology arising from traumatic events, in comparison to intraoral radiographs [12].

Radiographic equipment

X-ray units are not a common part of dental practice in Serbia. Only one third of the participants answered having this unit in their workplace and a similar result was obtained when discussing if dentists had routine access to a panoramic unit. A survey of general practitioners in England and Wales [13] says all dentists had access to an intraoral radiographic unit and 61% had routine access to a panoramic unit. The majority of practices within the North Queensland [7] region reported using intraoral radiographic equipment (96.8%) as well.

Digital intraoral receptors require less radiation dose to produce images as compared to conventional films. The results from an Indian study showed 38.7% of dentists used this system while still remaining adherent to analog receptor system [14]. The most preferred x-ray units by Serbian pediatric dentists are digital x-ray units.

In our research, basic knowledge about the age of their x-ray units, cone and collimator types, kilovolts and milliamperes proved to be rather limited, with the prevalence of dentists that did not have answers to these questions. Knowledge, attitudes and practices of North Queensland dentists [7] showed that most radiographic equipment appeared to be greater than 6 years old. In approximately two thirds of the cases (61.3%), the dentists used tube voltage of 65–70 kVp, and half of the responders a tube current of under 10 mA. A total of 73.4% of participants in a study in India [14] were not aware of the type of collimator used in their x-ray equipment. Of all respondents in a research about the use of radiation dose reduction techniques, 50.5% use long cones either exclusively or in combination with short cones [15]. Only one quarter of Serbian dentists who participated in the research answered they used exposure badges, whereas 88.8% of Australian dentists [7] reported wearing exposure badges while in their practice.

To ensure the optimum exposure condition, quality assurance tests of dental x-ray units should be performed. In India, Atomic Energy Regulatory Board (AERB) mandates that quality assurance tests of dental x-ray units should be carried out every two years by certified professionals. Despite this fact, a study has shown only 36.8% of the dentists were aware of this fact [14]. Some researches show that 92.1% of the dentists record periodic services at approximately 12 or 24 months (31%) [6], whereas the largest number of our respondents did not even answer this question.

Radiographic techniques

Panoramic imaging (also called pantomography) is a technique for producing a single image of the facial structures that includes both the maxillary and the mandibular dental arches and their supporting structures. Panoramic images are most useful clinically for diagnostic problems requiring broad coverage of the jaws. Panoramic imaging is often used as the initial evaluation image that can provide the required insight or assist in determining the need for other projections [4]. A notably high percent of pediatric dentists in Serbia answered that they do not make an OPG for every patient, which is similar to the results from a Turkish study [16], which states that only 8.9% of their respondents take panoramic radiographs on patients' first visits.

Intraoral radiographic (imaging) examinations are the backbone of imaging for a general dentist. Intraoral images can be divided into three categories: periapical projections, bitewing projections, and occlusal projections. Periapical radiographs should show all of a tooth, including the surrounding bone [4]. This method is often used by pediatric dentists in Serbia, but the results of this study show the largest number of the participants (38.9%) make 0–10 periapical radiographs per week. This result was also obtained by dentists in Turkey having the same answer (62.3%) [16].

A study in Ontario Canada [17] showed that on average, dentists reported making 27% of their patients' radiographs, whereas 82.5% of dentists in Turkey [16] reported that they took the radiographs themselves. The results of our research pointed out that only 8.8% of pediatric dentists in Serbia take radiographs themselves.

The majority of dentists in New Queensland [7] (79.4%) reported varying the exposure time depending on the place of interest, with the average exposure time being less than 0.16 seconds. Adjusting the exposure time according to the location of a tooth is not a common practice in Serbia.

The use of a film holder with a beam-aiming device reduces the number of overlapping contact points and improves the image quality, minimizing interpretation errors [4]. American Dental Association states dental professionals should not hold the film while exposing patients to x-rays. General dental practitioners in England and Wales [13] mostly use film holders, but pediatric dentists in Serbia do not have this habit. It can be observed that many dentists taking part in an Indian study [14] using digital radiography system do hold sensors for their patients in fear of damaging costly sensors, which is actually dangerous and could lead to damages that are hidden and have a long latent period to reveal themselves. Unfortunately, 7.9% of our participants still use the method of holding the film with their finger while taking radiographs.

Radiation protection

Dental operatories should be designed and constructed to meet the minimal shielding requirement of the state regulations; this requires consultation with a qualified expert. This recommendation states that walls must be of sufficient density or thickness that the exposure to non-occupationally exposed individuals. In most instances, it is not necessary to line the walls with lead to meet this requirement. Every effort should be made so that the operator can leave the room or take a position behind a suitable barrier or wall during the exposure. The thyroid gland is more susceptible to radiation exposure during dental radiographic exams given its anatomic position, particularly in children. Therefore, every precaution should be taken to minimize radiation exposure, and protective thyroid collars should be used whenever possible [4]. A study from Finland says the use of lead shields reduced the fetal dose by 39%–97% and reduced the breast dose by 22%–99% [8]. Leaded aprons and thyroid shields that contain lead or other materials are patient-protective equipment, which minimizes the exposure to scattered radiation [18]. Thyroid shielding with a leaded thyroid shield or collar is strongly recommended for children and pregnant women, as these patients may be especially susceptible to radiation effects [18–20]. In Turkey, a study detected only 30% of the participants answered the walls of the x-ray room were covered with lead, and even a smaller number stated they use lead aprons (8.7%) and thyroid collars (3.7%). Protective barriers were used by 11.2% of dentists participating in the study [16]. A study about the knowledge of oral radiology among Swedish dentists [21] states that 93.4% of examinees absolutely agree that it is necessary to protect a thyroid gland during intraoral radiology. The present study showed that 39.7% of dentists in Serbia have a lead-lined x-ray room, 46.8% use protective barriers, 46.4% use lead aprons and 40.5% use thyroid collars while taking radiographs.

Conclusion

Based on the conducted survey and the obtained results, it is concluded that attempts should be made in order to upgrade and update dentists' overall knowledge about the use of radiology in pediatric dentistry. The research showed a large number of pediatric dentists did not know the answers to the questions in the survey. This might be due to the fact that most pediatric dentists in Serbia do not own radiological equipment. This, however, is not an excuse for not knowing the basic indications and preventive measures in radiology. We should aspire to educate pediatric dentists, both by getting better undergraduate and postgraduate knowledge. Establishing guidelines for intraoral and extraoral radiography is a top priority. Protocols for radiographic equipment should be renewed and modified for the overall needs of pediatric dentists in Serbia.

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The application of artificial intelligence algorithms for testing the correlation between the state of oral health and adolescent behavior concerning oral health

Primena algoritama veštačke inteligencije za ispitivanje korelacije stanja oralnog zdravlja i ponašanja adolescenata u vezi sa oralnim zdravljem

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Abstract

Background/Aim. A period of adolescence is characterized by turbulent emotional, physical and physiological changes. There are numerous risk factors that may endanger the oral health of adolescents as the influence of parents reduces, while the influence of the environment and peers increases. Therefore, the main aim of this study was to determine the behavior of adolescents concerning oral health, using a new statistical method – artificial intelligence algorithms. **Methods.** In the first part of the survey, data on the behavior of adolescents related to oral health were collected. Hiroshima University Dental Behavioral Inventory (HU DBI) questionnaire was used, and additionally expanded with three questions. The second part of the study included clinical examination. The research was conducted at the Faculty of Dentistry in Pančevo, Serbia. The first and second grade high school students were selected for the observation unit.

The total sample consisted of 374 students (128 males and 246 females). We applied a special programming language called Python for parsing data, creating a database in digital form, processing data by standard statistical methods and through the Singular Value Decomposition (SVD) method. **Results.** The artificial intelligence algorithms clustered the respondents into two groups, based on their responses from the HU DBI questionnaire. Thus, the quality of the method and the need for analysis of this type in dental studies are demonstrated and proven. **Conclusion.** Based on the results obtained through artificial intelligence algorithms, we could conclude that respondents should rather be clustered into characteristic groups and analyzed than divided and observed according to sex, as it is the intuitive division.

Key words:
mouth; health; intelligence, artificial; adolescence; algorithms.

Apstrakt

Uvod/Cilj. Period adolescencije karakterišu burne emocionalne, fizičke i fiziološke promene. Javljaju se brojni faktori rizika koji mogu ugroziti oralno zdravlje adolescenata jer se uticaj roditelja smanjuje, a raste uticaj okoline i vršnjaka. Stoga je glavni cilj istraživanja bio da se utvrdi ponašanje adolescenata u vezi sa oralnim zdravljem, pri čemu je primenjena nova statistička metoda – algoritmi veštačke inteligencije. **Metode.** U prvom delu istraživanja, prikupljeni su podaci o ponašanju adolescenata u vezi sa oralnim zdravljem. Korišćen je upitnik Univerziteta u Hirošimi (Hiroshima University Dental Behavioral Inventory – HU DBI), koji je proširen sa tri pitanja. Drugi deo istraživanja obuhvatio

je klinički pregled. Istraživanje je sprovedeno na Stomatološkom fakultetu u Pančevu, Srbija. Za jedinicu posmatranja izabrani su učenici prvog i drugog razreda srednje škole. Veličina ukupnog uzorka bila je 374 ispitanika (128 ispitanika muškog pola i 246 ispitanika ženskog pola). Za analizu podataka, pravljenje baze podataka u digitalnoj formi, obradu podataka standardnim statističkim metodama i metodom Dekompozicija na singularne vrednosti [*Singular Value Decomposition* (SVD)], korišćen je Piton (*Python*) programski jezik. **Rezultati.** Algoritmi veštačke inteligencije klasterifikovali su ispitanike u dve grupe, na osnovu njihovih odgovora iz HU DBI upitnika. Na ovaj način se pokazao i dokazao kvalitet metode i potreba za analizama ovog tipa u stomatološkim studijama. **Zaključak.** Na osnovu rezultata dobijenih primenom algoritama veštačke inteligencije mogli

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smo zaključiti da bi bilo logičnije da se ispitanici klasteri-fikuju u karakteristične grupe i zatim analiziraju, nego da se dele i posmatraju po polu, jer je to intuitivna podela.

Introduction

The World Health Organization (WHO) defines adolescents as individuals who go through a period of growth and development. This phase, between 10 and 19 years of age, begins after childhood and lasts until adulthood [1]. Adolescence is characterized by the acceleration of physical growth, as well as changes in psychological development. The adolescent population is a vulnerable group with regard to preserving oral health. In the period of turbulent emotional, physical and physiological changes there are many risk factors that can endanger the oral health of adolescents. During this period, the influence of parents reduces, while the influence of the environment and peers increases. Despite the common knowledge about health risks, many adolescents adopt high-risk behaviors, such as smoking, alcohol, or the use of narcotics [2].

In order to determine the real needs of adolescents in terms of oral health and to set future goals of preventive and therapeutic programs, numerous public health and epidemiological studies have been conducted. In this way, large amounts of data are obtained that can determine the existence of a link between socio-demographic factors, habits, oral health behavior and the state of oral health [3]. Modern technology has devised ways to efficiently store data in order to take the least possible amount of space and to preserve the main information. The large increase in the production of multimedia data implies constant development and further optimization of various compression algorithms [4], like digital image compression [5]. The basic concept of the Singular Value Decomposition (SVD) algorithm is based on the principles of decreasing dimensionality through decomposition onto singular values [6] in order to increase the degree of image compression [6, 7].

The main objectives of this study were focused on identifying behavior of adolescents concerning oral health and the objective state of oral health, as well as on the correlation of oral health behavior and actual clinical oral health condition. One of the main goals was to compare the results obtained with standard statistical methods and application of artificial intelligence algorithms, which until now have not been applied to this type of analyses in dental research.

Methods

A special programming language called Python was applied for parsing data, creating a database in digital form, and processing data by standard statistical methods and through the SVD [8].

The SVD algorithm provides a simple approximation strategy for the original matrix by which the digital image is represented, using a matrix of smaller dimensions [9]. After sorting the singular values in a descending order, it is possible to keep the first "k" largest, and to set the others to zero. The result is a new "k" matrix, which depends on the value of "k", a good approximation of the original matrix, in terms of the smallest squares [10]. The choice of the value of the matrix rank, which, after applying the SVD algorithm presents the representation of the original image matrix, is a compromise between achieving a certain desired degree of compression and retaining the acceptable quality of the digital image [9]. According to input matrix "A", the computer program classifies the respondents into groups by a cluster method, thanks to logical machine learning (artificial intelligence).

To assess the impact of different forms of behavior on oral health, the Serbian version of the Hiroshima University Dental Behavioral Inventory (HU DBI), expanded with three questions, was applied. The original questionnaire was developed by Kawabata et al. [11]. It contains twenty questions that primarily relate to oral hygiene habits. All questions have two possible answers (I agree / disagree). A quantitative assessment of attitudes and behaviors related to oral health is possible based on the established total number of adequate responses, with a maximum score of 12. Bigger score means more adequate attitudes and behaviors in relation to oral health [12]; one point is assigned to each answer "I agree" to questions 4, 9, 11, 12, 16 and 19, as well as for each answer "I do not agree" for questions 2, 6, 8, 10, 14 and 15. Scoring any question, which relates to a particular attitude or behavior in the HU DBI questionnaire, is based on analytical research within which the statistical model has been developed [13].

The examinations were performed at the outpatient clinic, with artificial lighting, using a dental probe and a mirror. The total number of healthy, carious, extracted and sealed teeth for each respondent was determined [Decayed, Missing Filled Teeth (DMFT) index]. The criteria for setting caries diagnosis was the breakdown of the continuity of the tooth enamel (the presence of cavities).

The research was conducted at the Faculty of Dentistry in Pančevo. The observation unit was comprised of students of the first and second grade of high school who, as patients, visited the Faculty of Dentistry in Pančevo, Serbia. The total sample consisted of 374 students (128 males and 246 females). All of them with their parents agreed to participate in this anonymous research.

Results

The average number of diseased teeth calculated for the whole sample was 4.27 ± 0.24. For the probability level $p = 0.95$ we ascertained that in the basic sample it ranged from 3.78 to 4.76. The DMFT index values ranged from 0 (for both sexes) to 17 in boys or 15 in girls. The distribution of DMFT Eudex by the number of respondents is graphically presented in Figure 1.
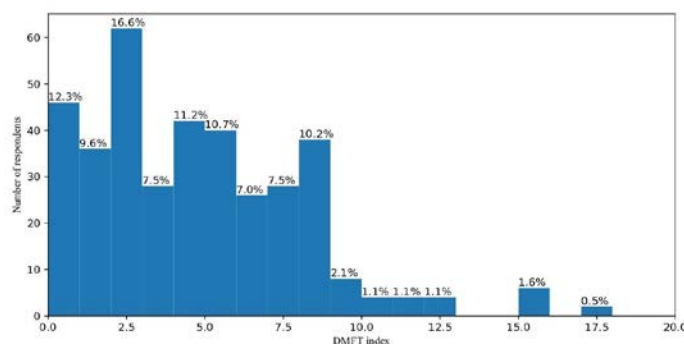
Fig. 1 – Distribution of the Decayed, Missing, Filled Teeth (DMFT) Index.

Table 1

Behavior concerning oral health according to the Hiroshima University Dental Behavioral Inventory (HU DBI)

Parameter	Males	Females	Total
Number of respondents	128	246	374
Minimum value	5	3	3
Maximum value	9	9	9
Average value	7.14	6.854	6.95
SE	0.154	0.106	0.087
SD	1.223	1.166	1.194
CV (%)	17.128	17.02	17.174
95% CI			
lower limit	6.832	6.642	6.777
upper limit	7.449	7.065	7.127
Significance of differences in average values			
$t = 1.561$			
$p = 0.120$			

SE – standard value error; SD – standard deviation; CV – coefficient of variation; CI – confidence interval; t – value for Student's t-test; p – value of statistical significance.

Concerning distribution between genders, with a probability of $p = 0.95$, it may be expected that the average values of the DMFT index range would be from 3.31 to 4.97 in males and from 3.73 to 4.94 in females. There were 46 respondents who did not have any carious, extracted and sealed teeth, and whose DMFT index was 0. Consequently, the number of persons with the DMFT index > 0 was 328 (87.7%).

There was no difference in the responses of boys and girls ($p > 0.05$); so the hypothesis that there is no difference between males and females was confirmed (Table 1). In almost one third of the questions (7 out of 23) in the HU DBI, respondents gave identical answers in more than 90% of cases (Table 2; Figure 2).

It is interesting that 22% of the respondents considered it inevitable to get artificial teeth at an old age. A large number of adolescents (95%) were concerned about the possible presence of halitosis and were constantly checking for their unpleasant breath, while a third of the respondents (31%) were concerned with the color of teeth, while 71%

Table 2

Percentage of agree and disagree with the HU DBI items

HU-DBI Item	Agree (%)	Disagree (%)
1. I go to see the dentist at least once a year.	72	28
2. My gums bleed when I brush my teeth.	6	94
3. I am worried about the color of my teeth.	31	69
4. I've noticed some white sticky deposits on my teeth.	10	90
5. I think that I cannot help having false teeth when I am old.	22	78
6. I think my teeth are getting worse despite my daily brushing.	7	93
7. I brush each of my teeth carefully.	47	53
8. I have never been professionally taught how to brush.	31	69
9. I think I can clean my teeth without using toothpaste.	9	91
10. I often check my teeth in a mirror after brushing.	96	4
11. I worry about having bad breath.	95	5
12. It is impossible to prevent gum disease with tooth brushing alone.	60	40
13. I put off going to the dentist until I have a toothache.	20	80
14. I have used a dye to see how clean my teeth are.	3	97
15. I use a toothbrush with hard bristles.	44	56
16. I don't feel I've brushed well unless I brush with strong strokes.	37	63
17. I feel I sometimes take too much time to brush my teeth.	62	38
18. I have had my dentist tell me that I brush very well.	81	19
19. I am satisfied with the appearance of my teeth.	71	29
20. I brush my teeth twice a day or more.	90	10
21. I use dental floss every day.	20	80
22. I use mouthwash on regular basis.	32	68
23. I smoke cigarettes every day.	3	97

HU DBI – Hiroshima University Dental Behavioral Inventory.
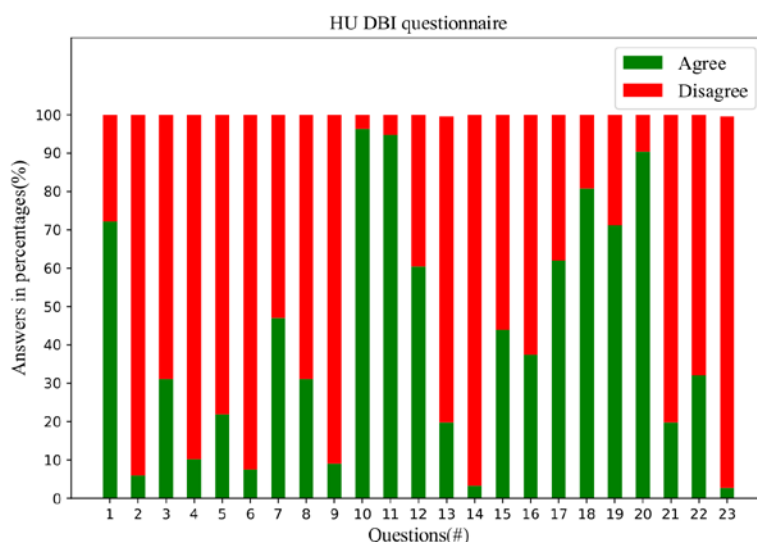
Fig. 2 – Distribution of the answers to questions from the Hiroshima University Dental Behavioral Inventory.

were satisfied with the appearance of their teeth. Although a large number of respondents used a hard toothbrush (44%), gingival bleeding during brushing was seldom (6%). A possible explanation was a fact that children mostly do not use strong/rough brushing movements (63%). A large number of respondents (62%) thought that they need to spend too much time for basic tooth wash. More than half of the respondents (60%) said that it is impossible just to brush teeth to prevent gingival inflammation. Most respondents did not consume cigarettes, which represents a health-safe behavior.

In the group of questions related to the HU DBI, the answers were assigned the following colors (Figure 3): I disagree – purple; I agree – yellow.
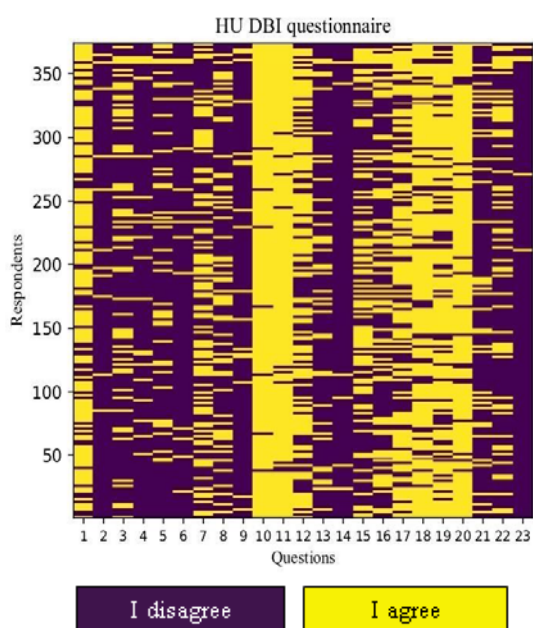
Fig. 3 – Hiroshima University Dental Behavioral Inventory (HU DBI) – Initial matrix.

By determining the most important element of the initial matrix, we got a new matrix (Figure 4), which, with the accuracy greater than 80%, showed that 47% of the respondents answered identically 4 questions from the HU DBI questionnaire with affirmative answer: Question 10 – After washing my teeth, I often check in a mirror how clean my teeth are; Question 11 – I'm worried/checking for an unpleasant smell out of my mouth; Question 18 – My dentist told me to wash my teeth well; Question 20 – I wash my teeth two or more times a day.
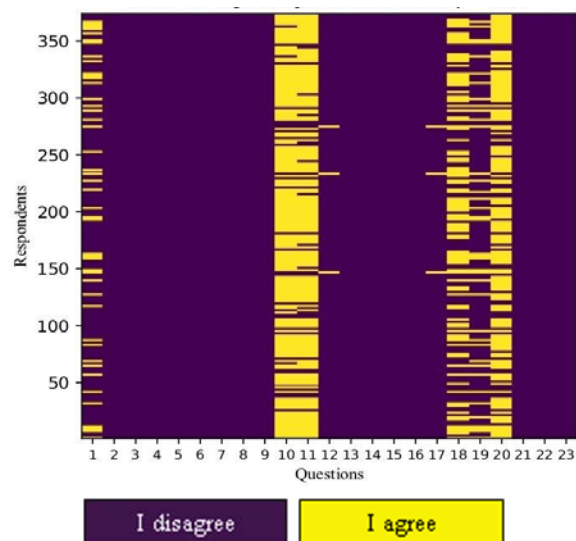
Fig. 4 – Hiroshima University Dental Behavioral Inventory (HU DBI) – Sigma importance 1, accuracy > 80%.

The artificial intelligence algorithms clustered the respondents into two groups ($p < 0.05$) regarding the answers "I agree" (Group 1) and "I disagree" (Group 2); so the hypothesis that there is a statistical significance between these two groups was confirmed (Figure 4, Table 3). The scale on the right side of the graph (Figure 4) represents the

Table 3

Decayed, Missing, Filled Teeth (DMFT) in respondents clustered into two HU DBI groups

Parameter	Group 1	Group 2	Total
Number of respondents	176	198	374
Minimum value	0	0	0
Maximum value	12	17	17
Average value	3.62	4.84	4.27
SE	0.32	0.36	0.24
SD	2.95	3.56	3.34
CV (%)	81.32	73.54	78.28
95% CI			
lower limit	2.99	4.12	3.78
upper limit	4.26	5.56	4.76
Significance of differences in average values			
$t = -2.508$			
$p = 0.013$			

HU DBI – Hiroshima University Dental Behavioral Inventory; Group 1 – respondents who answered "I agree" to questions number 10, 11, 18 and 20 from the HU DBI; Group 2 – respondents who answered "I disagree"; SE – standard value error; SD – standard deviation; CV – coefficient of variation; CI – confidence interval; t – value for Student's t-test; p – value of statistical significance.

equivalent color to the group to which the respondents belong: "Exact" (Group 1) – yellow; "Incorrect" (Group 2) – purple. Through the analysis of questions 10, 11, 18 and 20, we got an impression that these respondents were seriously concerned about the health of their teeth and took care of their mouth and teeth (Table 3). These results indicate that it would be interesting to examine the clinical condition of their oral health (DMFT index).

Through the analysis of basic statistical parameters for these two groups of respondents, one could notice a significant difference in the average values of the DMFT index (3.62/4.84) between these two groups of respondents. The respondents who were particularly concerned for the condition of their mouth and teeth had a significantly lower DMFT index and hence a better clinical state of oral health than the other group of respondents. Also, the average value for the D (decayed) component of the DMFT index was lower for the Group 1 (D = 1.69), compared to the Group 2 (D = 2.15), the M (missing) component was almost the same (0.33 / 0.32), while the number of filled (F) teeth was significantly lower in the Group 1 (F = 1.60) compared to the Group 2 (F = 2.00). By comparing these results with the results obtained with the basic statistical methods, we found the largest number of answers "I agree" in relation to the following questions: Question 10 – After washing my teeth, I often check in a mirror how clean my teeth are (96%); Question 11 – I'm worried/checking for an unpleasant smell out of my mouth (95%); Question 18 – My dentist told me to wash my teeth well (81%); Question 20 – I wash my teeth two or more times a day (90%).

Discussion

The state of oral health of adolescents is characterized by the presence of diseased teeth in most respondents (87.7%). At the level of the overall sample, the caries index average value was 4.27. According to literature data, the average value of this index in German 15-year-olds is 1.8 [14], in Greece 3.19, in Slovenia 4.3, in Bosnia 6.6 and in Serbia 5.5 [13–17].

In Central and Eastern European countries, the increased prevalence of caries in school children and adolescents is associated with inadequate application of preventive measures and the lack of organized health promotion activities with younger age groups [18]. Also, high prevalence of caries in developing countries can be attributed to the fact that the system of dental healthcare in these countries is in transition [19, 20]. Serbia, unfortunately, belongs to countries with insufficient health education activities and the healthcare system is primarily oriented towards therapy and not the prevention of oral diseases. Therefore, a high DMFT index value in the sample of 15-year-olds is not surprising.

The lifestyle of adolescents is directly and indirectly related to the development of caries [21]. Studies in the Netherlands and Australia have proved the influence of parents on the development of oral hygiene behavior of children - the transfer of knowledge, but also the control over the health behavior [22].

A survey conducted on the territory of the Republic of Serbia in 2013 showed that there is a significant correlation between good oral hygiene of parents and practice of controlling children habits and good oral health [23].

The respondents of both genders who gave 9 correct answers, out of possible 12 that are scored in the HU DBI questionnaire, filled out the questionnaires with focus. The standard deviation was much higher than the standard error value, plus the coefficient of variation was large enough, which all leads to the conclusion that we had a satisfactory sample of respondents. More recently, this questionnaire has been used in a research in which the attitudes and behaviors related to the oral health of dentists and dental hygienists around the

world have been compared [24]. It has been found that there are significant differences among students from different countries and cultural groups, as well as among students in relation to the grade of their studies [25].

Many oral health studies suggest that one of the reasons for neglecting oral hygiene in adolescence is that young people feel that they have no time to thoroughly brush their teeth [26], which we have noted in this study, too. Namely, 60% of respondents stated that they need too much time for basic tooth care.

Generally, it has been established that adequate behaviors in relation to oral health are associated with a better oral health status of adolescents [27]. The DMFT index of 4.27 is not at all satisfactory, indicating that adolescents have greater care of oral health but do not work as they should, do not use adequate pastes in the right way. Certainly, the most important component is the diet that is not treated as the key risk factor in our country. Research has clearly indicated that significant preventive effects are missing when taking free sugar up to 7 times a day even in good oral hygiene and with the use of fluoride pastes [28]. Additionally, brushing teeth without adequate fluoride paste also has no preventive effect [29].

A large number of researches mark the school as a very convenient place for conducting public health education campaigns, as children spend most of their time at school. The Oral Health Education (OHE) program in Bangladesh, conducted by educated dentists in primary schools, 6 months after the implementation, showed a significant increase in knowledge, changes in children's behavior and more frequent visits to dentists after the education [30]. Numerous results show that oral health significantly worsens after leaving primary school.

Gathering medical information and storing these data in the future (with the protection of patient privacy) should help early identification of risk patients and application of better treatment modalities. Accordingly, these possibilities should become reality [31, 32].

Multivariate analysis is by far the greatest power of artificial intelligence as it makes intelligent decisions, such as the human mind, based on the data from the photographic memory from the hard disk. No analysis is needed through emotions, and there is no negative attention deficit. Artificial intelligence does not need sleep and does not get tired after focusing on one topic for too long. At the same time, it benefits from a massive parallel processing. With enough memory and processing power, artificial intelligence could keep the medical records of the entire family tree and use it to quickly search the database with relevant diagnostic information and simultaneously call the banks of medical and social resources [32]. At present, it is not trusted enough to be completely autonomous.

Further research in the field of machine learning should be increased. It would be a good idea to integrate them into clinical practice as much as possible because, in this way, science can benefit greatly from this technology in the future. This type of research is also a basis for identifying health risks that could be followed by the use of electronic medical records [31, 32].

Conclusion

Habits related to oral health and determined by the HU DBI questionnaires are, in the case of the tested sample, predictors of oral health. In order to inform teenage children about the importance of oral health and to adopt healthy behaviors concerning oral hygiene and proper nutrition, educational interventions are needed. In studies based on the HU DBI, it is recommended to determine whether there is a group of respondents who gave affirmative answers to all four questions (Questions 10, 11, 18, and 20), since the algorithm of artificial intelligence identifies these subjects as a separate cluster with better oral health than respondents who did not give affirmative answers to these 4 questions.

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New gold pincer-type complexes induce caspase-dependent apoptosis in human cancer cells *in vitro*

Novi kompleksi zlata pincer-tipa indukuju kaspaza-zavisnu apoptozu u humanim ćelijama raka *in vitro*

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Abstract

Background/Aim. The use of cisplatin as a chemotherapeutic opened the door to the new metal-based drug research. New complexes containing metals such as platinum, palladium, ruthenium and gold have recently been analyzed as potential antitumor agents. The aim of the study was to investigate the cytotoxicity of Au(III) complexes with pincer-type ligands against cervical carcinoma cells (HeLa), breast cancer cells (MDA-MB-231 and 4T1) and colon carcinoma cells (HCT116 and CT26), as well as to examine the type and mechanism of cell death that these complexes induced in cancer cells. **Methods.** The cytotoxicity of Au(III) complexes was investigated by MTT assay. The apoptosis of the treated cancer cells was measured by flow cytometry and applying Annexin V/7AAD staining. The expressions of active proapoptotic protein Bax, antiapoptotic protein Bcl-2 and the percentage of cells containing cleaved caspase-3 in the treated cancer cells were determined by flow cytometry. **Results.** Complex 1 showed the most potent antitumor ef-

fect on HeLa cells, both compared to other two examined gold complexes and compared to cisplatin. The IC_{50} values on HeLa cells after 72 hours were 1.3 ± 0.4 μM, 3.4 ± 1.3 μM, 5.7 ± 0.6 μM, 26.7 ± 6.5 μM for complexes 1, 2, 3 and cisplatin, respectively. Complex 1 also exhibited the highest cytotoxicity against MDA-MB-231 and HCT116 cells compared to other tested compounds. The results of Annexin V/7AAD staining showed that all three gold complexes induced apoptosis in the treated cells. Our Au(III) complexes induced apoptosis by caspase-dependent mechanism, but we did not observe that the activation of an internal pathway of apoptosis occurred in the treated cancer cells. **Conclusion.** According to the results of our *in vitro* study, all three gold compounds, and especially complex 1, are promising candidates for a new generation of anticancer drugs.

Key words:
antineoplastic agents; apoptosis; caspases; enzyme assays; gold, compounds; neoplasms; toxicity, tests.

Apstrakt

Uvod/Cilj. Upotreba cisplatine kao citostatika je otvorila vrata za istraživanje novih lekova koji u svojoj strukturi sadrže neki od metala. Novi kompleksi koji sadrže metale poput platine, paladijuma, rutenijuma i zlata se od nedavno ispituju kao potencijalni antitumorski lekovi. Cilj rada bio je da se ispita citotoksičnost Au(III) kompleksa sa ligandima tipa pincer protiv ćelija karcinoma grlića materice (HeLa), ćelija raka dojke (MDA-MB-231 i 4T1) i ćelija karcinoma kolona (HCT116 i CT26), kao i tip i mehanizam ćelijske smrti koji ti kompleksi indukuju u ćelijama raka. **Metode.** Citotoksičnost

Au(III) kompleksa je ispitivana pomoću MTT testa. Apoptoza tretiranih ćelija raka je merena protočnom citometrijom i primenom bojenja Aneksin V/7AAD. Ekspresija aktivnog proapoptotičnog proteina Bax, antiapoptotskog proteina Bcl-2 i procenat ćelija koje sadrže aktivnu kaspazu-3 u tretiranim ćelijama raka određena je protočnom citometrijom. **Rezultati.** Kompleks 1 je pokazao najsnažniji antitumorski efekat na HeLa ćelije, kako u poređenju sa drugim ispitivanim kompleksima zlata, tako i u poređenju sa cisplatinom. Vrednosti IC_{50} kompleksa zlata na HeLa ćelije nakon 72 sata bile su 1,3 ± 0,4 μM, 3,4 ± 1,3 μM, 5,7 ± 0,6 μM, 26,7 ± 6,5 μM za komplekse 1, 2, 3 i cisplatinom, redom. Kompleks 1 je takođe

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pokazao najvišu citotoksičnost prema MDA-MB-231 i HCT116 ćelijama u poređenju sa drugim testiranim jedinjenjima. Rezultati bojenja aneksinomV/7AAD pokazali su da sva tri kompleksa zlata indukuju apoptozu u tretiranim ćelijama. Naši kompleksi Au(III) indukovali su apoptozu mehanizmom koji je zavisio od kaspaze, ali nismo pokazali da je u tretiranim ćelijama raka došlo do aktivacije unutrašnjeg puta apoptoze. **Zaključak.** Prema rezultatima naše *in vitro* studije, sva tri jedinjenja zlata, a posebno kompleks 1, obećavajući su kandidati za novu generaciju antikancerogenih lekova.

Ključne reči:
antineoplastici; apoptoza; kaspaze; testovi, enzimski; zlato, jedinjenja; neoplazme; toksičnost, testovi.

Introduction

In the United States, it is assumed that about 40% of adults will be diagnosed with cancer at a certain age [1]. This is a worrying fact, but finding a cure for cancer is not easy. There are major differences between tumors of various tissues and organs in cellular morphology, tumor aggressiveness and treatment of tumors [1–3]. Such differences exist even among the tumors of the same organ, such as breast tumors [3]. Even in the course of a treatment, tumor cells are modified and may become resistant to the therapy, which even further complicates the treatment [4]. Therefore, one universal cure for cancer will most likely never be found and testing of a substance that has chemotherapeutic potential against two or three types of tumors *in vitro* cannot provide sufficiently precise results.

Deregulation in apoptotic cell death machinery is the main characteristic of cancer [5]. Apoptosis is the process of cell degeneration that is not associated with inflammation and damage to surrounding healthy cells, and therefore it is a more favorable mechanism for reducing the number of tumor cells compared to necrosis [5, 6]. Apoptosis happens spontaneously in malignant tumors, often noticeably delaying their growth, and it is increased in tumors responding to irradiation, chemotherapy, high temperature and hormone ablation [6]. Apoptosis alteration is responsible not only for tumor development and progression but also for tumor resistance to therapies [5]. On the other hand, much of the present attention in the process stems from the finding that it can be regulated by certain protooncogenes and the p53 tumor suppressor gene [5–7]. Two proteins located in the cytoplasm of the cells, B-cell lymphoma protein 2 (Bcl-2)-associated X (Bax) and Bcl-2, are the activator and the inhibitor of apoptosis, respectively. It has been described, that Bax and Bcl-2, and their ratio, are predictive markers in different cancers [8]. Also, the significance of other molecules included in the apoptosis, such as caspases, has also been previously reported [9].

The use of cisplatin as a "standard" chemotherapeutic opened the door to the new metal-based drug research [10]. New complexes containing metals such as platinum, palladium, ruthenium and gold have recently been analyzed as potential antitumor agents [10–12]. Also, organometallic gold compounds occupied an important place in various anticancer researches due to their exceptional chemical characteristics with respect to gold coordination complexes [13]. Actually, many researchers have discovered that they could be utilized to create exceptionally capable metal-based drugs with potential relevance in the treatment of cancer [14].

In order to evaluate whether Au(III) compounds with pincer-type ligands might be used as possible anticancer agents, three new monofunctional Au(III) pincer complexes with three bispyrazolate ligands such as 2,6-bis(5-*tert*-butyl-1*H*-pyrazol-3-yl)pyridine (H$_2$L^{tBu}), 2,6-bis(5-*tert*-butyl-1-methyl-1*H*-pyrazol-3-yl)pyridine (Me$_2$L^{tBu}), and 2,6-bis((4*S*,7*R*)-1,7,8,8-tetramethyl-4,5,6,7-tetrahydro-1*H*-4,7-methanoindazol-3-yl)pyridine (Me$_2$*L) had been synthesized. The newly synthesized complexes, namely [Au(H$_2$LtBu)Cl]Cl$_2$ (1), [Au(Me$_2$LtBu)Cl]Cl$_2$ (2) and [Au(Me$_2$*L)Cl]Cl$_2$ (3) were characterized by elemental analysis, spectroscopic techniques (IR, UV-Vis, 1D and 2D NMR) and mass spectrometry (MS) methods: MALDI TOF and ESI Q-TOF. These three gold(III) complexes were also tested against three types of cancer cells (A549, A375, LS-174) *in vitro* [15].

However, these Au(III) complexes were not tested against other types of cancer cells and therefore further analyses were necessary. The aim of our research was to examine whether these Au(III) compounds with pincer-type ligands might be utilized as potential antitumor agents. Therefore, we examined the antitumor potential of all three Au(III) complexes against cell lines of cervical carcinoma (HeLa), breast cancer (MDA-MB-231 and 4T1) and colon carcinoma (HCT116 and CT26). We also included mouse carcinoma cells in our research (4T1 and CT26) in order to prove future research potential of these complexes for *in vivo* studies. We investigated their cytotoxic effect against five types of cancer cells, the type and mechanism of cell death that these complexes induce in cancer cells.

Methods

Synthesis of Au(III) pincer complexes with three bispyrazolate ligands

The synthesis and characterization of 2,6-bis(5-*tert*-butyl-1*H*-pyrazol-3-yl)pyridine (H$_2$L^{tBu}), 2,6-bis(5-*tert*-butyl-1-methyl-1*H*-pyrazol-3-yl)pyridine (Me$_2$L^{tBu}), and 2,6-bis((4*S*,7*R*)-1,7,8,8-tetramethyl-4,5,6,7-tetrahydro-1*H*-4,7-methanoindazol-3-yl)pyridine (Me$_2$*L) were discussed in detail in the previously published article [15]. The newly synthesized complexes, namely [Au(H$_2$LtBu)Cl]Cl$_2$ (1), [Au(Me$_2$LtBu)Cl]Cl$_2$ (2) and [Au(Me$_2$*L)Cl]Cl$_2$ (3) were characterized by elemental analysis, spectroscopic techniques (IR, UV-Vis, 1D and 2D NMR) and mass spectrometry (MS) methods: MALDI TOF and ESI Q-TOF.

Preparation of drug solutions

Complexes 1, 2, 3 and cisplatin were measured, dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific,

UK) at the concentration of 40 mM and then filtered through filters with a pore diameter of 0.22 mm. Afterward, the compounds were diluted in the complete medium so that the final concentration of DMSO was never greater than 0.5% (v/v). Dilution series of all compounds were prepared at the concentrations of 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM and 100 μM. All solutions used in the experiments were prepared exclusively on the day of the experiment, in order to avoid any potential modification in the chemical structure of the compounds tested. The control population consisted of cells that were not treated with the test substances and to which the same amount of DMSO, in comparison to the treated cells, was added. Blanks were microtiter plate wells that did not contain any cells or media.

Cell cultures

The study examined the effects of the Au(III) pincer complexes 1, 2 and 3, as well as cisplatin against five types of tumor cells: HeLa (human cervical cancer cells), MDA-MB-231 (human breast cancer cells), 4T1 (mouse breast cancer cells), HCT116 (human colon carcinoma cells) and CT26 (mouse colon carcinoma cells). All cells used in this study were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). Cells were cultured in a complete medium that was prepared as follows: DMEM (Dulbecco's Modified Eagle's Medium-High Glucose, D5796, Sigma Aldrich, Germany) and was supplemented with 10% FBS (Fetal Bovine Serum, Sigma Aldrich, Germany), 1% Penicillin-Streptomycin (Penicillin-Streptomycin, P4333, Sigma Aldrich, Germany), and 1% non-essential amino acids (MEM Non-essential Amino Acid Solution, M7145, Sigma Aldrich, Germany). The cells were maintained in 25 cm^2 flasks (Thermo Fischer Scientific, US) that were stored in CO_2 incubator at absolute humidity and 5% CO_2 at the temperature of 37ºC. Every 2–3 days, the cells were passaged in order to be used in all experiments in their exponential (Log) growth phase.

MTT assay

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) (MTT) assay is the colorimetric test used to examine the viability of cells. The basic principle of this assay is based on the ability of viable or living cells to reduce yellow tetrazolium bromide (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to the purple formazan [16]. Namely, living cells contain NAD(P)H-dependent oxidoreductase enzymes that have this ability, so the purple color of the cellular suspension after the incubation period reflects the amount of viable cells. To be precise, in each well of 96-wells microtiter plate, 100 μL of a suspension containing 3 × 10^3 cells in the exponential growth stage was added. The cells were incubated in the atmosphere containing 5% CO_2 and at 37ºC for 24 hours, and then different concentrations (0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM) of the examined complexes or cisplatin were added. As we have previously mentioned, we only added complete medium containing the appropriate amount of DMSO in control wells. Each concentration of the investigated substances was tested in triplicate and in three independent experiments. After 72 hours of the incubation of tumor cells in the atmosphere containing 5% CO_2 and at 37ºC, supernatant was extracted from each well and 200 μL of the tetrazolium bromide solution in the medium (0.05 mg/mL) was added. After another 2–3 hours of the incubation under the previously mentioned conditions, the supernatant was again removed from each well, and 150 μL of DMSO was added to dissolve the crystals of violet formazan. The plates were then shaken in the dark for 15 minutes at room temperature and the intensity of the purple color in each well was measured using the plate reader (Zenyth 3100, Anthos Labtec Instruments, Austria) by analyzing the absorbance at 595 nm. The percentage of viable cells was calculated by dividing the value of the readout absorbance in the wells that contained the treated cells with the average absorbance value measured in the wells of the untreated cells, and the ratio thus obtained was multiplied by 100.

% of the viable cells = [(absorbance of the treated cell – absorbance of blank) / (absorbance of the untreated cell – absorbance of blank)] * 100

% of the cytotoxicity of the compound = 100% – % of the viable cells

All cytotoxicity results were presented graphically as the arithmetic mean of the cytotoxicity of the tested substances from all three repeated experiments and triplicates ± standard deviation (SD). The values that reduce the treated cell viability by 50% relative to the control (IC_{50} values) were calculated using Microsoft Office Excel 2010, via logarithm-transformed dose-response data, previously obtained by MTT assay.

Annexin V/7AAD assay

The type of cell death induced by Au(III) pincer complexes was tested by flow cytometry and using Annexin V/7AAD staining according to the manufacturer's recommendation [Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D (7-AAD) Apoptosis Kit, BD Biosciences]. To be precise, two colors were used, Anneksin V, which binds to the residues of phosphatidyl serine and a viable color, 7-AAD. In viable cells that did not start apoptosis, the residues of phosphatidyl serine are turned to the inside of the cell membrane, so that there is no binding of Anneksin V to the residues of phosphatidyl serine. When initiating the apoptosis process, the parts of the cell membrane are drawn to the outside, so the cells that entered apoptosis bind Anneksin V. Such cells are grouped as Annexin V positive on the results of flow cytometry assay. On the other hand, 7-AAD is a color that binds the nucleus and color that does not pass through the cell membrane, so the cells that are 7-AAD positive are in fact the cells whose integrity of the cell membrane has been compromised. Such cells are either in the late stage of apoptosis or are necrotic. Therefore, it is assumed that the Annexin V(-)/7-AAD(-) cells are viable, Annexin

V(+)/7-AAD(-) are the cells at an early stage of apoptosis, Annexin V(+)/7-AAD(+) are the cells in the late stage of apoptosis and the necrotic cells are Annexin V(-)/7-AAD(+).

Therefore, we added 1 mL of cell suspension containing 10^6 cells/mL of complete medium in each well of 24-well plates. After 24 hours of incubation in the atmosphere containing 5% CO_2 and at 37 ℃, the medium was removed and 1 ml of the medium containing the half maximal inhibitory concentration (IC_{50}) of the tested substances was added. Each substance was tested in triplicate and in three independent experiments. The control population consisted of cells supplemented with complete medium with the same concentration of DMSO as in the treated cells. The cells were then incubated for 24 hours in order to detect substance-induced cellular changes [17]. After the incubation in the atmosphere containing 5% CO_2 and at 37 ℃, the cells were trypsinized, washed three times in ice-cold PBS (Phosphate Buffer Saline) and 1×10^5 cells were dissolved in 100 μL of binding buffer (BD Biosciences, USA). Then, 10 μL of Annexin-V-FITC and 20 μL of 7-AAD were added to the cells, followed by a 15-minute incubation at room temperature in the dark and the addition of 400 μL of binding buffer. The cells were then stirred and evaluated on the flow cytometer (The Cytomics FC500 Series, Beckman Coulter). At least 15000 events per sample were analyzed. The results were analyzed using FlowJo vX.0.7 and presented as the arithmetic means of the results obtained in three independent experiments and triplicates ± SD. We analyzed statistical significance between the percentages of cells found in early and late apoptosis against the percentage of necrotic cells for each test substance and for each type of tumor cells [18, 19].

Analysis of key proteins involved in the apoptosis process

The next step in our research was to examine the mechanism by which the process of apoptosis in the treated cells was activated. We wanted to determine if there was a change in the amount or activity of the key proteins involved in the apoptosis process. Therefore, we examined the cellular levels of the proapoptotic protein active-Bax, the antiapoptotic protein Bcl-2, and the percentage of the cells in which caspase-3 was active [18, 19]. We compared the populations of the cells treated with the half maximal inhibitory concentration (IC_{50}) of the tested substances (experimental group) to the population of the untreated cells (control group). Every type of examined cells was seeded in five 25 cm^2 flasks, i.e. four for the investigated compounds and one for the control group of cells. After 24 hours of incubation in the atmosphere containing 5% CO_2 and at 37 ℃ and absolute humidity, the media was replaced in all cell culture flasks. In four flasks, we added a complete growth medium supplemented with the previously determined half maximal inhibitory concentration (IC_{50}) of complexes 1, 2, 3 and cisplatin. In one flask the complete growth medium was added and the cells from this flask were the control population of the cells. The cells were then incubated for another 24 hours in the atmosphere containing 5% CO_2 and at 37 ℃ and absolute humidity in order to

detect substance-induced cellular changes [17]. Afterward, the cells from all the flasks were trypsinized and from every cell culture flask were divided into nine tubes, i.e. three for active-Bax staining, three for Bcl-2 staining and three for active caspase-3 staining. All cells were washed three times in PBS, fixed and permeabilized according to manufacturer's instructions (Fixation and Permeabilization Kit, eBioscience). The cells we isolated for Bcl-2 staining were supplemented with primary antibody for Bcl-2 (mhbcl01, Life technologies) in the ratio of 1:1,000 for 15 min. Permeabilized HeLa cells were also incubated with primary antibody for active-Bax (N20, sc-493; Santa Cruz Biotech Inc.) and for activated caspase-3 (#9661, Cell Signing Technology) for 30 minutes and at the concentration of 1:1,000. Subsequently, these two groups of cells (for active-Bax and caspase-3) were incubated with the secondary, goat FITC-labeled antibody (goat anti-rabbit IgG FITC Ab6717-1, Abcam) for 30 minutes and at the concentration of 1:2,000. Afterward, all cells were washed in PBS and analyzed by flow cytometer FC500 (Beckman Coulter, US). At least 15,000 events per sample were analyzed. The mean fluorescence intensities (MFI) for Bcl-2 and Bax were calculated as the ratio of the measured fluorescences for Bcl-2 and Bax against the fluorescence of the isotype control and represent the concentrations of Bcl-2 and active-Bax in stained cells. Values for active caspase-3 are presented as the percentages of cells that emit fluorescence to active caspase-3. The experiment was repeated three times.

Statistical analysis

The distributions of the obtained data were evaluated for normality using the Shapiro-Wilk test. The values of MTT and Annexin assays were presented as mean ± SD. The values of apoptotic proteins were presented as medians due to large standard deviations and distribution of data that was not normal. All experiments were performed in triplicates and in three separate repetitions. Commercial SPSS version 20.0 for Windows was used for statistical analysis. Statistical evaluation was performed by Student's *t*-test for paired observations, or one-way ANOVA depending on data distribution. *P* values less than 0.05 were considered to indicate a statistically significant difference.

Results

The cytotoxicity of complex 1 and its ligand was partially tested on the cell lines of colon carcinoma (LS-174), lung carcinoma (A549) and melanoma (A375) cell lines. The results of this study have shown that complex 1 exhibited stronger antitumor effect on all three types of tumor cells tested compared to cisplatin [15]. Since some compounds may show delayed toxicity, it is concluded that the analysis of cytotoxicity should be performed after an interval of at least 48 hours [20]. Therefore, we chose to analyze cytotoxicity after 72 hours, similarly to the previous experiments [21–23].

The results of MTT assay showed dose-dependent cytotoxic effects against all tested cancer cells (HeLa, MDA-MB-231, 4T1, HCT116 and CT26) *in vitro* (Figure 1).
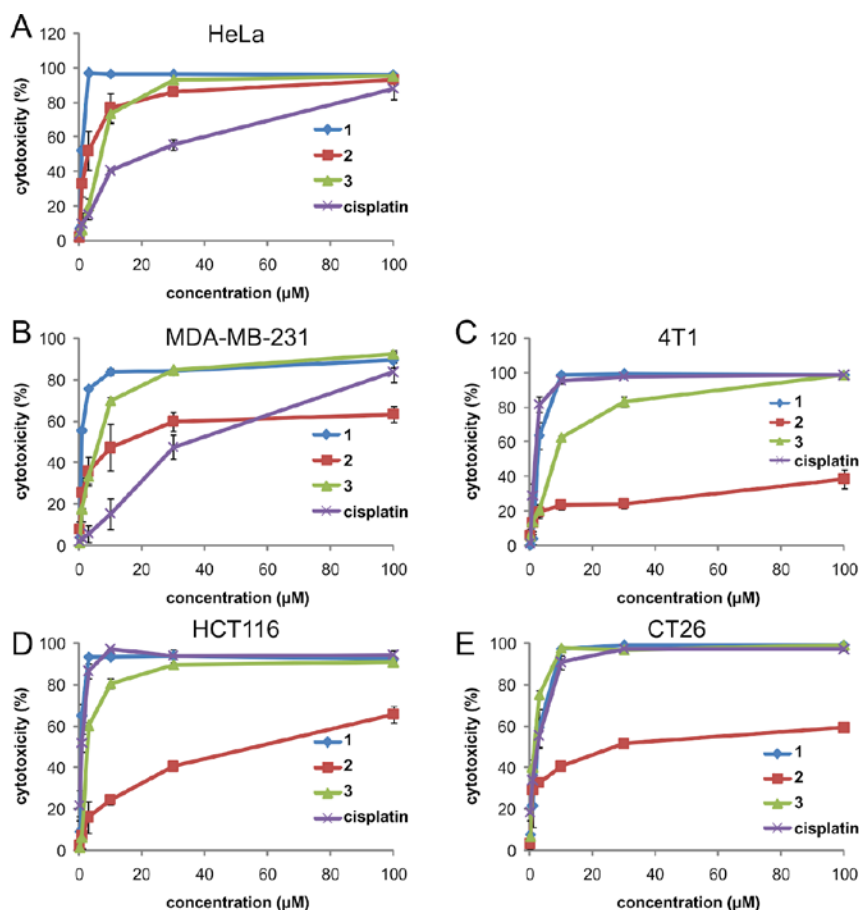
Fig. 1 – Gold (III) complex cytotoxic activities against cancer cells.
MTT assay results after 72 hours of the incubation of complexes 1, 2, 3 and cisplatin
against HeLa cells (A), MDA-MB-231 cells (B), 4T1 cells (C), HCT116 cells (D) and
CT26 cells (E). The results are presented as mean ± standard deviation.

Table 1
IC$_{50}$ values (µM) for complexes 1, 2, 3 and cisplatin after 72 h drug exposure

Cancer cells	1	2	3	cisplatin
HeLa	1.3 ± 0.4^a	3.4 ± 1.3	5.7 ± 0.6	26.7 ± 6.5
MDA-MB-231	1.6 ± 0.3	15.1 ± 2.3	5.4 ± 0.5	30.8 ± 6.1
4T1	1.7 ± 0.2	> 100	6.2 ± 0.4	1.8 ± 0.2
HCT116	0.7 ± 0.2	53.9 ± 4.7	4.1 ± 0.4	1.6 ± 0.2
CT26	4.3 ± 0.3	26.04 ± 2.1	6.2 ± 0.3	2.6 ± 0.3

The results are presented as mean ± standard deviation determined from
the results of MTT assay in three independent experiments.
IC – inhibitory concentration.

By further analysis of the results obtained by the MTT test, as described in material and methods section, we calculated the IC$_{50}$ values that more accurately showed the cytotoxicity of the compounds tested. Complex 1 showed the most potent antitumor effect on HeLa cells, both compared to the other two examined Au(III) complexes and compared to cisplatin ($p < 0.05$). The IC$_{50}$ values on HeLa cells after 72 hours were 1.3 ± 0.4 µM, 3.4 ± 1.3 µM, 5.7 ± 0.6 µM, 26.7 ± 6.5 µM for complexes 1, 2, 3 and cisplatin, respectively (Table 1).

The antitumor effects of our gold(III) complexes were also tested against human (MDA-MB-231) and mouse (4T1)

breast cancer cells. All three gold(III) complexes showed stronger antitumor effects on both breast cancer cell lines compared to cisplatin ($p < 0.05$, Table 1). Complex 1 exhibited the highest cytotoxicity against MDA-MB-231 cells compared to other tested compounds ($p < 0.05$). The IC$_{50}$ value of complex 1 against MDA-MB-231 cells was 1.6 ± 0.3 µM, which was significantly lower compared to complexes 2 and 3 (15.1 ± 2.3 µM and 5.4 ± 0.5 µM for complexes 2 and 3, respectively; $p < 0.05$). All three complexes displayed significantly increased cytotoxicity of MDA-MB-231 cells compared to cisplatin whose IC$_{50}$ value against MDA-MB-231 cells was 30.8 ± 6.1 µM ($p < 0.05$).

Complex 1 exhibited a similar cytotoxic effect against the mouse breast cancer cells 4T1 as cisplatin ($p > 0.05$). The IC_{50} values for complex 1 and cisplatin were 1.7 ± 0.2 μM and 1.8 ± 0.2 μM, respectively (Table 1). Complexes 2 and 3 displayed significantly decreased cytotoxicity against 4T1 cells compared to cisplatin and the IC_{50} values for complexes 2 and 3 were > 100 μM and 6.2 ± 0.4 μM, respectively ($p < 0.05$, Table 1).

The most cytotoxic agent against human colon cancer cells HCT116, was complex 1 displaying twice as high cytotoxicity as cisplatin ($p < 0.05$; 1 vs. 2, 1 vs. 3 and 1 vs. cisplatin). The IC_{50} value for complex 1 and cisplatin against HCT116 cells was 0.7 ± 0.2 μM and 1.6 ± 0.2 μM, respectively. Complexes 2 and 3 exhibited less potent cytotoxic effects against HCT116 cells than cisplatin ($p < 0.05$, Table 1). It is worth noticing that our gold(III) complexes showed stronger cytotoxicity against the human MDA-MB-231 cells compared to the mouse 4T1 breast cancer cells ($p < 0.05$). Also, when we analysed MTT assay results of our gold(III) complexes against colon carcinoma cells, an identical phenomenon occurred ($p < 0.05$; 1(CT26) vs. 1(HCT116), 2(CT26) vs. 2(HCT116), 3(CT26) vs. 3(HCT116)). In this case, complexes 1, 2, and 3 showed weaker cytotoxicity against CT26 mouse colon carcinoma cells than against HCT116 human colon cancer cells. All three complexes exhibited lower cytotoxicity against CT26 cells compared to cisplatin ($p < 0.05$, Table 1).

We showed that our gold(III) complexes, and especially complex 1, displayed strong antitumor effects *in vitro* against all three types of tested human cancers cells, i.e. cervical cancer cells, breast cancer cells and colon cancer cells. Therefore, the next step of our research was to investigate the mechanism, or the type of cell death resulting in a decreased viability of the treated cells.

The results of Annexin V/7AAD staining showed that the apoptosis was induced by our gold complexes and cisplatin in all five types of the cancer cells tested (Figure 2, $p < 0.05$). In all cases, less than 4% of the total population of the cells were necrotic, while the rest of the non-viable cell population was in different stages of apoptosis. In general, a higher percentage of cells entered early apoptosis phase, and a slightly lower percentage of cells had already entered the late stage of apoptosis (Figure 2).

Consequently, the next step of our research was to investigate whether our gold complexes influenced the cytoplasmic concentration of antiapoptotic protein Bcl-2, the ac-
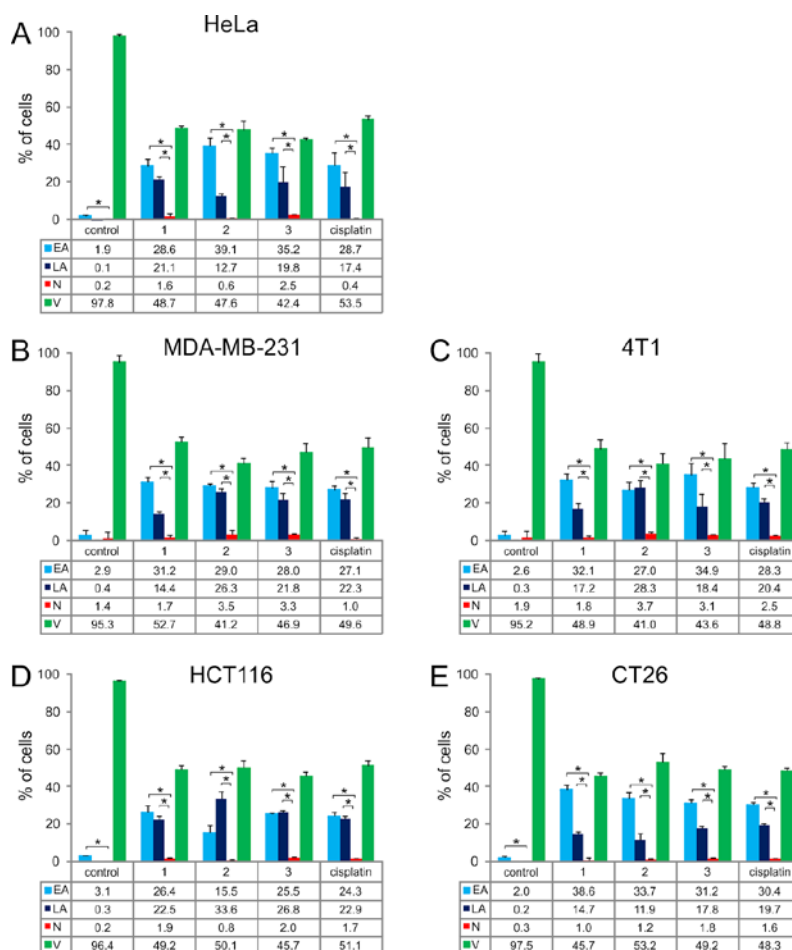
Fig. 2 – Annexin V/7AAD assay results after the treatment with inhibitory concentration (IC)$_{50}$ values of complexes 1, 2, 3 and cisplatin against HeLa cells (A), MDA-MB-231 cells (B), 4T1 cells (C), HCT116 cells (D) and CT26 cells (E). The results are presented as mean ± standard deviation. Control cells are untreated cells. EA – early apoptosis; LA- late apoptosis; N – necrosis; V – viability.

tivation of the proapoptotic protein Bax and the activation of the caspase cascade in HeLa cancer cells. We decided to investigate these events in HeLa cells considering that all three complexes exhibited the strongest cytotoxicity against this type of cancer cells, and these results were presented in Figure 3.

The results of our research have shown that all three gold complexes and cisplatin insignificantly reduced the amount of antiapoptotic protein Bcl-2 (Figure 3A, $p > 0.05$). Although this decrease was not statistically significant, it appeared as a noticeable trend. In addition, we showed that there was no statistically significant change in the activation of proapoptotic protein Bax in the groups of cancer cells cultivated in the presence of Au(III) complexes (Figure 3B, $p > 0.05$). However, in cisplatin-treated cells, a statistically significant increase of active-Bax was detected in comparison to the untreated cells (Fig. 3B, $p < 0.05$). If we observe Bcl-2/Bax ratio, there was also no significant change in the values following the effects of complexes 1–3 in comparison to control (Figure 3C, $p > 0.05$). Quite the opposite, cisplatin-treated cells exhibited a statistically significant increase of Bcl-2/Bax ratio in comparison to the untreated cells (Figure 3C, $p < 0.05$). On the other hand, a statistically significant increase in the amount of active caspase-3 in the cells treated with gold(III) complexes 1–3 and cisplatin in relation to the control population of the untreated cells was noted (Figure 3D, $p < 0.05$).

Discussion

Seeking new solutions to increase selectivity and specificity of chemotherapy in cancer cells has attracted much attention in science, recently. It was a widespread opinion that the cytotoxic effects of metal complexes are the result of direct damage to nuclear DNA [24]. However, gold(III) complexes exert their cytotoxic activities through mechanisms that are considerably diverse from those of platinum drugs [24] and the molecular mechanisms and targets of gold(III) complexes are still not precisely defined.

Hence in this work, three new gold(III) complexes with different hydro/lipophilic properties were evaluated for their anticancer activity *in vitro*. When we observed cytotoxic activities of our gold(III) complexes and cisplatin against all five types of the tested cancer cells, we discovered that complex 1 showed the strongest cytotoxicity in comparison to other complexes and compared to cisplatin in HeLa, MDA-MB-231, 4T1 and HCT116 cells. It is important to point out that complex 1 exhibited stronger cytotoxic effects than cisplatin against all three types of the human cancer cells: HeLa, MDA-MB-231 and HCT116 cells. The results of our research are in agreement with the results of other authors [25–27] who also synthesized gold(III) complexes that are significantly more effective against cancer cells in comparison to cisplatin. However, cisplatin showed statistically higher cytotoxicity against CT26 cells compared to complex 1.
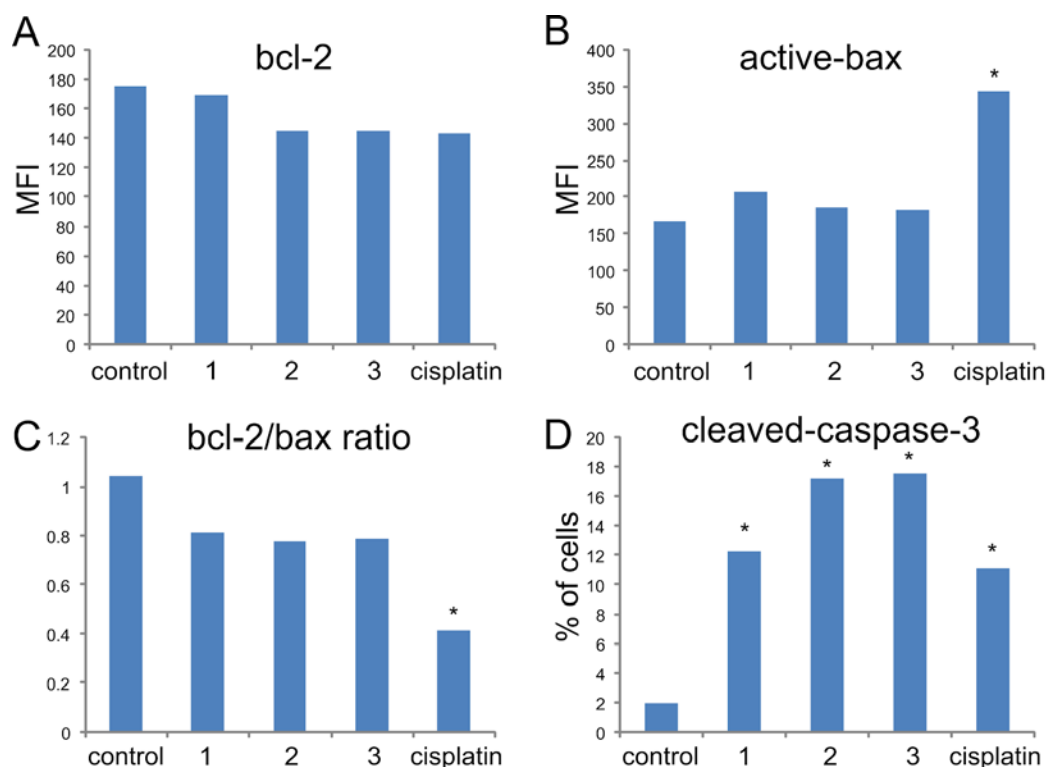
Fig. 3 – Gold (III) complexes induce apoptosis of HeLa cells via caspase-dependent pathway.
(A) MFI (mean fluorescence intensity) values for antiapoptotic protein Bcl-2 of the untreated cells (control) or the cells treated with inhibitory concentration (IC)$_{50}$ values of complexes 1, 2, 3 or cisplatin;
(B) MFI values for active proapoptotic protein Bax of the untreated cells (control) or the cells treated with IC$_{50}$ values of complexes 1, 2, 3 or cisplatin; (C) Bcl-2/Bax ratio for both untreated and treated HeLa cells;
(D) The percentages of cells displaying fluorescence for cleaved caspase-3.
The results are presented as medians (*$p < 0.05$ compared to the untreated cells).

The results of our study showed that gold(III) complexes exhibit strong anticancer activity *in vitro*, which is in agreement with the results of some previous studies [25–27]. However, our gold complexes demonstrated stronger cytotoxicity against human cancer cells *in vitro* compared to gold complexes of some other authors. Williams et al. [25] synthesized seven new cyclometalated Au(III) complexes with five of them bearing an acridine moiety attached via (N^O) or (N^N) chelates, acyclic amino carbenes (AAC) and N-heterocyclic carbenes (NHC). However, the Williams et al. [25] complexes showed significantly lower cytotoxicity compared to our complexes, which was at least two-fold less in comparison to IC_{50} value of our complex 1 against MDA-MB-231 cells. Only complex 11 synthesized by Williams et al. [25] demonstrated an IC50 value against MCF-7 breast cancer cells comparable to our results. The complexes of Bertrand et al. [26] were biotin, 17α-ethynylestradiol and benzimidazole-based (NHC)AuCl conjugates connected by linkers of variable length. Bertrand et al. [26] investigated the cytotoxicity of their complexes against MCF-7, MDA-MB-231, A549, HCT116 and HEK-293 cancer cells. However, our complexes exhibited 3.5-fold stronger cytotoxicity *in vitro* compared to gold complexes of Bertrand and associates [26]. The series of lipophilic [AuIII(C^N^C)(NHC)]+ complexes synthesized by conjugation of an N-heterocyclic carbene (NHC) ligand to the [AuIII(C^N^C)]+ moiety, showed promising in vitro cytotoxicity towards a panel of cancer cell lines with IC50 values spanning between 0.17 and 1.2 mM [27]. These results are in line with the results of our study and show promising *in vitro* results of Au(III) complexes against cancer cells, in general. Li et al. [22] synthesized a series of cyclometalated gold(III) compounds [Aum-(C^N^C)mL]n+ (m = 1–3; n = 0–3; HC^N^CH=2,6-diphenylpyridine) by ligand substitution reaction of L with N-donor or phosphineligands. The cytotoxicity of their. Au(III) complexes was examined against HeLa cells under the same conditions in comparison to our study. Their IC_{50} values were similar or even significantly lower in comparison to the results that we obtained, and the proposed mechanism that decreased the viability of the treated cells was apoptosis.

The results of recent studies have pointed toward the induction of apoptosis as a major cytotoxic mechanism of gold(III) complexes against cancer cells [22, 28]. Apoptosis is mediated by two main pathways, an extrinsic pathway, which involves cell surface receptors, and an intrinsic pathway via mitochondria and the endoplasmic reticulum. The results of our research were completely in agreement with the results of other studies, where it was also shown that complexes of gold displayed cytotoxicity against the tested cancer cells by the induction of apoptosis [26]. This is very important because substances acting cytotoxically by the induction of apoptosis do not induce changes in surrounding healthy tissue; there is no process of inflammation or other adverse effects [6].

Therefore, the next step in our research was to examine the mechanism of apoptosis in the group of treated cancer cells. It has already been shown that the substances acting through the mitochondrial, internal pathway of apoptosis changed the activity or concentration of proapoptotic and antiapoptotic proteins [18, 19]. In addition, it has already been shown that gold complexes could induce apoptosis by activating the mitochondrial pathway of apoptosis [26, 29]. The activation of caspase-8 is typical for the external (receptor) pathway of apoptosis, while the increased active Bax and/or decreased Bcl-2 is rather connected with the internal (mitochondrial) pathway. In both cases, downstream activation of caspase-3 occurs. Afterward, caspase-3 initiates apoptotic DNA fragmentation by proteolytically inactivating the DFF45/ICAD protein complex [30].

The results of our research indicated that most probably there had been no induction of apoptosis due to the effect of complexes 1–3 through the internal pathway of apoptosis. Further researches are necessary in order to confirm the following assumption, but we may suggest that the activation of the external pathway of apoptosis is the most probable cause of Au(III) complex-induced apoptosis [6, 31]. On the other hand, we undoubtedly concluded that all three gold(III) complexes induced apoptosis by activating the caspase cascade, as there was a statistically significant increase in the amount of active caspase-3 in the cells treated with IC_{50} values of gold(III) complexes 1–3 compared to the untreated cells ($p < 0.05$).

Although it has been shown that gold(III) complexes have a different mechanism of action in comparison to cisplatin, the precise mechanism of cytotoxic activity of gold(III) complexes has not yet been fully clarified.

Conclusion

It has been shown that gold(III) complexes could induce apoptosis by activating the internal pathway of apoptosis and activating the caspase cascade. The results of our research showed that gold(III) complexes tested induced apoptosis by the caspase-dependent mechanism, but we did not observe that an activation of the internal pathway of apoptosis occurred in treated cancer cells. The most probable cytotoxic mechanism of the investigated gold(III) complexes was the activation of the external pathway of apoptosis, but this assumption must be proven. However, all three investigated gold(III) complexes, especially complex 1, showed strong cytotoxicity against human cancer cells *in vitro* and induced apoptosis by caspase-dependent mechanism. It is therefore necessary to further investigate the mechanism of cytotoxicity of these complexes. According to the results of our *in vitro* study, and if some further *in vivo* investigations show promising results, complex 1 may be a good candidate for a new generation of anticancer drugs.

Conflict of interest

The authors declare no conflict of interest.

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The association between the frequency of dental visits and independent factors among the adults aged 20 years and over in Serbia

Povezanost između učestalosti poseta stomatologu i nezavisnih činilaca među odraslim stanovništvom starosti 20 i više godina u Srbiji

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Abstract

Background/Aim. Regular check-ups with a dentist are an important component of general oral hygiene habits. In addition to regular visits to a dentist, this includes the ongoing knowledge upgrade in the field of oral health as well as the application of preventive measures recommended by the selected dentist. The aim of this study was to determine the link between dental visits and independent sociodemographic factors and smoking in the adult population in Serbia. **Methods.** This cross-sectional study represents an analysis of 2013 National Health Survey for the population of Serbia (without the data on Kosovo and Metohija population). The study included 13,404 adults of 20 years of age and older. The mean age of participants was 51.7 years, including 7,221 (53.9%) females and 6,183 (46.1%) males. In order to determine possible predictors of a dental visit, a multivariate logistic regression model was implemented. A visit to a dentist was a dependent variable, while the independent variables were: sex, age, marital status, type of settlement, region, education, employment status, Wealth Index and smoking status. **Results.** Significant differ-ences were observed between categories of dental visit and all independent variables except marital and smoking status. In the multivariate model, the odds of visiting a dentist in the period "12 months or longer" vs. "in the last 6 months" were the highest among older respondents [odds ratio (OR) = 1.03; 95% confidence interval (CI) = 1.02–1.04], from a rural area (OR = 1.17; 95% CI = 1.03–1.32), with a low (OR = 2.55; 95% CI = 2.12–3.07) and middle education level (OR = 1.76; 95% CI=1.54–2.00), the unemployed (OR=1.20; 95% CI = 1.06–1.37), those who belong to poorer (OR = 1.30; 95% CI = 1.08–1.54) or the poorest class (OR = 1.71; 95% CI = 1.38–2.12) and smokers (OR = 1.13; 95% CI = 1.01–1.26). **Conclusion.** The study demonstrated that sociodemographic factors and smoking are important factors related to a visit to a dentist. This study can help to advance regular visits to a dentist and programs of health education focusing on oral health and smoking cessation as well.

Key words:
adults; oral health; dentists; office visit; sociological factors; smoking; serbia.

Apstrakt

Uvod/Cilj. Redovne kontrole kod stomatologa su važna komponenta opštih oralnih higijenskih navika. Pored redov-nih kontrola kod stomatologa, podrazumeva se i redovno un-apređivanje u oblasti oralnog zdravlja, kao i primena preven-tivnih mera po preporuci stomatologa. Cilj ove studije bio je utvrđivanje povezanosti između posete stomatologu i nezavisnih socio-demografskih faktora i pušenja kod odrasle populacije u Srbiji. **Metode.** Istraživanje predstavlja analizu podataka dobijenih u okviru Nacionalnog istraživanja zdravlja stanovništva Srbije koje je sprovedeno 2013. godine kao studija preseka na reprezentativnom uzorku odraslog stanov-ništva Srbije (bez podataka o stanovništvu Kosova i Metohi-je). Studijom je bilo obuhvaćeno 13 404 ispitanika starosti 20 godina i više. Prosečna starost ispitanika bila je 51,7 godina, uključujući 7 221 (53,9%) ženu i 6 183 (46,1%) muškarca. Prediktori učestalosti posete stomatologu analizirani su multi-varijantnom logističkom regresijom. Poseta stomatologu je bi-la zavisna promenljiva, dok su nezavisne promenljive bile: pol, starost, bračni status, tip naselja, region, obrazovanje, radni status, indeks blagostanja i pušački status. **Rezultati.** Ustanovljene su značajne razlike između kategorija posete stomatologu i svih nezavisnih promenljivih, osim bračnog i pušačkog statusa. Rezultati multivarijantnog modela pokazali su da su šanse za posetu stomatologu u periodu "12 meseci ili duže" u odnosu na "u poslednjih 6 meseci" bile najveće kod starijih ispitanika [odds ratio (OR) = 1,03; 95% confidence interval (CI) =1,02–1,04], onih koji žive u naseljima van grada (OR = 1,17; 95% CI = 1,03–1,32), sa niskim (OR = 2,55; 95% CI =

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2,12–3,07) i srednjim nivoom obrazovanja (OR = 1,76; 95% CI = 1,54–2,00), nezaposlenih (OR = 1,20; 95% CI = 1,06–1,37), onih koji pripadaju siromašnijoj (OR = 1,30; 95% CI = 1,08–1,54) ili najsiromašnijoj klasi (OR = 1,71; 95% CI = 1,38–2,12) i pušača (OR = 1,13; 95% CI = 1,01–1,26). **Zaključak.** Socio-demografski faktori i pušenje su važni faktori vezani za posetu stomatologu. Ova studija može biti od pomoći u unapređenju redovnih poseta stomatologu, kao i programa zdravstvenog vaspitanja usmerenog na oralno zdravlje i prestanak pušenja.

Ključne reči:
odrasle osobe; usta, zdravlje; stomatolozi; zdravstvena ustanova, poseta; socijalni faktori; pušenje; srbija.

Introduction

Regular check-ups with a dentist are an important component of general oral hygiene habits. In addition to regular visits to a dentist, this includes the ongoing knowledge upgrade in the field of oral health as well as the application of preventive measures recommended by the selected dentist [1]. Furthermore, monitoring dental visits is important because it represents a basis for predicting the costs distributed for dental health care annually [2], recognizing the differences in oral health, and assessing the impact of changed economic conditions and health policies [3]. Dental professionals are convinced that frequent check-ups allow a disease to be detected and treated in time, but they are the only ones who can adequately assess the most appropriate interval between check-ups [4], and the most commonly recommended revisiting period is in 6 months [5].

By the 1990s, in the Republic of Serbia (as a part of the former Yugoslavia) the health care system was financed through a compulsory social insurance, but the access to health care was a constitutional right of all citizens [6]. However, the last decade of the 20th century in Serbia was marked by wars, sanctions of the international community and the negative consequences they caused, which led to the collapse of all segments of society, including the health care system [7]. The Republic Health Insurance Fund (RHIS), financed by mandatory taxation of employers and employees provides dental health care only through public dental services. Since 2005, RHIS for adult population has covered only emergency dental care and provision of acrylic complete and partial dentures for patients older than 65 years [8]. Those changes significantly affected the accessibility and provision of dental health care [9]. In 2006, the total number of dental visits in Serbia decreased compared to the previous year by 44%, and in 2007 it was reduced by additional 11%. The number of dental cavity intervention services and cavity complication interventions has significantly decreased after 2005. For example, in 2007 there were 59% fewer cavity fillings than in 2005. Furthermore, there was a reduction in the treatment of cavity complications, as much as 64% fewer treatments in 2007 compared to 2005 [7]. According to the 2013 National Health Survey in Serbia, only 26.9% of the respondents had their chosen dentist in the government-owned institution, while 31% reported having a dentist in private practice [10].

To explain the determinants of health care use, the Andersen's behavioral model was usually applied. The predisposing factors include demographic characteristics, such as age, gender, education, marital status, type of settlement and health beliefs. Financing and organizational factors are considered to serve as conditions enabling services utilization (income/financial situation, insurance, usual source of care, availability of health-related information, affordability of medical care). Need factors include perceived need for health care (evaluated health status and perceived need and self-rated health) [11].

An additional factor related to the use of dental services is smoking. Moreover, smokers have lower rates of dental care utilization, despite the fact that tobacco use is a risk factor for tooth loss [12] and oral squamous cell carcinoma [13].

The aim of this study was to determine the link between dental visits and independent sociodemographic factors and smoking in the adult population in Serbia.

Methods

Study design and sampling

This study represents an analysis of the 2013 National Health Survey for the population of Serbia (without the data on Kosovo and Metohija population), which was carried out by the Ministry of Health of the Republic of Serbia. The study protocol was approved by the Ethical review board of the Institute of Public Health of Serbia. A stratified two-stage representative sample of the population of Serbia was used for this study. The sample was selected to provide statistically reliable estimates at the national level and at the levels of 4 geographical regions of Serbia (The Province of Vojvodina, Belgrade, Central and West Serbia, South and East Serbia). Within 10,089 of all registered households in the Republic of Serbia in the census 2011, 6,500 households were randomly selected for the research sample (3,960 urban and 2,540 rural households) and interviewed during October-December 2013. The interviews and measurements were carried out in each household by teams of two trained interviewers and a healthcare worker. Informed, written consent was obtained from all respondents. The household response rate was 64.4%. Of the total 16,474 registered members of the household aged 15 years and over, 14,623 were interviewed giving a response rate of 88.9%. Among the people who agreed to be interviewed, 13,756 accepted to fill a self-administered questionnaire (response rate 94.1%) [10]. For the purpose of this study, we analyzed the data on the participants aged 20 years or over, the total of 13,404 adults for whom dental visits data were available (91.7% of all interviewed respondents). The mean age of participants was 51.7 years.

Study variables

A dental visit (in the public and in private sector) was a dependent variable, and it was assessed within 3 categories (in the last 6 months, 6–12 months, 12 months or longer). In addition, a series of demographic and socio-economic variables po-

tentially related to a dental visit were included: sex (female or male), age (20–34, 35–44, 45–54, 55–64, and 65 years and over), type of settlement (urban or rural), marital status (living with or without a partner), education level (low, middle or high), and employment status (employed, unemployed or inactive). In addition, the households and respondents were classified according to Wealth Index (Demographics and Health Survey Wealth Index) into five socio-economic categories: poorest, poorer, middle class, richer and the richest class [14]. Smoking status was assessed as never smoked, past smoker and smoker.

Statistical analysis

Statistical analyses, bivariate and multivariate logistic regressions were used to analyze the data. In order to assess the differences between groups, the χ^2-test and ANOVA were used where appropriate. To determine possible predictors of a dental visit, the multivariate logistic regression model was implemented for all categories of dental visits. The analysis was done using the statistical software package SPSS 21, including the weight factor ("weight on"). This factor was used for the correction of disproportionate size of the sample and adjustment of the collected data.

Results

Almost two thirds of participants had a partner. The highest percentage of the respondents (54.3%) had middle education, and 28.9% had low education. Only one in three was employed (33.2%) and more than two fifths (43.8%) were poor. The distribution of demographic, socioeconomic characteristics and the smoking status of participants are presented in Table 1.

Table 1

The distribution of demographic, socioeconomic characteristics and the smoking status of the sample and description of study population across categories of dental visit (Survey 2013)

Variable	n (%)	< 6 months n (%)	6–12 months n (%)	≥ 12 months n (%)	p
Total	13,404 (100)	2,445 (18.3)	1,851 (13.8)	9,108 (67.9)	
Sex					
female	7,221 (53.9)	1,431 (19.8)	1,065 (14.8)	4,725 (65.4)	
male	6,183 (46.1)	1,014 (16.4)	786 (12.7)	4,383 (70.9)	< 0.0001[a]
Age (years), mean ± SD	51.7 ± 17.3	44.8 ± 15.9	44.2 ± 16.0	55.1 ± 16.9	< 0.0001[b]
Age (years)					
20–34	2,713 (20.2)	782 (28.8)	609 (22.4)	1,322 (48.8)	< 0.0001[a]
35–44	2,158 (16.1)	516 (23.9)	397 (18.4)	1,245 (57.7)	
45–54	2,291 (17.1)	421 (18.4)	336 (14.6)	1,534 (67.0)	
55–64	2,839 (21.2)	420 (14.8)	268 (9.4)	2,151 (75.8)	
65+	3,403 (25.4)	306 (9.0)	241 (7.1)	2,856 (83.9)	
Marital status					
living with a partner	8,771 (65.4)	1,556 (17.7)	1,219 (13.9)	5,996 (68.4)	0.119[a]
living without a partner	4,633 (34.6)	889 (19.1)	632 (13.6)	3,112 (67.3)	
Type of settlement					
urban	7,554 (56.4)	1,615 (21.4)	1,198 (15.8)	4,741 (62.8)	< 0.0001[a]
rural	5,850 (43.6)	830 (14.1)	653 (11.1)	4,367 (74.8)	
Region					
Belgrade	2,850 (21.2)	2,850 (21.2)	472 (16.5)	1,763 (61.9)	< 0.0001[a]
Vojvodina	3,299 (24.6)	3,299 (24.6)	359 (10.9)	2,363 (71.6)	
Šumadija and West Serbia	4,089 (30.5)	4,089 (30.5)	567 (13.9)	2,853 (69.7)	
South and East Serbia	3,166 (23.6)	3,166 (23.6)	453 (14.4)	2,119 (67.0)	
Education					
low	3,868 (28.9)	357 (9.2)	262 (6.7)	3,249 (84.1)	< 0.0001[a]
middle	7,281 (54.3)	1,437 (19.7)	1,103 (15.1)	4,741 (65.2)	
high	2,255 (16.8)	651 (28.9)	486 (21.5)	1,118 (49.6)	
Employment					
employed	4,438 (33.1)	1,069 (24.1)	838 (18.9)	2,531 (57.0)	< 0.0001[a]
unemployed	3,076 (22.9)	582 (18.9)	435 (14.1)	2,059 (67.0)	
inactive	5,890 (44.0)	794 (13.5)	578 (9.8)	4,518 (76.7)	
Wealth Index					
poorest class	3,004 (22.4)	303 (10.1)	216 (7.2)	2,485 (82.7)	< 0.0001[a]
poorer class	2,865 (21.4)	462 (16.1)	329 (11.5)	2,074 (72.4)	
middle class	2,670 (20.0)	487 (18.2)	400 (15.0)	1,783 (66.8)	
richer class	2,507 (18.7)	573 (22.9)	422 (16.8)	1,512 (60.3)	
richest class	2,358 (17.5)	620 (26.3)	484 (20.5)	1,254 (53.2)	
Smoking status					
never smoked	5,620 (45.6)	1,014 (18.0)	822 (14.6)	3,784 (67.4)	0.123[a]
former smoker	2,358 (19.2)	475 (20.1)	317 (13.4)	1,566 (66.5)	
smoker	4,330 (35.2)	820 (18.9)	584 (13.5)	2,926 (67.6)	

[a]χ^2-test; [b]ANOVA.

There was a higher percentage of males (70.9%) than females (65.4%) that visited a dentist in a 12-month period or longer and the mean age was significantly higher among the study respondents who visited a dentist in the same period. The majority of participants reported visiting a dentist in the period of 12 months or longer and this pattern is the same for all age categories. In the urban area and Belgrade region only every fifth study participant visited a dentist every 6 months, and in a rural region every seventh. Among those who reported visiting a dentist in the period of 12 months or longer, the highest percentage belonged to the participants with a low education level (84.1%), inactive participants (76.7%), and those who belonged to the lowest socio-economic class (82.7%). Significant differences were observed between the categories of dental visits and all dependent variables, excluding marital status and smoking (Table 1).

The results of bivariate and multivariate logistic regression analysis on the association between a dental visit and sociodemographic and lifestyle factors are presented in Table 2. In category visit to a dentist ≥ 12 months vs. < 6 months, using a bivariate analysis, we observed that demographic factors (age, sex, type of settlement and regions), socioeconomic factors (education, employment and Wealth Index) and smoking status were significantly associated with a dental visit. In the multivariate model, we found that demographic factors (female gender, age, rural area), socioeconomic factors (low and middle education level, Wealth Index), smoking status (smokers and former smokers) were significantly associated with a dental visit. Results showed that the odds of visiting a dentist in the period "12 months or longer" vs. "in the last 6 months" were the highest among older respondents (OR = 1.03), with a low (OR = 2.55) and middle (OR = 1.76) education level, the unemployed (OR = 1.20), the respondents who belonged to poorer (OR = 1.30) or the poorest class (OR = 1.71) and smokers (OR = 1.13).

Table 2

Predictors of dental visit – bivariate[#] and multivariate logistic regression analysis (Survey 2013)

Variables	Type of logistic regression analysis	6–12 months vs. < 6 months (1.909 vs. 2.549) OR (95% CI); *p*	≥ 12 months vs. < 6 months (8.817 vs. 2.549) OR (95% CI); *p*
Age	bivariate	1.00 (0.99–1.01); 0.270	1.04 (1.03–1.05); 0.000
	multivariate	1.00 (0.99–1.01); 0.591	1.03 (1.02–1.04); 0.000
Sex	bivariate		
female		0.94 (0.84–1.06); 0.361	0.67 (0.61–0.74); 0.000
male		1*	1*
Sex	multivariate		
female		1.02 (0.89–1.17); 0.700	0.70 (0.63–0.78); 0.000
male		1*	1*
Marital status	bivariate		
living with a partner		1.11 (0.98–1.26); 0.098	0.99 (0.89–1.09); 0.837
no partner		1*	1*
Marital status	multivariate		
living with a partner		1.09 (0.95–1.26); 0.209	1.03 (0.92–1.15); 0.524
no partner		1*	1*
Type of settlement	bivariate		
urban		1*	1*
rural		1.02 (0.90–1.16); 0.678	1.76 (1.60–1.93); 0.000
Type of settlement	multivariate		
urban		1*	1*
rural		1.08 (0.92–1.28); 0.313	1.17 (1.03–1.32); 0.014
Region	bivariate		
Belgrade		1*	1*
Vojvodina		0.80 (0.68–0.95); 0.012	1.38 (1.22-1.57); 0.000
Central and West Serbia		1.09 (0.92–1.28); 0.287	1.45 (1.27–1.64); 0.000
South and East Serbia		1.00 (0.84–1.19); 0.962	1.21 (1.06–1.39); 0.004
Region	multivariate		
Belgrade		1*	1*
Vojvodina		0.87 (0.72–1.05); 0.162	0.95 (0.82–1.09); 0.479
Central and West Serbia		1.17 (0.97–1.41); 0.094	1.01 (0.88–1.18); 0.800
South and East Serbia		1.08 (0.89–1.31); 0.436	0.85 (0.73–0.99); 0.048
Education	bivariate		
low		0.96 (0.78–1.18); 0.710	3.87 (3.32–4.50); 0.000
middle		1.01 (0.88–1.17); 0.798	2.17 (1.93–2.43); 0.000
high		1*	1*
Education	multivariate		
low		0.96 (0.74–1.23); 0.757	2.55 (2.12–3.07); 0.000
middle		0.99 (0.85–1.16); 0.978	1.76 (1.54–2.00); 0.000
high		1*	1*

Employment	bivariate		
employed		1*	1*
unemployed		0.94 (0.81–1.09); 0.430	1.50 (1.33–1.68); 0.000
inactive		0.97 (0.83–1.13); 0.704	1.13 (1.01–1.28); 0.032
Employment	multivariate		
employed		1*	1*
unemployed		0.97 (0.82–1.14); 0.743	1.20 (1.06–1.37); 0.004
inactive		1.06 (0.89–1.26); 0.505	1.08 (0.94–1.24); 0.246
Wealth Index	bivariate		
poorest class		0.89 (0.72–1.10); 0.303	3.14 (2.68–3.68); 0.000
poorer class		0.90 (0.75–1.08); 0.265	1.93 (1.67–2.22); 0.000
middle class		1.05 (0.88–1.25); 0.560	1.63 (1.42–1.88); 0.000
richer class		0.94 (0.80–1.11); 0.469	1.20 (1.05–1.37); 0.007
richest class		1*	1*
Wealth Index	multivariate		
poorest class		0.89 (0.67–1.20); 0.467	1.71 (1.38–2.12); 0.000
poorer class		0.91 (0.72–1.14); 0.419	1.30 (1.08–1.54); 0.004
middle class		1.00 (0.82–1.23); 0.939	1.16 (0.99–1.37); 0.063
richer class		0.92 (0.76–1.11); 0.405	1.04 (0.90–1.21); 0.555
richest class		1*	1*
Smoking status	bivariate		
never smoked		1*	1*
former smoker		0.81 (0.68–0.96); 0.016	0.81 (0.71–0.92); 0.001
smoker		0.84 (0.73–0.96); 0.013	1.18 (1.06–1.32); 0.001
Smoking status	multivariate		
never smoked		1*	1*
former smoker		0.82 (0.69–0.97); 0.027	0.84 (0.73–0.96); 0.015
smoker		0.87 (0.75–1.00); 0.063	1.13 (1.01–1.26); 0.033

#**Adjusted on age; *Reference category.**

OR – odds ratio; CI – confidence interval.

Discussion

This study was based on the analysis of the data of the third consecutive national health survey (2000, 2006, 2013) and we identified several factors associated with the last visit to a dentist in a period longer than 12 months. The highest percentage of the respondents in this survey (67.9%) reported visiting a dentist 12 months ago or longer. Only one in three (32.1%) respondents visited dentist less than one year ago, which is slightly higher than in the 2006 survey (30.7%). However, the 2000 survey level (when the distribution of the visits to a dentist in the past 12 months was 35.1%), was not reached [15]. According to these data, Serbia is far from northern European Union countries: the Netherlands (83%), Germany and Luxembourg (77%), Sweden (71%), but close to Romania (34%) and Hungary (35%) [16].

We also observed that the higher the age of the respondents is, the higher the chance is that they will visit a dentist less frequently, which is confirmed by other studies [17]. However, in Sweden [18] the situation is reversed, and the reason for such contradictory results can be explained by the increasing presence of natural teeth in people over 60 years of age, as well as a developed awareness of the importance of oral hygiene habits among the respondents.

No association between marital status and visits to the dentist was found in our study, while the results of Lee et al. [19] showed that in adults aged 65 or older being married was associated with higher odds of dental care utilization.

Women were less likely to visit a dentist in the period longer than 12 months compared to men (OR = 0.70). According to the literature, women more regularly visit a dentist than men [20], which also confirms the survey results by developed countries, such as Germany and the United States [21, 22]. In a cross-sectional study in Turkey [23], women had more visits to a dentist compared to men during a previous 12-month period. Such results can be explained by the fact that women, due to a higher level of awareness of health problems [23], their role in society, as well as hormonal differences [24], are more likely to regularly visit a dentist than men.

It has long been known that the majority of the rural population belong to a lower economic status and education level. They also have lower access since dental services often tend to be located in wealthy urban neighborhoods [25, 26]. In regard to the place of residence in our research, we noted that respondents who live in a rural area tend to visit a dentist once in a 12-month-period or longer more frequently in comparison to the city residents. This trend has been confirmed by a study conducted in the United States on the population aged 18 years and older, where rural residency was shown as an independent factor associated with lower dental care utilization [22]. The research showed that the population of a rural area is most likely to visit a dentist only when they experience acute pain [27, 28].

Our findings revealed that the quintiles of the welfare, education level and employment status are significant predictors of rare dental visits. Education is a measure of intellectual level, and also an important and stable predictor of soci-

oeconomic status for most adults, unlike the employment status and income, which are strongly influenced by economic fluctuations [29, 30]. In our study, as well as in National Health Survey in Serbia 2006 [15], the highest percentage of the respondents have completed a secondary school, while there is the smallest percentage of those with higher education. Our study showed that the respondents with secondary education were twice as likely to visit dentists in the period of 12 months or longer, when compared to the highly educated individuals, and odds increase among the respondents with a lower level of education. Similarly to the results of our study, the study of behavioral risk factors among the adult US population from 1995 to 2008 showed that those with a higher education level reported a significantly higher number of dental visits in the last 12 months compared to the population with a secondary and lower education level [31]. The same situation is in Europe, as demonstrated by a cross-sectional study conducted in 11 European countries on the adult population aged 50 years and over [32]. A study that included the residents of the 24 European countries has defined the level of education as the most important factor of dental health care services use in terms of sex, age, marital status, and working status, as well as the number of available dentists [33].

The unemployed in Serbia are less likely to use health care services [34]. When it comes to the dental health service, both state and private, the unemployed in Serbia were 1.20 times more likely than the employed to visit a dentist in the period of 12 months or longer. In line with our results, the US adults who reported being unemployed in contrast to the employed had greater odds (OR = 1.174) of not having a dental visit in the last 12 months [22]. Following the economic crisis affecting Iceland in 2008, the unemployed women were nearly twice as likely to visit a dentist in the period of 12 months or longer than before the crisis began [35].

One of the most common causes of irregular visits to a dentist is the cost of dental services, that is, the financial constraints resulting from a bad financial situation of a household [36]. The expenses relating to dental services vary between countries and depend on the legislative regulation of dental health care [18, 37]. The estimates of spending money on dental health care in low and middle income countries showed that dental health care can be a considerable burden on households, to the extent of preventing the expenditure on basic necessities [38]. Since the onset of the economic crisis in 2008, the standard of living in Serbia has been gradually decreasing, and consequently, the poverty rate increased from 6.1% in 2008 to 8.9% in 2014 [39]. In our study, the respondents who, according to the welfare quintile, belong to the poor economic class were 1.30 to 1.71 times more likely to visit a dentist in the period of 12 months or longer in relation to the richest. The study based on the data from 13 European countries [40] and the data from the United States [41] confirms the positive correlation of the household material condition regarding quintile of welfare and personal earnings with a

dental visit. The results of the study in China indicate that the poor are not only less likely to seek dental care, but they make less frequent dental visits than the rich [42]. According to the results of the research by Wamala et al. [43], financial limitations dominated as the main reason for refraining from seeking a dental treatment.

Based on our results, smoking is a significant predictor of dental visits. Former smokers had lower odds of visiting a dentist in the period longer than 12 months. From this attitude ex-smokers have towards dental visits, it can be concluded that quitting this form of risky behavior is connected with an increased awareness of the importance of oral health as a component of the overall health. On the other hand, smokers were more likely to have a dental visit in the period longer than 12 months compared to non-smokers. Our finding was consistent with the results of other authors reporting that current smokers were more likely to delay routine dental visits [44] and less likely to report visiting a dentist within the past year than non-smokers [45].

Our study had several limitations. First, a visit to a dentist was self-reported with possible recall bias. Second, we were unable to examine other factors associated with a dental visit, such as dental insurance, number of dental caries, periodontal diseases or community water fluoridation. Third, our study was cross-sectional. Therefore, we cannot infer causality. Cross-sectional studies are not relational, and cannot determine causal relationships between different variables. It should be noted that, in addition to the demographic, socioeconomic factors and smoking, there are other factors that are known or suspected to affect a dental visit that could be subject to examination in a future research. These are psychological factors (fear, psychosocial issues) and factors related to the community (relations between people, social support).

Conclusion

Nevertheless, this study demonstrated that sociodemographic and lifestyle factors are also important factors related to the visit to a dentist. The results of the socioeconomic status in relation to the visit to a dentist suggest the existence of inequalities. It is necessary to implement policies and programs aimed at improving accessibility of dental health care, particularly among the socially disadvantaged adults in Serbia.

Acknowledgement

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CD11C immunopositive cells in the human fetal vermiform appendix

CD11C-imunopozitivne ćelije u crvuljku fetusa čoveka

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Abstract

Introduction/Aim. A vermiform appendix is an abdominal organ which contains the elements of gut-associated lymphoid tissue and carries out important immunological functions as a reservoir of intestinal microbial flora. It also has a role in the normal development of gut-associated lymphatic tissue. The aim of this study was to examine the distribution of dendritic cell marker CD11c in the human fetal vermiform appendix from the 13th to the 23rd week of development. **Methods.** The material in this study consisted of 28 human fetal vermiform appendixes from the 13th to the 23rd week of gestagion. The tissue samples were routinely processed to obtain paraffin blocks, and 5 μm thick tissue sections were stained with hematoxylin and eosin, and with rabbit monoclonal antibody against CD11c antigen and mouse monoclonal antibody against desmin. **Results.** The first CD11c immunopositive cells appear in the 14th week of development. They are present in the mucosa/submucosa and are interconnected via their cytoplasmic processes. Around these cells, a small number of lymphocytes can be seen. The first lymphoid aggregations appear in the 16th week of development, and lymphocytes are organized around the network made of CD11c immunopositive cells. From the 18th week of development, the lymphoid aggregations are organized in the form of primary lymphoid follicles, containing an extensive network made of CD11c immunopositive cells. **Conclusion.** CD11c immunopositive cells appear first in the process of primary lymphoid follicle generation and have a role in forming a lattice which will serve as the basis for lymphocyte migration.

Key words:
fetus; appendix; dendritic cells; leukocytes; biomarkers; antigens; cd11.

Apstrakt

Uvod/Cilj. Crvuljak je abdominalni organ koji sadrži limfno tkivo u submukozi i ima značajnu imunološku ulogu kao rezervoar intestinalne mikrobijalne flore. Takođe, ima ulogu u normalnom razviću limfnog tkiva pridruženog mukozi gastrointestinalnog trakta. Cilj rada je bio da se ispita distribucija dendritskog ćelijskog markera CD11c u humanom fetalnom crvuljku od 13. do 23. nedelje razvića. **Metode.** Materijal istraživanja su činila 28 humana fetalna crvuljka od 13. do 28. nedelje gestacijske starosti. Tkivni uzorci su rutinski obrađeni do parafinizovanih kalupa, sa kojih su pravljeni preseci debljine 5 μm koji su zatim bojeni hematoksilinom i eozinom, kao i zečijim monoklonskim antitelom na CD11c i mišjim monoklonskim antitelom na dezmin. **Rezultati.** Prve CD11c-imunopozitivne ćelije se pojavljuju u 14. nedelji razvića. Smeštene su u mukozi/submukozi i međusobno su povezane citoplazmatskim produžecima. Oko ovih ćelija se uočava mali broj limfocita. Prvi agregati limfnog tkiva se uočavaju u 16. nedelji razvića, a limfociti su organizovani oko mreže koju prave CD11c-imunopozitivne ćelije. Od 18. nedelje razvića limfni agregati se organizuju u vidu primarnih limfnih folikula u kojima je prisutna ekstenzivna mreža sačinjena od CD11c-imunopozitivnih ćelija. **Zaključak.** CD11c-imunopozitivne ćelije se pojavljuju prve u procesu formiranja primarnog limfnog folikula gde imaju ulogu u formiranju mreže koja će služiti kao osnova za migraciju limfocita.

Ključne reči:
fetus; apendiks; ćelije, dendritične; leukociti; biološki pokazatelji; antigeni; cd11.

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Introduction

The vermiform appendix is an abdominal organ, classified as a part of the large intestine, which was considered for a long time to be a vestigial remnant of the caecum, without any specific function in humans, especially having in mind that it is not involved in the processes of digestion and intestinal peristalsis[1]. However, some more recent studies have shed a different light on the function of this organ, suggesting the importance of its immunological roles, especially regarding its being a haven for microbial flora of the intestines, releasing it in cases when its repopulation is required [1]. This is especially important in cases when the normal intestinal flora has been destroyed after some viral or bacterial infections. Furthermore, it is believed that the presence of bacterial flora in the vermiform appendix is important for tolerance induction against the indigenous flora, as well as for the stimulation and normal development of gut-associated lymphoid tissue [2].

The vermiform appendix is the intestinal derivative of the midgut. The development of the vermiform appendix is closely related to the midgut development, which can be summarized to take place in three phases [3]. In phase one, which occurs during the sixth week of development, the midgut elongates considerably, which results in the formation of a hairpin-shaped loop. Due to insufficient space in the embryo, this loop extends into the extraembryonic coelom of the umbilical cord forming the physiological umbilical hernia [3]. During this phase, the intestinal loop rotates 90 degrees around the superior mesenteric artery. The phase two occurs during the tenth week of development and is characterized by back-positioning of the intestinal loop inside the embryonic cavity, closure of the physiological umbilical hernia and by additional rotation of the intestinal convolute for 180 degrees [3, 4]. During the third phase that occurs in the twelfth week of gestation, the midgut is fixated in the peritoneal cavity. The bud-like complex of the early caecum and appendix appears during the phase two of midgut development on the right-hand side of the upper abdominal cavity [3–5]. The process of colon elongation consequently leads to the descent of caecum and appendix, as they become finally positioned in the right iliac fossa [3, 4]. The *appendix vermiformis* can be observed at the eighth week of gestation, while the first accumulations of the lymphatic tissue develop during the weeks 14 and 15, at first as a few lymphatic cells located below the epithelium [3, 5]. Lymph nodules appear approximately during the 4th and 5th months of development and they continue to increase, with a peak in the 28th week of development. The components of gut-associated lymphoid tissue (GALT) continue to grow up to puberty [3, 5, 6]. The colonization of vermiform appendix with bacterial flora begins approximately two weeks after birth [2].

The lymphoid tissue of vermiform appendix consists of multiple solitary lymphoid follicles, whose cellular content comprises B lymphocytes and a small number of T lymphocytes, dendritic cells and macrophages [7].

Some authors suggest that the nerves of the enteric nervous system (ENS) might play a role in the regulation of immunological functions of the gut-associated lymphoid tissue, including the vermiform appendix [8]. The immune system and ENS interact with each other and some studies have revealed that nerves can act as inflammation modulators in the intestinal tissue [9]. Moreover, it has been reported that higher levels of neuropeptides in the vermiform appendix might provoke acute abdominal pain without any signs of acute appendicitis [10].

Bearing in mind the importance of lymphoid tissue in the vermiform appendix, the aim of this paper was to examine the expression of CD11c antigen in its tissue during the second trimester of fetal development. CD11c is one of the classical markers of dendritic cells, but is also expressed on the neutrophils, macrophages and some B lymphocytes [11, 12].

Methods

The material consisted of 28 vermiform appendixes of the fetuses from the 13th to the 23rd week of gestational age (Table 1). The material was obtained from the Clinic of Pathology, Clinical Centre Niš, Serbia, after spontaneous miscarriages or abortions performed for medical reasons. None of the fetuses included in this study had any congenital malformations or gastrointestinal disorders. Gestational ages were estimated by the anatomical criteria according to the Carnegie Staging system and the parameters of crown-rump length, head circumference and foot length. The study was approved by the Ethics Committee of the University of Niš, Faculty of Medicine, and was performed according to the guidelines of the Declaration of Helsinki.

Table 1

The gestational age and number of vermiform appendix samples used as a material

Gestational age (week)	Number of samples
13	2
14	3
15	3
16	2
17	3
18	3
19	3
20	2
21	2
22	3
23	2

All vermiform appendix samples were fixed in 10% buffered formalin and routinely processed to paraffin blocks. From each paraffin block, 5 µm-thick sections were obtained using a Leica microtome. The obtained paraffin sections were deparaffinized (in the thermostat at 64°C and xylene) and rehydrated in a series of descending concentrations of alcohol (100%, 96%, and 75%) and distilled water. The sections were stained with hematoxylin and eosin (HE), and immunohistochemically by using the rabbit monoclonal antibody against CD11c antigen (Abcam, ab52632, 1 : 100) and the mouse monoclonal antibody against desmin (Dako, M0760). The incubation with the antibody was performed overnight at 4°C. As a visualization system, EnVisionFLEX, HighpH (Agilent, K8000/8002) was used. The photo documentation, used for microscopic analysis, was obtained using an Olympus BX50 light microscope equipped with a Leica DFC295 digital camera (Leica Microsystems, Germany).

Results

The analysis of HE sections of vermiform appendix samples showed that their histological structure corresponded well to the week of gestational age. In the 13th week of development, all the layers of the wall (mucosa, submucosa, muscularis and serosa) were present on all the examined samples. All vermiform appendixes had the intestinal glands with crypts present in their mucosa; however, the *lamina muscularis mucosae* was absent. The *lamina muscularis mucosae* appeared first in the 18th week of development in the form of scattered smooth muscle cells between the mucosa and submucosa. The muscular layer was composed of well-developed circular and thin longitudinal sublayers (Figure 1A). Until the 23rd week of development, the lamina muscularis mucosae was well developed and the longitudinal muscle sublayer became thicker (Figure 1F).

Lymphatic tissue or CD11c immunopositive cells were
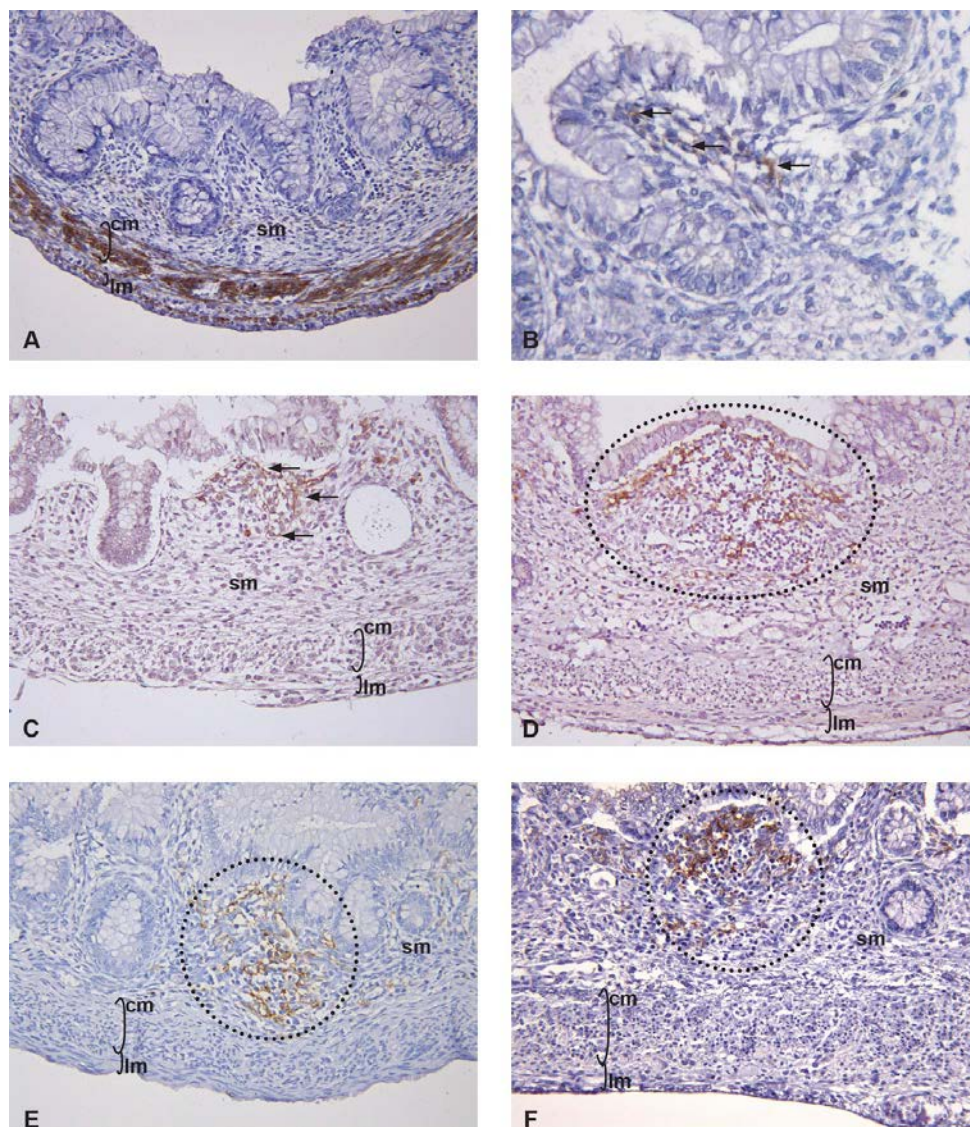
Fig. 1 – A) Appendix vermiformis in the 16th week of development. Circular and longitudinal muscular sublayers are stained with antibody against desmin (sm-submucosa, cm-circular muscle sublayer, lm – longitudinal muscle sublayer, x250); B) Mucosa/submucosa of the appendix vermiformis in the 14th week of development. Arrows point to the few, scarce CD11c immunopositive cells (immunohistochemical staining with CD11c antibody, x500); C) Appendix vermiformis in the 16th week of development. Arrows point to the CD11c immunopositive cells (sm-submucosa, cm-circular muscle sublayer, lm–longitudinal muscle sublayer. immunohistochemical staining with CD11c antibody, x250); D) Appendix vermiformis in the 18th week of development. Lymphoid tissue aggregation is encircled. Within the lymphoid aggregation CD11c immunopositive cells form a network (sm-submucosa, cm-circular muscle sublayer, lm – longitudinal muscle sublayer; immunohistochemical staining with CD11c antibody, x250); E) Appendix vermiformis in the 20th week of development. Primary lymphoid follicle containing an extensive network of CD11c immunopositive cells is encircled (sm-submucosa, cm-circular muscle sublayer, lm – longitudinal muscle sublayer; immunohistochemical staining with CD11c antibody, x250); F) Appendix vermiformis in the 23rd week of development. Primary lymphoid follicle is encircled (sm-submucosa, cm-circular muscle sublayer, lm – longitudinal muscle sublayer. immunohistochemical staining with CD11c antibody, x250).

absent in the samples in the 13th week of development. Rare CD11c immunopositive cells were first observed in the vermiform appendixes in the 14th week of development (Figure 1B). These cells were seen in small groups with focal distribution in the mucosa/submucosa layer. Their morphology revealed the presence of cytoplasmic processes with whom they were interconnected. CD11c immunopositive cells were surrounded by rare, scarce lymphocytes that were not seen in the areas between the groups of CD11c immunopositive cells. The first lymphocyte aggregations, resembling the primary lymphoid follicles, were observed in the samples in the 16th week of development (Figure 1C). The lymphocyte aggregations were organized around the network made by CD11c immunopositive cells. The number of CD11c immunopositive cells was markedly higher and the network they form was more extensive. As the development continued, the number of CD11c immunopositive cells and lymphocytes gradually increased (Figure 1D). Until the 20th week, the primary lymphoid follicles were completely formed (Figures 1E, F). CD11c immunopositive cells were present inside the lymphoid follicle, where they formed a network around which the lymphocytes were situated.

Discussion

The morphology of the vermiform appendix from the 13th to the 23rd week of development showed that all the layers of its wall were present, as well as the intestinal glands and two sublayers in its muscular layer. The *lamina muscularis mucosae* was observed only in the samples from the 18th week of development. The longitudinal muscle sublayer was very thin in the 13th week of development, but it became thicker and both muscle sublayers were clearly visible until the 23rd week. These findings were in accordance with the results of other authors who studied the development of this organ [13, 14]. However, in the literature there has been different information concerning the appearance of lymphatic tissue in its mucosal/submucosal layer [15–17]. Our findings suggest that the first lymphocytes can be observed already in the 14th week of development, and that lymphoid aggregations are clearly visible in the 16th week. CD11c immunopositive cells were first observed in the 14th week of development. According to their morphology, these cells corresponded to the dendritic cells in the gut-associated lymphoid tissue. Dendritic cells are a normal constituent of the lymphoid follicles and have a crucial role in facilitating the immunological function of gut-associated lymphoid tissue. They are interconnected *via* their cytoplasmic processes and are present in the form of a lattice in the locations where the future lymphoid follicles will develop. The available data show that CD11c immunopositive dendritic cells are largely present in the gut-associated lymphoid tissue [18]. However, due to a complex molecular profile of these cells, they have still not been fully characterized. Another problem with the examination of dendritic cells lies in the fact that they are comprised of more cellular subtypes which differ in their expression of their molecular markers. Summers et al. [19] showed that three out of five DC subtypes in the palatine tonsil, found both in diffuse lymphatic tissue and germinal centers, expressed CD11c. Experimental models suggest that the formation of primary lymphoid follicles is a multistep process that involves both B lymphocytes and dendritic cells [20–22]. The first step in this process appears to depend on the secretion of $LT\alpha_1\beta_2$ by B lymphocytes, which is responsible for the formation of a network of dendritic cells [22, 23]. When the network of dendritic cells is formed, the migration of B lymphocytes is facilitated and the quantity of lymphatic tissue increases, thus forming a morphologically distinctive primary lymphoid follicle [22].

Conclusion

Our results give the morphological evidence that a network of CD11c immunopositive cells of the future primary lymphoid follicle arises before the appearance of lymphoid aggregates in the vermiform appendix, suggesting thus that these cells are crucial in the process of lymphoid follicle generation.

Acknowledgement

Conflict of interest

The authors declare that they have no conflict of interests.

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Thymectomy immediately after myasthenic crisis

Timektomija neposredno po završetku miastenične krize

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Abstract

Introduction. Myasthenic crisis (MC) refers to rapid progression of myasthenic weakness accompanied by ventilatory and bulbar dysfunction. Since there is no single standard accepted in the treatment of every patient in myasthenic crisis, we report our experience in the treatment of such a patient. **Case report.** We report a 22-year-old male patient with clinical, pharmacological, neurophysiological, and immunological diagnosis of seropositive generalized myastenia gravis (MG) of unstable course. During the first 6 months of his disease, three deteriorations were registered, and the last one developed into a myasthenic crisis. The patient was intubated. Previous anticholinergic and imunosuppresive therapies were withdrawn, and he was treated with plasma exchange and human immunoglobulins during the crisis. After thymectomy, performed 3 weeks after extubation, the patient was stable. **Conclusion.** Progress in treatment of MC over the last few decades has dramatically improved its prognosis. Thymectomy is useful in the prevention of MC recurrence.

Key words:
**myasthenia gravis; diagnosis; thymectomy; critical
illness; treatment outcome.**

Apstrakt

Uvod. Miastenična kriza dovodi do brze progresije miastenične slabosti koja je udružena sa respiratornom i bulbarnom disfunkcijom. Kako nije strogo prihvaćen nijedan standard u lečenju svih bolesnika u krizi, u radu smo prikazali naša iskustva u lečenju jednog takvog bolesnika. **Prikaz bolesnika.** U radu je prikazan bolesnik, star 22 godine, sa klinički, farmakološki i imunološki dijagnostikovanom seropozitivnom generalizovanom miastenijom gravis nestabilnog toka. Tokom prvih 6 meseci bolesti registrovana su tri pogoršanja, dok se poslednje razvilo u miasteničnu krizu. Bolesnik je intubiran, ranija antiholinesterazna i imunosupresivna terapija su korigovane, a on je, tokom krize, lečen izmenama plazme i humanim imunoglobulinima. Posle timektomije, izvršene 3 sedmice posle ekstubacije, bolesnik je bio stabilan. **Zaključak.** Napredak u lečenju miastenične krize tokom poslednjih decenija dramatično je poboljšao prognozu miastenične krize. Timektomija je korisna u prevenciji ponavljanih miasteničnih kriza.

Ključne reči:
**miastenia gravis; dijagnoza; timektomija; kritična
stanja; lečenje, ishod.**

Introduction

Myasthenic crisis (MC) is one of the most urgent conditions in neurology. The definition of MC is best formulated by a group of experts for myasthenia gravis (MG) who defined it as a condition in which the patient is vitally threatened by rapid progression of the disease, ventilatory and bulbar dysfunction [1]. Therefore, respiratory insufficiency accompanied by exacerbation of the disease is a sufficient criterion for the diagnosis of MC in patients with MG.

Myasthenic crisis is the state of delayed postoperative extubation for more than 24 hours after the operation because of respiratory failure in patients with MG [2]. Since there is no single standard accepted in the treatment of every MG patient because of heterogenity of the disorder.

According to individual approach to each patient with MG, we report our experience with a MG patient in crisis.

Case report

The patient, a 22-year-old male, experienced the first manifestation of the disease early in 2016. It was a transient episode of double vision, and the disease was not identified at that time. The patient had syndromic diagnosis, and was treated with parenteral corticosteroids for three days, with complete recovery from neurological deficit.

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A year and a half later, the ambiguities followed by semiptosis, speech difficulties, and weakness in the proximal arm muscles, manifested unprovoked.

At that time, a clinical, pharmacological, neurophysiological and immunological diagnosis of seropositive (acetylcholine receptor antibody 5.8 nmol/L) and generalized MG was established.

After initiation of corticosteroid therapy, the patient experienced a deepening of myasthenic weakness, primarily in the form of oculo-bulbar dysfunction. Because of that, we performed a series of five plasma exchange (PE) therapies instead. Thereupon, the patient achieved a complete but short remission. The planned thymectomy was not performed. Over the next two and a half months, there was a development of more pronounced weakness in eyelids, a transient episode of dysphagia and difficulty chewing, as well as neck muscles weakness.

An increase in oral dosage of corticosteroids and pyridostigmine bromde (Mestinon®) resulted in some improvement, but without complete recovery. However, after the next series of five additional PE, the neurological deficit withdrew again for a short time. Two months later, in February of 2018, there was again an increase in generalized weakness, but this time with occasional suffocation, mostly at night.

The patient was hospitalized in February of 2018, when two PEs were performed, with a significant but incomplete recovery. PE was suspended because the patient became febrile (38.8°C). An antibiotic and antipyretic therapy was administered, and haemoculture was taken. After initial improvement, existing weakness of cervical musculature increased. The patient also experienced difficulty in swallowing and speaking, weakness of eye muscles, and episodes of shortness of breath, together with increased secretion in the nose and the mouth, and a decrease in saturation (PCO_2 41 mmHg/51 mmHg PO_2), because the patient was intubated.

It was concluded that it was a MC and an anticholinergic therapy was suspended, but a parenteral corticosteroid therapy and intravenous human immunoglobulin (IVIg) therapy at a dose of 0.4 g/kg body weight was started and continued for 5 consecutive days.

The patient was intubated for in the next 10 days when was regularly monitored for vital parameters. Laboratory blood and urine tests were performed. After stabilization of his general condition and normalization of muscle strength, the patient was extubated.

The patient had a transient episode of strabismus and nasal speech lasting two days, a week after he was extubated, which spontaneously resolved. Thymectomy was performed three weeks after extubation via video-assisted thoracoscopic surgery (VATS), with previous antibiotic preparation and a one-day administration of IVIg.

The surgery was completed without complications. The patient was able to breathe spontaneously immediately after the surgery. His vital parameters were stable and his neurologic status was almost normal, with no significant muscle weakness and persistent fatigue.

Histopathological examination pointed to hyperplasia of the thymus. In the next 6 months, the patient experienced no clinical deterioration.

Discussion

We present a patient with an unstable preoperative course of MG. Initiation of corticosteroid therapy precipitated myasthenia-related increase in weakness and it was the reason for PE therapy, with temporary effects. Infectious syndrome appeared third: the most difficult deterioration of the disease, accompanied by respiratory failure in MC. According to the literature [3], about one-fifth of patients with MG experience crisis during their life, usually within the first year of illness. The interval from disease onset to first MC was in the range 0.5–60 months (median interval 6 months), and most of them (60.6%) experienced recurrent (≥ 2) episodes. Most of these patients were acetylcholine receptor antibody-positive (72.7%). Similar to the above, our patient had seropositive generalized MG, and he developed MC within one year since the onset of clear symptoms of the disorder.

Among the precipitating factors for a MC, cited in the literature (infections and sepsis, surgical procedures, initiation of treatment with corticosteroids or rapid tapering of it, exposure to drugs that may increase myasthenic weakness, pregnancy, reaction to iodinated contrast), infection is the most common [2, 4]. In our patient, initiation of corticosteroid therapy was accompanied by increasing weaknesses, and infectious episodes precipitated MC in his case.

The authors do not agree on cholinesterase inhibitors and MC; according to some, they are recommended in the crisis [5], while most are of the opinion that they should be discontinued [6, 7]. Cholinesterase inhibitors were discontinued in our patient during artificial ventilation, and we believe that in this way it is easier to repair acetylcholine receptors of skeletal muscles, and to provide a better response to the cholinesterase inhibitors upon their re-introduction.

All authors agree that MG patients need PE, IVIg, corticosteroids, immunosuppressants (especially azathioprine), and lately, a monoclonal antibody therapy with rituximab as an additional treatment for MC has also been suggested [8]. Plasma exchange during an MC was significantly associated with early extubation [3] as PE can rapidly eliminate the pathological autoantibodies. We used PE as a superior immunomodulatory therapy in the case of our patient, but it had to be replaced with IVIg because of his fever.

Although guidelines for extubation in patients without MG are clear (vital capacity ≥ 15 mL/kg, maximal inspiratory pressure ≤ 20 cm H_2O, expiratory pressure > 40 cm H_2O, and tidal volume ≥ 5 mL/kg) [9] there is a lack of strict criteria concerning when and how to safely extubate patients in MC because of their fluctuating weakness. The decision to extubate MG patients relies mostly on the clinical judgment of the neurologist. Our patient was extubated at the moment when muscular power tests for all of his skeletal muscles that could be assessed yielded good results. The decision proved right because clinical remission was maintained after extubation.

Duration of ventilation during a MC is an important indicator of treatment efficacy: early extubation is defined as ventilation support for < 7 days; prolonged ventilation is defined as the requirement for mechanical ventilation for > 15 days [10]. A third of patients with MC achieved early extubation (≤ 7 days), and only a quarter of patients needed prolonged ventilation (> 15 days). Younger patients with PE tended to undergo successful early extubation, while older male patients with atelectasis tended to have poor outcomes [3].

As for recurrence of MC, because the patients who underwent thymectomy had significantly fewer MC episodes and a longer duration between MC attacks [3], we decided to perform thymectomy in our patient immediately after stabilization of his condition, 3 weeks after extubation. This decision turned out to be correct because the patient was stable after thymectomy. That our decision was not premature is confirmed by the experience of other authors, who performed thymectomy on their patient during MC, when the patient was still on artificial respiration [11].

That myasthenia crisis is not exclusively associated with thymoma is proven by the fact that thymoma is seen only in a quarter of patients in crisis [3]. The histopathological findings in our patient pointed to thymic hyperplasia.

Progress in the recognition and treatment of MC over the past few decades has dramatically improved the prognosis of MC, and decreased the mortality rate from 75% to the current rate of less than 5% [2, 9].

Conclusion

Patients in MC require accommodation in the intensive care unit, intubation, recognition and treatment of triggers, and correction of previous therapy. Since thymectomy is useful in prevention of MG recurrence, it was performed immediately after extubation in our patient. After thymectomy the patient was stable.

There is a need for individual approach to each patient with MC.

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Foudroyant middle-ear pneumococcal inflammation with meningoencephalitis in a six-year-old girl

Fudroajantno pneumokokno zapaljenje srednjeg uva sa meningoencefalitisom kod šestogodišnje devojčice

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Abstract

Introduction. Intracranial complications of *otitis media* still occur, despite great progress in the treatment of all forms of inflammation. These are serious conditions which are still life-threatening and require a fast and accurate diagnosis and an adequate treatment. We present an illustrative case of an acute, foudroyant, pneumococcal, suppurative *otitis media* with infection spreading into endocranium and development of meningoencephalitis and otic hydrocephalus. **Case report.** A 6-year-old girl was admitted to the University Children's Clinic in Belgrade, Serbia, because of fever, headache, vomiting and disorder of consciousness. Computed tomography scan of the endocranium and temporal bone revealed brain edema and hypodense content in the left mastoid and tympanic cavity. The diagnosis of acute *otitis media* with meningoencephalitis was made and we started with intensive antibiotic treatment. Lumbar puncture and hemoculture confirmed a pneumococcal infection. Otosurgical treatment was conducted,

too, due to an inadequate reaction to the conservative treatment. Firstly, left mastoidectomy with the implantation of a ventilation tube was performed, followed by a radical tympanomastoidectomy, because there was no improvement. Three weeks after the second operation, a magnetic resonance imaging of the endocranium was performed and an otic hydrocephalus was diagnosed. A neurosurgical operation was performed on the same day with the setting of the ventriculoperitoneal shunt. **Conclusion.** Intracranial complications of acute *otitis media* in children are extremely rare and they require a multidisciplinary treatment. Surgical treatment of the ear should not be postponed and the choice of the type of otosurgical intervention should be individually adapted. Audiological and neurological complications of the disease are frequent and they further prolong and impair the treatment.

Key words:
otitis media, suppurative; meningoencephalitis; hydrocephalus; surgery, operative; antibiotics.

Apstrakt

Uvod. Intrakranijalne komplikacije zapaljenja srednjeg uva još uvek se javljaju, uprkos velikom napretku u lečenju svih oblika zapaljenja. Predstavljaju ozbiljna stanja koja i dan danas ugrožavaju život bolesnika i zahtevaju brzu i preciznu dijagnostiku i adekvatno lečenje. Prikazujemo ilustrativan slučaj akutne, pneumokokne, supurativne upale srednjeg uva fudroajantnog toka sa širenjem infekcije u endokranijum i razvojem meningoencefalitisa i hidrocefalusa. **Prikaz bolesnika.** Šestogodišnja devojčica hospitalizovana je na Univerzitetskoj dečjoj klinici u Beogradu, Srbija, zbog visoke febrilnosti, glavobolje, povraćanja i pogoršanja stanja svesti. Kompjuterizovana tomografija endokranijuma i temporalne kosti pokazala je edem mozga i hipodenzni sadržaj u celulama levog mastoida i kavuma timpani. Postavljena je

dijagnoza akutnog zapaljenja srednjeg uva sa meningoencefalitisom i započeta je intenzivna antibiotska terapija i mehanička ventilacija na odeljenju intenzivne nege. Lumbalna punkcija i hemokultura pokazale su pneumokoknu infekciju. Zbog neadekvatnog odgovora na konzervativni tretman i pogoršanja opšteg stanja, devojčica je operisana i načinjena je implantacija aeracione cevčice sa mastoidektomijom levo. Nakon toga došlo je do inicijalnog poboljšanja opšteg stanja, da bi od desetog postoperativnog dana stanje ponovo počelo da se pogoršava. Zbog toga je odlučeno da se načini radikalna timpanomastoidektomija levo koja je i urađena četrnaestog postoperativnog dana. Dvadeset drugog dana nakon druge operacije urađena je magnetna rezonanca endokranijuma i dijagnostikovan je otički hidrocefalus koji je neurohirurški zbrinut istog dana postavljanjem ventrikuloperitonealnog šanta. **Zaključak.** Intrakranijalne komplikaci

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je akutnog zapaljenja srednjeg uva kod dece ekstremno su retke. Lečenje dece sa ovim komplikacijama je multidisciplinarno. Hirurško lečenje uva ne treba odlagati, a izbor tipa otohirurške intervencije treba se individualno prilagoditi. Audiološke i neurološke komplikacije bolesti su česte i do-datno produžavaju i otežavaju lečenje.

Ključne reči:
otitis medija, supurativni;.meningoencefalitis; hidrocefalus; hirurgija, operativna; antibiotici.

Introduction

Acute *otitis media* (AOM) is one of the most common infectious diseases in the world. More than 80% of children experience the AOM at least once before the age of 3, and 40% of children in the world experience six or more recurrences by the age of seven [1]. The most common causes of the AOM are *Streptococcus pneumoniae* and *Haemophilus influenzae* [2].

Despite great progress in the treatment of all forms of infectious *otitis media* (OM), complications still occur. The frequency of complications significantly decreased in the antibiotic era, as well as the need for the surgical treatment. Intracranial complications (ICC), particularly meningitis, intracranial abscesses, lateral sinus thrombosis and hydrocephalus, are still life-threatening conditions and require a fast and accurate diagnosis and an adequate and aggressive treatment. These complications are mainly caused by the chronic OM (about 80% of cases), but they are significantly less frequently caused by the AOM [2, 3]. The incidence of ICC in patients with AOM is under 1%, while the incidence of meningitis, which is the most common ICC, is between 0.002% and 0.3%. In the pre-antibiotic era, ICC incidence was up to 6% [4, 5]. The common symptoms of the ICC are: fever, headache, nausea and vomiting, and a disorder of consciousness may also occur [2, 3]. A particular problem in early diagnosis of the ICC of AOM is the fact that local otoscopic finding is not always typical, especially in small children. A quarter of children with otogenic meningitis experience headache and high temperature as the most common symptoms with an unconvincing otoscopic finding for acute inflammation [6].

Modern radiographic methods – computed tomography (CT) and magnetic resonance imaging (MRI) have made the diagnosis of complications faster and more precise. The sensitivity of CT with contrast in the detection of the ICC is 92.75% [7].

The ICC treatment is complex and involves a combination of an intensive antibiotic therapy and a surgical treatment. The intravenous use of third generation cephalosporins and glycopeptides is recommended for the conservative treatment of otogenic meningitis. Carbapenem involvement can also be considered [8]. Systemic antiedematous and anti-inflammatory corticosteroid therapy is still controversial. Patients receiving corticosteroids have 50% fewer neurological sequelae, but it has been found that corticosteroids do not affect the reduction in the incidence of deafness [9]. Despite all the measures taken in the treatment, the prognosis is sometimes uncertain [10].

A case of an acute, pneumococcal, suppurative OM with foudroyant course of the disease is presented with infection spreading into endocranium and the development of meningoencephalitis and hydrocephalus, as well as bilateral deafness as sequelae of the disease in the six-year-old girl.

Case report

A girl, aged 5 years and 7 months, was admitted to the University Children's Clinic (UCC) in Belgrade, Serbia, because of fever, headache and vomiting that lasted for two days before admission and a disorder of consciousness on the day of admission. The score on the Glasgow Coma Scale (GCS) was 8/15, body temperature (BT) was 39.9°C and the level of C-reactive protein (CRP) was 446 mg/L. Immediately after the admission, lumbar puncture was performed: cerebrospinal fluid was turbid, protein level 170 mg/dL, glucose level 10 mg/dL, white cell count 11,000/mm^3 with 97% of polymorphonuclear cells. The diagnosis of meningitis was made. Initial medical treatment started with the third generation cephalosporin (ceftriaxone 100 mg/kg *iv*/day), glycopeptide antibiotic (vancomycin 60 mg/kg *iv*/day), osmotic diuretic and systemic corticosteroids. CT scan of endocranium and temporal bone was performed in the next few hours and was shown brain edema and hypodense content in the left mastoid and tympanic cavity (Figure 1). On the next day, the state of consciousness deteriorated (GCS 5/15), and mechanical ventilation started. Barbiturates and benzodiazepines were introduced into the therapy due to the appearance of convulsions. Analysis of blood and cerebrospinal fluid showed that *Streptococcus pneumoniae* type 3 sensitive to ceftriaxone and vancomycin was the cause of the infection. An otorhinolaryngologist was consulted on the third day after the admission and the diagnosis of meningoencephalitis with AOM was made. An emergency otosurgical intervention was performed. The implantation of a ventilation tube with simple mastoidectomy was done. During the surgery, a purulent secretion was obtained after paracentesis (the swab was sterile). Intraoperatively, it was found that the *antrum mucosae* was edematous and that there was no pathological finding in the mastoid cells. The state of consciousness and the general state began to improve after the surgery. On the fifth postoperative day, the GCS was 12, body temperature (BT) was 36.5°C, the CRP level was 22 mg/L, the girl was extubated and spontaneous breathing was established. After three days, a control CT scan of endocranium and temporal bone was made and it showed that there was hypodensity in trepanated mastoid cavity, with the empty tympanic cavity and a tube in position. In the following days, the general condition began to deteriorate, the girl became febrile again and an increase in laboratory parameters of inflammation was registered.
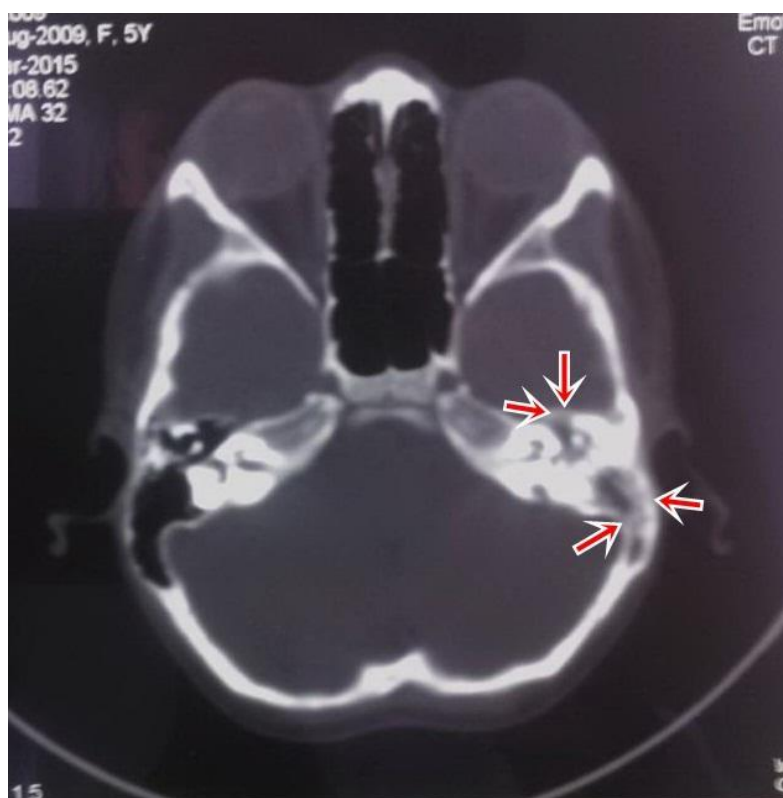
Fig. 1 – Computed tomography (CT) scan of endocranium and temporal bone reveals brain edema and hypodense content in the left mastoid and tympanic cavity.

It was decided to perform a radical otosurgical intervention: a modified radical tympanomastoidectomy, which was performed on the fourteenth postoperative day. During the operation, it was found that the mucosa of the mastoid cavity, perisigmoid and peridural mucosa, as well as mucosa in the mastoid antrum and attic was inflamed and thickened. The antibiotic therapy was changed, in addition to vancomycin (60 mg/kg *iv*/day) and barbiturate, carbapenem (meropenem 120 mg/kg *iv*/day) was administered postoperatively. Clindamycin 40 mg/kg *iv*/day was introduced into therapy too, in order to cover a possible concomitant anaerobic infection of temporal bone. The general condition began to improve again, the laboratory parameters of inflammation returned to the limits of reference values on the tenth day after the second operation, and BT was up to 38°C, which was interpreted as a central disorder of thermoregulation.

On the 22nd day after the second operation, MRI of the endocranium was performed and it showed the enlargement of the chamber system with encephalitic changes in the brain. It was decided to perform a neurosurgical operation on the same day and the external ventricular drain and convert with ventriculoperitoneal shunt was done due to otic hydrocephalus. A pediatric immunologist was consulted and after the immunological analysis, he concluded that it had been an "infection in an immunocompetent child". After three days, a control CT scan (Figure 2) was carried out and it showed a satisfying finding.

Fig. 2 – Computed tomography (CT) scan of endocranium and temporal bone reveals satisfying finding in the left mastoid and tympanic cavity.

The girl was discharged on the 49th day after the admission in the UCC in a good general condition, with aphasia and the inability to walk alone. Otomicroscopic finding of the left ear showed that the ventilation tube was in position, passable and without drainage, and that the mastoid cavity is dry, in the process of epithelialization. Auditory evoked potential test was performed and showed bilateral prolonged interpeak latencies and delayed absolute latencies of waves I, III, V. After the release from hospital, the patient continued with intensive physical rehabilitation with audiological control within a month.

Upon the discharge, the girl was vaccinated with a pneumococcal conjugate vaccine (PCV) 13 and evaluated audiologically. A profound bilateral sensorineural hearing loss with aphasia was identified as a consequence of encephalitis. Before the disease, the girl was a bilingual speaking child. CT scan of the temporal bone was performed nine months after the hospitalization, since the preoperative procedure for cochlear implantation (CI) showed that bilateral cochlear ossification had started. A CI was placed firstly in the right, untreated ear (10 months after the onset of the disease), and six months after that, another CI was placed in the left ear. Intensive hearing and speech rehabilitation started in the native language. Two years after the illness, the girl was completely rehabilitated and she began to attend an elementary school.

Discussion

Intracranial complications of AOM are extremely rare. The infection most commonly spreads from the middle ear directly into the endocranium through the bone weakened by osteomyelitis. Much less frequently, the infection also may be spread in the endocranium along preformed pathways, such as the oval and round windows. Intraoperative finding in the first operation showed that there was no pathological content in the mastoid cells, which indicates possible hematogenic spread of the infection from the middle ear to the endocranium, with the occurrence of a severe form of meningoencephalitis. Literature data also show that meningitis is often caused by hematogenic spread of the infection from the middle ear, and that the mastoid is not inflammated[11]. Some authors showed the possible role of silent OM in the development of meningitis in children. They showed considerable histopathological tissue changes of acute OM and inflammatory cells in the round window membrane and within the perilymph, the modiolus, and the cochlear aqueduct, suggesting the latter as likely portals from the inner ear to the meninges. This would be a silent route of infection from the middle ear to the endocranium[12].

The treatment of ICC should start as soon as the diagnosis is made, with high doses of antibiotics. When it comes to surgical engagement in the treatment, the recommendations are consistent – otosurgical treatment is postponed until the patient is neurologically and systemically stabilized. If there is a deterioration of the patient's clinical condition and the progression of infection despite the use of high doses of antibiotics, it is necessary to perform an ear operation as soon as possible in order to evacuate the primary infectious focus[2]. The state of consciousness and general condition of our patient was deteriorated despite an intensive antibiotic therapy, and therefore it was decided to perform an emergency otosurgical intervention. There was a dilemma about which surgical method is the right choice in this case. Gower and McGuirt[13] have conducted a survey that included 100 patients with ICC that occurred as a result of OM. The most common ICC by far has been meningitis that developed in 76 patients. There were five patients with hydrocephalus. They agree that ICC needs to be treated with paracentesis, but only

if there is no improvement in patient's condition after the use of antibiotics[14]. In contrast, Singh and Maharaj[14] suggest that all ICCs should be treated with cortical mastoidectomy, except for chronic OM with cholesteatoma which should be treated with radical mastoidectomy[14]. Felisati et al.[15] have administered an intensive antibiotic therapy that has been ineffective in three adult patients with otogenic meningitis, making a surgical treatment necessary. They recommend wall up mastoidectomy for patients with otogenic meningitis. Samuel and Fernandes[16] suggest an urgent otosurgical treatment, cortical mastoidectomy and myringotomy (with the implantation of ventilation tubes if necessary). In our case, the implantation of the ventilation tube and simple mastoidectomy led to a short-term initial improvement of the state of consciousness and general condition of the girl. A few days following the surgery, the condition began to deteriorate again, and there was a dilemma about whether to change antibiotics or, in addition, to make a more radical surgical intervention. In a retrospective eighteen-year study, Barry et al.[17] had 79 patients with otogenic ICC. Thirteen patients had meningitis, and AOM was diagnosed in 32 patients. They believe that the pre-antibiotic practice of performing mastoidectomy in the first 24 hours is inappropriate, although some authors still recommend this. They agree with Gower and McGuirt[13] that the urgent otosurgical intervention is indicated in patients with coalescent mastoiditis, as well as in patients who have a deterioration of clinical condition and the progression of infection during the first 48 hours of intensive antibiotic therapy. They suggest that paracentesis should be done first and if there is no improvement in the patient's condition, a mastoidectomy is indicated[17], which is also recommended by Slovik et al.[18]. Our patient has experienced an improvement in the condition after a more radical otosurgical intervention. Consequently, it raises the question of whether it has been necessary to perform an instant radical surgery intervention. Dudvarski et al.[10] had similar experience. They described a case of meningoencephalitis as a complication of AOM in an 11-year-old child, which is the first paper in Serbian literature that describes the case of AOM with ICC. The authors state that they first performed a mastoidectomy with the implantation of the ventilation tube, followed by a radical tympanomastoidectomy because there was no clinical improvement[19].

The leading cause of postnatal deafness in children is bacterial meningitis. Infection spreads from meninges to the cochlea, which leads to its fibrosis and ossification in up to 34% of children with bacterial meningitis. CI is challenging due to frequent cochlear ossification[19]. Cochlear ossification presents a surgical problem. There are solutions that can lead to a satisfactory outcome nowadays, but it is still desirable to perform CI at the earliest stage, before the cochlear ossification starts, in order to optimize the outcome of the treatment[20].

The AOM clinical course in this immunocompetent girl was fulminant although the infection had been caused by a low-invasive *Streptococcus pneumoniae* type 3. There are more than 90 immunologically distinct serotypes of *Streptococcus pneumoniae*. According to their invasive potential, they have been classified into serotypes with a high (sero-

types 1,5 and 7), intermediate (serotypes 4, 9, 14 and 18) and low invasive potential (serotypes 3, 6, 8, 15, 19, 23 and 33). Ahl et al.[21] have established that the septic shock is more frequently caused by *pneumococcus* type 3 rather than by *pneumococcus* type 14 that has an intermediate invasive potential. The results of their research have shown that serotypes with high invasive potential mostly cause the diseases in younger patients, unlike serotypes with a medium and low invasive potential that most frequently cause the diseases in the elderly patients with comorbidities[22], which was not the case with the girl. It is possible that the foudroyant course of AOM was caused by the fact that the girl had not been vaccinated with pneumococcoal vaccine (PCV). The first PCV, which contains the antigens of 7 different pneumococcal serotypes (PCV7) was licensed and introduced into the Immunization Schedule in the United States in 2000. PCV7 was included in the national immunization programs, or was recommended for routine vaccination, in a number of European countries, between 2006 and 2008[23]. This vaccine was replaced by a vaccine containing antigens of 6 additional pneumococcal serotypes (PCV13) between 2009 and 2011[24]. Since March 1, 2018, this vaccine has been included in the mandatory immunization calendar in Serbia.

The application of PCV7 has significantly reduced the percentage of nasopharynx colonization with PCV7 serotypes and also the frequency of AOM (in Finland by 6%, Israel by 17%, the UK by 19.8% and Sweden by 26%), as well as the frequency of repeated AOMs to 23%. By introducing PCV13, the incidence of AOM in Israel has been reduced by an additional 43% compared to the period when PCV7 was used (resulting in a total of 60% reduction in regard to pre-PCV period)[25, 26]. The application of PCV has also reduced the resistance of pneumococcus to antibiotics (vaccine serotypes), as well as the reduction of AOM complications. The incidence of pneumococcal acute mastoiditis in Australia has significantly reduced since PCV 7 has been introduced, and the incidence of total mastoiditis has significantly decreased, too, as well as the ICC incidence[25]. In Denmark, after the application of PCV7 and PCV13, the incidence of severe AOM forms decreased by almost 10%, while the incidence of AOM complications decreased by about 20%[26].

Conclusion

Meningoencephalitis is a very rare and unusual complication of AOM. Foudroyant course of the disease requires rapid diagnostics and a multidisciplinary treatment. It is necessary that a neurologist, radiologist, neurosurgeon, infectologist, clinical pharmacologist and physiatrist also have an active participation in the treatment, in addition to the pediatrician in intensive care and otorhinolaryngologist. Antibiotic therapy is the basis of the treatment, but otosurgical treatment is still indicated when there is no improvement in the patient's clinical condition after the administration of antibiotics. After a complete evaluation of patient's condition, it is necessary to correctly evaluate the time of the surgical treatment of the ear. The choice of otosurgical method should be adapted to each patient individually. Audiological and neurological complications are frequent and they further prolong and impair the treatment.

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Birth of a healthy child fathered by a man with Klinefelter's syndrome after preimplantation genetic testing

Rođenje zdravog deteta od oca sa Klajnfelterovim sindromom nakon preimplantacionog testiranja

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Abstract

Introduction. Most men with non-mosaic Klinefelter's syndrome (KS; 47, XXY) have azoospermia, and were until recently considered completely infertile. However, it has been confirmed that some non-mosaic patients had spermatozoa in the ejaculate, although severe oliogasthenoteratosoospermia was present in all of them. Although a high fertilization rate with an intracytoplasmic sperm injection (ICSI) procedure using sperm from a non-mosaic patient and the cumulative pregnancy rate following *in vitro* fertilization constitute 53%, the incidence of live births after ICSI using sperm of men with non-mosaic KS is very low: 1 in 8 cases. **Case report.** At the Clinical Centre of Vojvodina's Department of Gynecology and Obstetrics, a successful *in vitro* fertilization treatment was conducted using the sperm of a man with KS non-mosaic syndrome. Preimplantation genetic testing was performed for the embryo selection, and an euploid embryo was transferred in the subsequent natural cycle. Delivery of male newborn was spontaneous, vaginal, with head presentation, after 40 weeks and 3 days. The newborn's Apgar score was 10/10. His birth weight was 3,950 g, and his length was 55 cm. At 12 months of age, his psychomotor development was assessed as normal on the Brunet-Lézine psychomotor development scale. **Conclusion.** Due to an increased risk of chromosomal abnormalities (abnormalities of sex or autosomal chromosomes) in embryos from couples with KS males, preimplantation genetic testing is conducted to select a chromosomally normal embryo. This may help achieve pregnancy sooner, decrease the chance of multiple pregnancy and its potential complications, decrease the risk of miscarriage, and reduce the need for invasive prenatal diagnostic procedures and the risks of other complications, as well as the risk of late termination of pregnancy in case of pathological findings of the fetal karyotype using conventional diagnostic procedures.

Key words:
klinefelter syndrome; reproductive techniques, assisted; fertility; sperm injections, intracytoplasmatic; azoospermia, non-obstructive; preimplantation diagnosis.

Apstrakt

Uvod. Većina muškaraca sa nemozaičnim 47, XXY Klajnfelterovim sindromom (KS) su azoospermični i donedavno su smatrani potpuno neplodnima, sa izrazito nepovoljnom prognozom muškog fertiliteta. Međutim, potvrđeno je da neki muškarci sa nemozaičnim KS imaju spermatozoide u ejakulatu, mada je kod svih prisutan težak oblik oligoastenoteratozoospermije. Uprkos visokoj stopi fertilizacije sa postupkom intracetoplazmatske injekcije spermatozoida (ICSI) korišćenjem spermatozoida muškaraca sa nemozaičnim KS, kao kumulativnoj stopi fertilizacije koja iznosi 53%, ipak incidencija živorođenja kod njih nakon ICSI veoma je niska i iznosi jedan od osam slučajeva. **Prikaz bolesnika.** Na Klinici za ginekologiju i akušerstvo Kliničkog centra Vojvodine u Novom Sadu uspešno je sproveden postupak vantelesne oplodnje spermom muškarca sa nemozaičnim KS, uz odabir embriona preimplantacionim genetskim testiranjem. Euploidni embrion prenesen je u matericu majke u njenom narednom, prirodnom ciklusu. Porođaj je bio spontani, vaginalni, prezentacija potiljačna, u 40 + 3 gestacijskoj nedelji. Rođen je zdrav dečak, telesne mase (TM) 3 950 g i telesne dužine (TD) 55 cm. Njegov Apgar skor (AS) iznosio je 10/10. U uzrastu od 12 meseci, na Brune-Lezin-ovoj skali psihomotornog razvoja dečak je pokazao uredan psihomotorni razvoj. **Zaključak.** Zbog povišenog rizika od nastanka hromozomskih aberacija kod embriona (ab

normalnosti na polnim ili autosomalnim hormozomima) kod parova gde muškarac ima Klajnefelterov sindrom, primenom preimplantacionog genetskog testiranja bira se jedan euploidan embrion, čime se skraćuje vreme do ostvarivanja trudnoće, smanjuje se verovatnoća spontanog pobačaja, verovatnoća višeplodne trudnoće i njenih eventualnih komplikacija, čime se smanjuje potreba za invazivnim prenatalnim dijagnostičkim procedurama i rizici eventualnih, njima izazvanih komplikacija, kao i rizici

završetka trudnoće u kasnijoj gestaciji u slučaju patološkog nalaza kariotipa ploda klasičnim dijagnostičkim procedurama.

Ključne reči:
klajnfelterov sindrom; reprodukcija, asistirana, tehnike; plodnost; injekcije sperme, intracitoplazmatične; azoospermija, neopstruktivna; dijagnoza, preimplantacijska.

Introduction

Klinefelter's syndrome (KS) is one of the most common chromosomal aberrations of human sex chromosomes, often resulting in hypogonadism and male infertility – azoospermia or severe oligospermia. Accordingly, KS is one the most common genetic causes of male infertility, found in approximately 10% of all men who suffer from azoospermia. Epidemiological data show that KS has an estimated prevalence of between 1:400 and 1:1000 male births. The prevalence of KS was reported to be 0.1 to 0.2% in the general population and 0.15 to 0.17% in prenatally detected cases [1, 2].

Since KS may have variable phenotypic features, a large number of males remain undiagnosed until they are well into adulthood and have fertility problems. Nowadays, besides prenatal invasive procedures (chorionic villi sampling, amnio/cordocentesis), prenatal noninvasive testing is also available for detecting cell-free DNA in maternal blood in order to establish diagnosis of fetal chromosomal disorders in early pregnancy. In the Autonomous Province of Vojvodina, a high percentage of pregnant women (over 90%) and their partners choose the option of pregnancy termination when they receive prenatal diagnosis of KS and become familiar with its clinical presentation.

Azoospermia is diagnosed when no spermatozoa are detected upon microscopic evaluation (the absence of spermatozoa) in more than 90% of cases, although there is also a possibility of the emergence of oligospermia (low sperm count and motility), which provides a reasonable probability of parenthood by *in vitro* fertilization (IVF) [3].

At present, data in the literature show that spermatozoa can be found by a testicular sperm extraction technique (TESE) in about 40–50% of males with KS, with the incidence of pregnancy and live births in approximately 50% of cases [3].

Progress in the field of assisted reproduction techniques and the advent of intracytoplasmic sperm injection (ICSI) procedure improves the chances of normal fertilization and embryo development even in cases of oligozoospermia and azoospermia. In spite of the pathological karyotype (47, XXY), the retrieved spermatozoa can be used for the ICSI procedure in males with KS, enabling genetic fatherhood.

Both normal fertilization of the ovum and normal development of the embryo, with subsequent pregnancy and childbirth, were achieved involving both mosaic and non-mosaic KS patients, after successful sperm retrieval using the TESE procedure [4].

According to the data published in the 1990s, the use of the fluorescent *in situ* hybridization technique (FISH), 2.09–2.7% hyperdiploid sperm was found in males with mosaic and non-mosaic KS [4, 5]. There is little chance of passing extra chromosomes on to their offspring. Accordingly, healthy children were born after the TESE procedure with ICSI in cases of patients with non-mosaic KS [4, 5]. Moreover, cases of embryos derived from KS patients who have a non-mosaic 47, XXY karyotype were published in that period.

Before accessing the procedure itself, it is necessary for the couples to be able to obtain all the necessary information, as well as to be explained that sperm retrieval is necessary, and subsequently proceed to IVF. Since there is a likelihood of the occurrence of a pathological fetal karyotype, with the possibility of other chromosomal abnormalities [2–5, 6], couples are given the option of choosing preimplantation genetic testing or other prenatal procedures for the diagnosis of chromosomal aberrations if conception occurs.

Preimplantation genetic testing (PGT) and preimplantation genetic diagnosis (PGD) were initially used for early biopsy and analysis of normal, unaffected embryos prior to their implantation into the mother's womb, with the aim of achieving a higher rate of successful implantations and live births of healthy children. Initially, the PGT/PGD method involved the use of FISH technique on polar bodies, in which biopsied blastomeres or trophectoderm cells were seeded on the cell culture plate. New methods have been developed, such as array comparative genome hybridization (aCGH), which is currently applied at the Clinical Centre of Vojvodina's Department of Gynecology and Obstetrics, and at the Centre for Medical Genetics at the Child and Youth Health Care Institute of Vojvodina, Novi Sad, in addition to the next generation sequencing (NGS). Clinical application of aCGH technique detects whether there are quantitative differences in the number of copies of a DNA sequence in a DNA sample of the analyzed embryo, as well as the likelihood of detecting aneuploidy and unbalanced rearrangements of chromosomes [7, 8, 12, 13]. Balanced chromosomal rearrangements (balanced translocations or inversions, etc.), in which the amount of genetic material does not change, may not be detectable by CGH.

Case report

A couple (a 23-year-old woman and a 33-year-old man) was presented at the Centre for Reproductive Medicine at the Clinical Centre of Vojvodina for IVF on an outpatient basis. The man was diagnosed with non-mosaic KS (47, XXY) through the G-banding technique.

Examination findings revealed azoospermia, an increased follicle-stimulating hormone (FSH) level of 18.1, and the testosterone level of 5.0 pg/mL.

In vitro fertilization was performed at the Department of Gynecology and Obstetrics. The analysis of the man's ejaculated spermatozoa revealed non-progressive, low sperm count, poor motility, and morphological irregularities (cryptozoospermia). Simultaneously, the woman underwent ovarian stimulation consistent with the antagonist protocol, using 200 IU rFSH (Puregon, MSD). Consequently, 9 oocytes were retrieved, from which 7 reached metaphase II (MII) status. Following the ICSI procedure, 4 blastocyst stage embryos were retrieved by trophotoctoderm biopsy for PGT.

PGT showed the presence of one euploid blastocyst, which was subsequently transferred to the woman's uterus during a natural cycle.

Pregnancy was confirmed by the amount of β-human chronic gonodatropin (β-hCG) serum in the blood. During pregnancy, the patient refused any further prenatal testing, although advised. The course of pregnancy was uneventful.

Delivery was spontaneous, vaginal, with head presentation, after 40 weeks and 3 days. A healthy boy was born. His Apgar score was 10/10, birth weight was 3,950 g and his length was 55 cm. At 12 months of age, his psychomotor development was assessed as normal on the Brunet-Lézine psychomotor development scale.

Methods

The blastocyst biopsy involves opening the zona pellucida on the fifth cultivation day at the opposite side of the inner cell mass (ICM). After several hours of cultivation, trophectoderm cells begin to hatch out of the zona pellucida through the hole, and they can be used for analysis.

First, the biopsied cells of the embryo were rinsed with the washing medium (manufactured by Origio), and placed into the phosphate-buffered saline (PBS + PBP) solution. Then they were placed into special sterile 0.2 mL microreaction PCR tubes. Sterilization was accomplished through exposure to UV light. Samples were placed into microtubes which had been previously filled with 200 µL PBS + PVP solution, with one sample put in each tube. In addition to the microtubes containing the samples, a control microtube was prepared, containing only the aforementioned solution. All microtubes were labelled, including the control microtube. The samples thus prepared were transferred to the Molecular Genetics Laboratory at the Children and Youth Health Care Institute of Vojvodina, Novi Sad. The transfer was performed under stringently controlled conditions.

Array comparative genomic hybridization testing was performed in the reference genetics laboratory. Each DNA sample was placed on a genomic chip-slide and immobilized (Illumina assay). Each slide consisted of large-insert genomic clones covering all autosomes and sex chromosomes at the range of approximately 100–200 kb, providing a detailed chromosomal analysis. The analysis was carried out using laser scanning software that displayed the results in the form of an algorithm. The whole procedure of the microovary-based aCGH analysis, from biopsy to outcome assessment, was completed within 16 hours. Thus, if blastocyst biopsy is performed (after five days of cultivation), it is necessary to perform embryo cryopreservation during the aCGH analysis, until euploid blastocyst stage embryo transfer can take place.

Discussion

First pregnancy outcomes after PGD were reported in 1990. They were performed with the aim of preventing transmission of X-linked diseases [9]. Since then, PGD has been implemented in couples with both structural and numerical aberrations or gene disorders, to avoid transmission of genetic disease to their offspring. PGD involves the biopsy of embryos, nowadays typically performed on trophectodermal cells biopsied from blastocysts, with removal of 8–10 blastomers at the blastocyst stage. The European Society of Human Reproduction and Embryology (PGD consortium) was established to monitor extra-uterine pregnancy outcomes by recording the number of babies born as a result of IVF with PGD, in addition to collecting data from centres throughout the world [10]. The follow-up of the children born after PGD or PGT has confirmed that their growth and development up to 2 years of age is similar to the children whose conception was natural. [11].

In general, in mosaic and non-mosaic KS patients, the possibility of sperm retrieval is about 30–50%, although predictive value for sperm retrieval is higher in mosaic KS [12].

A majority of men with non-mosaic KS (47, XXY) have azoospermia, and until recently they were considered completely infertile. However, it has been confirmed that some non-mosaic KS patients have spermatozoa in the ejaculate, although severe oligoasthenoteratozoospermia is present in all of them. A high fertilization rate with the ICSI procedure in mosaic KS was reported in 1995 [13].

The cumulative pregnancy rate following IVF constitutes 53% [14]. However, the incidence of live births after ICSI where fathers had non-mosaic KS is very low, and clinical pregnancy rates were recorded in 7 out of 10 cases [15–17]. It has been described earlier that only normal 46, XY cells can complete the meiotic process, and therefore every spermatozoid produced by a KS patient [18] has a high probability of having an abnormal karyotype. Unequal number of X chromosomes can lead to cell death [19]. Literature suggests, however, that meiotic progression and sperm production are possible in men with non-mosaic KS.

PGT is now additional practice aiming to help couples with KS men to achieve pregnancy sooner. According to literature, 54% of embryos derived from KS patients were found to be euploid [20]. Detected abnormalities refer to the

presence of an extra chromosome or the absence of a particular chromosome, observed in triploid or tetraploid embryos. The results of PGT show that sex chromosome abnormalities are present in about 15% of embryos, which is significantly higher than in healthy general population (only 3%). Therefore, compared to other embryos, embryos derived from KS males have a higher proportion of pathological findings. Namely, a higher proportion of sex chromosome disomy in patients with KS [21] was observed. The close proximity of the 21st chromosome to the sex chromosome during chromosome segregation, in the process of meiosis, may lead to the failure in segregation of chromosome 21 if the seminal vesicles are not palpable or they are not prominent – which is the case with KS [21]. After all, the benefit of PGT in couples with KS males is debatable: there is no indication that embryos derived from KS patients show a higher prevalence of sex-chromosome aneuploidy [20]. Furthermore, results in ICSI cycles comparing non-PGT cycles with PGT cycles did not show significant differences in implantation, clinical pregnancy, and live birth rates per cycle [21].

The frequency of chromosomal abnormalities in spermatozoa is higher in men with KS, but up to date clarification has not been provided as to whether these abnormalities are the result of constitutional chromosome aberrations or the consequences of using testicular sperm extraction. Due to the increased risk of chromosomal abnormalities in embryos (abnormalities of sex or autosomal chromosomes) from couples with KS males, the use of PGT involves selecting a chromosomally normal embryo. This can help achieve pregnancy sooner, decrease the chance of multiple pregnancy and its potential complications, decrease the risk of miscarriage, reduce the need for invasive prenatal diagnostic procedures and the risks of other complications, as well as the risk of late termination of pregnancy in case of pathological findings concerning the fetal karyotype, using conventional diagnostic procedures. The selection of chromosomally normal embryos, based on preimplantation genetic screening (PGS), eliminates the chance of transferring embryos carrying chromosomal aberrations. PGS/ICSI was performed in 26 treatment cycles involving KS patients, which resulted in 8

pregnancies [20]. Thus, information about the possibility of achieving pregnancy can be provided to couples with KS males, in addition to providing them with information about prenatal diagnosis of KS.

Although PGT is highly accurate, no test is 100% reliable, and this is due to the mosaicism of the embryo biopsied. Couples need to be counselled about the necessity of performing further non-invasive or invasive testing during pregnancy. Both PGT and non-invasive prenatal testing (NIPT) are associated with false positive and false negative results due to trophoblast-derived mosaicism. Mosacism is present when an embryo contains two or more distinct cell lines. The increased sensitivity of the next-generation sequencing (NGS) technology can recognize it in preimplantation embryos. Mosaicism is the result of mitotic errors during embryo development. With the next-generation sequencing (NGS), 10–20% of PGT are mosaic. This can cause a false positive or false negative PGT result [22–25].

The first trimester combined screening test is the gold standard for calculating the risk for trisomy 21, 13 and 18, with a detection rate of 95% when nuchal translucency, nasal bone, ductus venous, and tricuspid valve blood flow are assessed. The management plan for patients undergoing IVF with PGS should be first trimester screening, followed by comprehensive counselling and support, and recommendation of non-invasive prenatal testing or invasive testing, depending on the findings. The role of the IVF specialist is to recommend the right test for the right patient.

After achieving pregnancy, with or without PGT, it is always advisable to confirm the results of PGT through other non-invasive or invasive prenatal tests, with amniocentesis providing a high diagnostic certainty.

So far there has been no indication that embryo biopsy causes an increased risk of adverse neonatal outcome [30].

Conclusion

PGT of embryos from couples with KS males is recommended in order to achieve pregnancy sooner, and enhance the chances of success.

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prof. dr sc. med. DRAGANA OBRADOVIĆ
(1966–2021)

Kada dan počne tužnom viješću, nakon šoka i nevjerice počinju sjećanja i naviru uspomene. Tog jutra, 27. jula dobio sam poruku od prof. dr Dragane Stamatović da je umrla Dragana Obradović. Pored svega što se može očekivati u ovo teško vrijeme, ja ni tada, ni sada ne mogu da povjerujem da osoba takve energije može da ode i napusti nas. Nažalost, tog jutra, 27.07.2021, posle duže bolesti preminula je prof. dr Dragana Obradović, nastavnik Medicinskog fakulteta Vojnomedicinske akdemije (VMA) Univerziteta odbrane u Beogradu i specijalista neurolog Klinike za neurologiju VMA.

Dragana Obradović je rođena 20. decembra 1966. godine, završila je VIII Beogradsku gimnaziju i Medicinski fakultet u Beogradu i bila je među najboljim učenicima i studentima. Specijalizaciju iz neurologije je upisala 1993. i završila 1997. godine sa odličnom ocjenom. Magistarski i doktorski rad je odbranila još za vrijeme trajanja specijalizacije. Prošla je sva izborna zvanja od asistenta do redovnog profesora. Svi ovi podaci i činjenice govore da je bila izvanredan đak, student, ljekar, neurolog, nastavnik i istraživač. I to nikada nije, niti će ikada biti upitno.

Predstavljala je poznato i priznato ime u neurološkoj zajednici Srbije i okruženja, a na polju multiple skleroze i u internacionalnim okvirima. Jedan je od pionira liječenja multiple skleroze matičnim ćelijama. Ovo je i potvrđeno velikim brojem naučnih članaka, predavanja, tribina, seminara i drugih publikacija. Poznato je njeno ogromno zalaganje za obezbjeđivanje terapije pacijentima sa multiplom sklerozom. Ovo su biografski podaci za ogroman respekt, ali ja imam potrebu i želim da se u ovom tekstu osvrnem i govorim o Dragani kao osobi, drugarici sa specijalizacije, koleginici, ljekaru kliničaru, prijatelju, ...

Koliko je Dragana zaista značila onima koji su je upoznali govore riječi iz telegrama profesorke Tončev:

„... Vest da se Dragana preselila u večnost ostavila me je bez reči. Nakon toga su nastupili tuga i praznina. Prelistavanje zajedničkih fotografija. Draganu sam prvi put srela 1998. Zapamtila sam njene krupne plave oči. Već na sledećem susretu sam bila Gocili, a ona Draganica. Šta nas je povezalo? Ne znam, ali su svi naši mnogobrojni susreti bili izuzetno prijatni. I kada smo slušale predavanja, i kada smo pričale o knjigama koje smo pročitale i kada smo bile u raznim komisijama, ispitivale studente i kada smo pričale o svojim pacijentima...i obilazile gradove i podsticale jedna drugu da još nešto kupimo. A najviše kada smo pričale o porodici. Kada je govorila koliko voli Denu i Slobu, njene roditelje. Njoj je Bog dao sve. Bila je pametna, lepa, obrazovana, elokventna, hrabra. Imala je integritet i prepoznatljiv stil. I definitivno se razlikovala od drugih, i ja sam mislila da joj niko i ništa ne može. Mislila sam i nadala se da joj ni bolest ne može ništa i nadala se novim susretima.... Na žalost njih neće biti, ali će ona i oni biti i u nama i sa nama dok postojimo....“

I na kraju želim da kažem ja ko je bila i biće meni prof. dr Dragana Obradović. Dragana i ja smo došli i započeli specijalizaciju isti dan 20. decembra 1993, na njen rođendan, više godina dijelili specijalizantsku i spacijalističku sobu, i od prvog dana smo započeli put koji je imao mnogo predivnih dionica, velikih uspjeha, stalnog druženja, neprekidne komunikacije, međusobne podrške... Bilo je i dionica kada smo se u nerazumijevanju razumijevali, a mene je njena

osobina da bude principijelna, hrabra, spremna da brani svoj stav ne kalkulišući o posljedicama, potpuno uvjerila da se radi o kompletno formiranoj osobi nestvarnih intelektualnih kapaciteta i beskrajnog šarma i duhovne i fizičke ljepote. I kada se nijesmo slagali, znao sam i znam, da je ona dio mog života, bila i biće, kao osoba, drug, prijatelj, oponent, kritičar, saradnik i ličnost za svako poštovanje. Bila je svoja, drugačija na najbolji mogući način, i ostavila je neizbrisiv trag i sjećanje kod svakog ko ju je upoznao. I kako sam pročitao u dirljivom tekstu njenog muža i životnog saputnika, čovjek se rađa ili za velika ili za mala djela. Dragani je bilo suđeno da se rodi za velika djela, to je činila punog srca i vjere do posljednjeg trena svog ovozemaljskog bitisanja.

Dragana će ostati zauvijek neraskidivi dio bitisanja ljudi koji su je voljeli, a kako kaže Meša Selimović: „Prijateljstvo se ne bira, ono biva ko zna zbog čega, kao ljubav“.

Neka je vječna slava prof. dr Dragani Obradović.

pukovnik prof. dr Ranko Raičević
Načelnik Klinike za neurologiju VMA i
Predsednik Društva neurologa Srbije

INSTRUCTIONS TO THE AUTHORS

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The text of the articles includes: **Introduction**, **Methods**, **Results**, and **Discussion**. Long articles may need subheadings within some sections to clarify their content.

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Results should be presented in logical sequence in the text, tables and illustrations. Emphasize or summarize only important observations.

Discussion is to emphasize the new and significant aspects of the study and the conclusions that result from them. Relate the observations to other relevant studies. Link the conclusions with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

References

References should be superscripted and numerated consecutively in the order of their first mentioning within the text. All the authors should be listed, but if there are more than 6 authors, give the first 6 followed by *et al*. Do not use abstracts, secondary publications, oral communications, unpublished papers, official and classified documents. References to papers accepted but not yet published should be cited as "in press". Information from manuscripts not yet accepted should be cited as "unpublished data". Data from the Internet are cited with the date of citation.

Examples of references:

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DiMaio VJ. Forensic Pathology. 2nd ed. Boca Raton: CRC Press; 2001.

Blinder MA. Anemia and Transfusion Therapy. In: Ahya NS, Flood K, Paranjothi S, editors. The Washington Manual of Medical Therapeutics, 30th edition. Boston: Lippincot, Williams and Wilkins; 2001. p. 413-28.

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: *Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG,* editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

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Primeri referenci:

Đurović BM. Endothelial trauma in the surgery of cataract. Vojnosanit Pregl 2004; 61(5): 491–7. (Serbian)

Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

Mladenović T, Kandolf L, Mijušković ŽP. Lasers in dermatology. In: *Karadaglić Đ*, editor. Dermatology. Beograd: Vojnoizdavački zavod & Verzal Press; 2000. p. 1437–49. (Serbian)

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: *Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG*, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

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