ВОЈНОСАНИТЕТСКИ ПРЕГЛЕД

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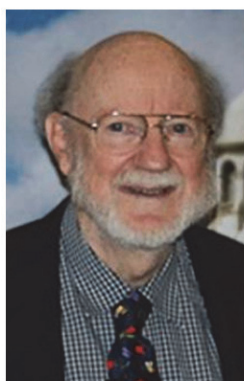
Vojnosanitetski pregled

William C. Campbell   Satoshi Ōmura   Youyou Tu

VOJNOSANITETSKI PREGLED

Prvi broj *Vojnosanitetskog pregleda* izašao je septembra meseca 1944. godine

Časopis nastavlja tradiciju *Vojno-sanitetskog glasnika*, koji je izlazio od 1930. do 1941. godine

IZDAVAČ
Uprava za vojno zdravstvo MO Srbije

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The Nobel Prize Laureates in Physiology or Medicine 2015 – William C. Campbell (born 1930, Remelton, Ireland), Satoshi Ōmura (born 1935, Jamanashi prefektur, Japan) and Youyou Tu (born 1930, Zhejiang Ningpo, China).
William C. Campbell and Satoshi Ōmura were awarded "for their discoveries concerning a novel therapy against infections caused by roundworm parasites", and Youyou Tu "for her discoveries concerning a novel therapy against Malaria". Their discoveries have provided humankind with powerful new means to combat these severe, debilitating diseases that affect hundreds of millions of people annually. The consequences in terms of improved human health and reduced suffering are immeasurable (see Editorial, p. 951- 2).

Ovogodišnji dobitnici Nobelove nagrade za medicinu: William C. Campbell (rođen 1930, Remelton, Irska), Satoshi Ōmura (rođen 1935, Jamanashi prefektura, Japan) i Youyou Tu (rođena 1930, Zhejiang Ningpo, Kina).
William C. Campbell i Satoshi Ōmura nagrađeni su za otkriće novog leka (avermektin) namenjenog lečenju teških infekcija prouzrokovanih parazitskim valjkastim crvima, a Youyou Tu za otkriće novog leka (artemizinin) za lečenje malarije. Njihova otkrića obezbedila su čovečanstvu nova sredstva za borbu protiv teških, iscrpljujućih bolesti koje pogađaju stotine miliona ljudi godišnje. Posledice ovih otkrića su nemerljive s aspekta smanjenja obolevanja i poboljšanja čovekovog zdravlja (vidi Uvodnik, str. 951-2).

The 2015 Nobel Prize Laureates in Physiology or Medicine

Dobitnici Nobelove nagrade za fiziologiju ili medicinu u 2015.

Dragan Mikić

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This year's Nobel Laureates in Medicine, William C. Campbell, Satoshi Õmura and Youyou Tu have developed therapies that have revolutionized the treatment of some of the most devastating parasitic diseases, such as river blindness (onchocerciasis) and lymphatic filariasis, two diseases caused by parasitic roundworms, and malaria caused by parasitic protozoa *Plasmodium*. These scientists have succeeded to develop novel anti-parasite therapies from bacteria and plants. Campbell and Õmura discovered a new drug, avermectin, the derivative which radically lowered the incidence of river blindness and lymphatic filariasis. Youyou Tu discovered artemisinin, a drug that has significantly reduced the mortality rates of patients suffering from malaria. The discovery of these two drugs has fundamentally changed the treatment of these parasitic diseases and significantly improved the global health of the world's population [1–6].

William C. Campbell is an American biologist and parasitologist, who got a job in the research laboratory of Merck, Sharp and Dohme, the pharmaceutical firm. He discovered that a component from one of Omura's *Streptomyces* cultures was very effective in killing off parasites in domestic animals. The bioactive agent was purified and named avermectin. Avermectin was subsequently chemically modified to ivermectin, which turned out to be highly effective in both animals and humans against a variety of parasites. Ivermectin was marketed as a drug for veterinary use in the early 1980s by pharmaceutical giant Merck and was extraordinarily profitable. In 1978 Campbell had suggested to his boss that, in addition to its success in veterinary medicine, the drug might also be effective in treating river blindness in humans. He and his colleagues persuaded Merck to continue research and, when that proved successful, to provide the drug for free, since the potential patients certainly could not afford to pay for it [1, 5, 7, 8].

Professor Satoshi Õmura is a Japanese microbiologist widely recognized as a world expert in the field of bioorganic chemistry, particularly for the discovery, development, biosynthesis and manipulation of useful chemicals derived from naturally-occurring microorganisms. He is an expert in isolating natural products, focused on a group of bacteria, Streptomyces which live in the soil known to produce a plethora of agents with antibacterial activities. From many thousand different cultures, he selected about 50 of the most promising, and one of these cultures later turned out to be Streptomyces avermitilis, the source of avermectin. The ivermectin, a dihydro derivative of avermectin was discovered and developed by collaborative research of Õmura and Merck Company as a macrolide anthelmintic antibiotic. It is widely held that the discovery and use of ivermectin represents the greatest public health intervention of the last quarter of the 20th century and can be regarded as on a par with the discovery and use of penicillin [4, 7, 8].

The eradication programs for river blindness and lymphatic filariasis, currently being orchestrated by the WHO, are based primarily on the use of ivermectin. Ivermectin has been donated by the Merck Company and the Kitasato Institute since 1987, and the drug is being administered to around 300 million people annually. It is envisaged that river blindness will be eliminated globally by 2025 and lymphatic filariasis by 2020 [8].

Professor Youyou Tu is a Chinese medical scientist, pharmaceutical chemist, and educator. She is the first Chinese Nobel laureate in physiology or medicine, and the first Chinese citizen to receive the Nobel Prize in natural sciences. She was born, educated and carried out research exclusively in China. 1 Youyou Tu is best known for discovering artemisinin (also known as qinghaosu) and dihydroartemisinin, used to treat malaria, which saved millions of lives. Her discovery of artemisinin and its treatment of malaria are regarded as a significant advance of tropical medicine in the 20th century and health improvement for people from developing countries in South Asia, Africa, and South America [6, 9, 10].

Malaria was traditionally treated by chloroquine or quinine, but with declining success. By the late 1960s, efforts to

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eradicate malaria had failed and the disease was on the rise. At that time, Youyou Tu in China revisited the ancient literature and discovered clues that guided her in her quest to successfully extract the active component from *Artemisia annua*. She was the first to show that this component, later called artemisinin, was highly effective against the malaria parasite, both in infected animals and in humans. Furthermore, Youyou Tu volunteered to be the first human subject. "As a head of this research group, I had the responsibility" she said. Artemisinin represents a new class of antimalarial agents that rapidly kill the malaria parasites at an early stage of their development, which explains its unprecedented potency in the treatment of severe malaria. When used in combination therapy, it is estimated to reduce mortality from malaria by more than 20% overall and by more than 30% in children. For Africa alone, this means that more than 100 000 lives are saved each year [6, 9, 10].

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Assessment of the periodontal health and community periodontal index in the Army of Serbia

Procena periodontalnog zdravlja i periodontalnog indeksa kod pripadnika Vojske Srbije

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Abstract

Background/Aim. Promotion of oral health in military population is not only a significant component of general health, but also of the military readiness and represents the strategic orientation of each country. The basic task of military dentistry is to provide oral health of military personnel and to enable their operational readiness at the optimal level. The aim of the study was to assess the periodontal condition in Serbian military population using the community periodontal index of treatment needs (CPITN), and the influence of general life habits and local risk factors on periodontal health. **Methods.** This prospective cross-sectional pilot study was conducted on 101 examinees at the mean age of 38.94 ± 11.63 years who had dental check-ups at the Dental Clinic of the Military Medical Academy in Belgrade. All the categories of military personnel aged 20–64 years were divided into five groups. The frequency distribution of general and local factors on periodontal health, oral hygiene index, and the assessment of the mean number of sextants by CPITN compared to age were examined. **Results.** The examinees at the age of 51–60 years had the best oral hygiene index (0.95 ± 0.65), whereas the oldest population had the worst (1.63 ± 0.42). Only one person (5.6%) at the age group of 51–60 years had a completely healthy periodontium. Observed in relation to the age groups, the mean values of sextants increased linearly, but in general population, the most frequent CPITN categories were in sextant with the periodontal pockets 4–5 mm (score 3). **Conclusions.** Compared to the results from other countries shown by the World Health Organization, the periodontal condition in our examinees is below the average. The appropriate preventive program preparation and its implementation are needed, including primarily the appropriate training on oral hygiene, as well as education based on periodontal disease prevention and treatment.

Key words:
oral health; oral hygiene; periodontal index; military personnel; serbia.

Apstrakt

Uvod/Cilj. Unapređenje oralnog zdravlja kod vojnih lica nije samo deo opšteg zdravlja, već isto tako i vojna spremnost, a predstavlja i strategijsku orijentaciju svake zemlje. Osnovni cilj vojne stomatologije jeste da obezbedi oralnu higijenu vojnog personala i omogući njihovu operativnu spremnost na optimalnom nivou. Cilj studije bio je da se proceni stanje parodoncijuma pripadnika Vojske Srbije koristeći *Community Periodontal Index of Treatment Needs* (CPITN), kao i uticaj osnovnih životnih navika i lokalnih oralnih faktora rizika na parodontalno zdravlje. **Metode.** Urađena je prospektivna pilot studija preseka kod 101 ispitanika prosečne starosti $38,94 \pm 11,63$ godina, koji su pregledani u Klinici za stomatologiju Vojnomedicinske akademije u Beogradu. Svi ispitanici bili su starosti 20–64 godina i podeljeni u pet grupa. Ispitivana je učestalost distribucije opštih i lokalnih faktora na parodontalno zdravlje, indeksa oralne higijene, kao i procena srednjeg broja sekstanata pomoću CPITN u odnosu na godine starosti. **Rezultati.** Ispitanici starosti 51–60 godina imali su najbolji indeks oralne higijene ($0,95 \pm 0,65$), dok su najstariji imali najlošiju ($1,63 \pm 0,42$). Samo je jedan (5,6%) ispitanik starosti 51–60 godina imao zdrav parodoncijum. Posmatrano prema starosnim grupama, srednje vrednosti sekstanata linearno su se povećavale, a na nivou cele populacije najveća vrednost CPITN bila je kod ovih sa parodontalnim džepovima dubine 4–5 mm (skor 3). **Zaključak.** U odnosu na rezultate iz drugih zemlja prema podacima Svetske zdravstvene organizacije, stanje parodoncijuma kod naših ispitanika je ispod proseka. Zbog toga je neophodno implementirati i preduzeti preventivni program, uključujući osnovnu obuku iz oralne higijene, kao i obrazovanje iz prevencije i lečenja oboljenja parodonciuma.

Ključne reči:
usta, zdravlje; usta, higijena; periodontalni indeks; kadar, vojni; srbija.

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Introduction

Promotion of oral health in military population is not only a significant component of general health, but also of the military readiness and represents the strategic orientation of each country. The basic task of military dentistry is to provide oral health of military personnel and to enable their operational readiness at the optimal level [1].

Good oral health of military personnel could reduce the number of urgent dental interventions and absence from military activities, contributing to the security of whole military formation [2]. Various general factors such as nutritive state, smoking, use of alcohol, oral hygiene, stress, etc, make a basic foundation of the most common risk factors [3] which have an important role in dental disease prevention [4]. The primary task of the World Health Organization (WHO) is the collection of epidemiological data related to oral health and morbidity. Periodontal diseases, including gingivitis and periodontitis are considered one of the most common diseases in total population and if they are not treated, they can cause teeth loss [5]. Contrary to gingivitis, where a key role has dental plaque which is the main cause, the occurrence of periodontitis is more complex, because it includes not only microorganisms from subgingival dental plaque, but also the host immune response.

Periodontal health data in Europe [6], as well as in military population [7–9] are nowadays largely available in literature, whereas the frequency of periodontal diseases among Serbian military personnel and a long-term trend of periodontal health among them have never been examined before. Current asseessment of periodontitis among military personnel could establish the foundation to promote periodontal health, develop preventive strategies and establish a periodontitis treatment plan and program.

Data on the community periodontal index of treatment needs (CPITN) [10], which are kept in the WHO bank, include examination data from more than 50 countries. They show a significant presence of periodontal disease in population (5–20%) in non-industrial countries [11]. The WHO CPITN is a practical and significant procedure for assessing periodontal treatment needs in one community [12], and it is used in many studies for these purposes [7, 13].

The aim of this study was to collect data related to the experiences of periodontal disease, lifestyle, habits, oral hygiene and periodontal indicators which should assess the frequency and possible risk factors of periodontitis among military personnel in Serbia. Besides, the aim is to study the realtionship between periodontal state and independent sociodemographic variables as well as to compare periodontal condition of this study group with the population in similar studies of other countries, including their military personnel [8].

Data on periodontitis frequency, influence and severity could help in presenting the importance of this disease and its consequences. These data can be used as guidelines for a practical periodontal preventive program and projection of periodontal disease treatment in our country, both for military personnel and general population.

Methods

In the prospective cross-sectional study, 101 subjects were examined at the Clinic for Dental Medicine of the Military Medical Academy in Belgrade.

The study lasted for 60 days (March – April 2014) and comprised all the categories of military personnel, both males and females at the age of 20–64 years. This study included all examinees who came to the Clinic in order to have some dental intervention (convenience sample). Excluding criteria of the study were: totaly edentulous patients, pregnacy, systematic infections of oral cavity, and neuropsychological diseases. Including factors were: status of military personnel, and sufficient number of teeth in the upper and lower jaw ($\geq$ 20). After being informed on the reasearch by their dentist, all examinees signed a volunteer Consent Form (CF) for participation in the study. A positive opinion on the ethics of the study was given by the Ethical Committee of the Military Medical Academy (May 23, 2012).

The study consisted of a close-ended survey that was filled in by a subject, and clinical examination carried out by a periodontist.

The survey contained 21 questions related to: general habits of patients (diet, consumption of carbonated drinks, simple sugars, alcohol, smoking); personal habits in oral hygiene (frequency of teeth brushing, duration of teeth brushing, the use of dental floss, interdental brushes and mouthwashes); personal opinion about the impact of oral and dental diseases on overall health status.

A specialist's examination included clinical examination of teeth and periodontium with a periodontal probe (WHO guidelines), and the dental mirror.

After clinical examination of teeth, the following indices were determined: soft debris index according to Green Vermillion (DI-S) by which it determines the presence of soft debrison of the buccal and lingual surfaces of the three representative teeth of the lower and upper jaw, scoring being on the scale of 0–3 [14]; simplified calculus index (CI-S) by which it determines the presence of calculus of the three representative teeth of the lower and upper jaw, scoring being on the scale of 0–3 [14]; oral hygiene index (Simplified Oral Hygiene Index – OHI-S) which is the sum of the first two indices DI-S i CI-S [14]; to evaluate the state of the periodontium and the needs for therapy, CPITN was used, which was recommended by experts of the WHO. Examination was conducted by using a periodontal probe on the sextants, scoring being on the scale of 0–4. In each sextant, all teeth should be cheked, and only the highest value, for each sextant is scored and recorded [10].

Statistical analysis of the data was done with the statistical software package, IBM SPSS Statistics version 19. All variables were presented as frequency of certain categories, while statistical significance of differences were tested by the χ^2-test. Continuous variables were summarized as means and standard deviations. The normality of the data was assessed using Kolmogorov-Smirnov test. significance of a difference between continual variables with three and more groups was tested by nonparametric Kruskal-Wallis analysis. A relation-

ship between variables was tested by Pearson's or Spearman's coefficient of correlation. All the analyses were estimated at $p < 0.05$ level of the statistical significance.

Results

A total of 101 subjects (78 males and 23 females) were included in the study, and the mean age of this population was 38.94 ± 11.63 years. Most of them (67.3%) were married. More than half of the subjects (63.4%) in this study had a high school education and 31.7% of them had the bachelor degree. Their demographic data are shown in Table 1.

Table 1
Sociodemographic characteristics of the participants in the study

Characteristics of the patients	Values
Gender, n (%)	
male	78 (77.2)
female	23 (22.8)
Age (years), x̄ ± SD (min-max)	38.94 ± 11.63 (21–64)
Marital status, n (%)	
married	68 (67.3)
single	32 (32.7)
Educational attainment, n (%)	
primary school	2 (2.0)
high school	64 (63.4)
higher school	3 (3.0)
faculty	32 (31.7)

x̄ – mean; SD – standard deviation.

For the purpose of showing the relationship of ages and other parameters as well as for the subsequent discussion, the category of military population was divided into five groups according to age (Figure 1).
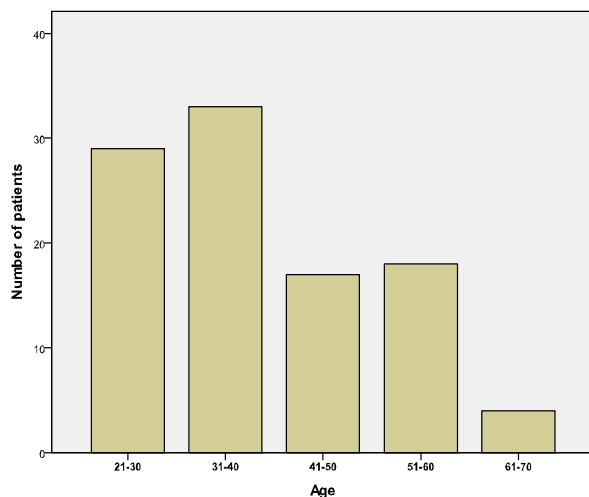
Fig. 1 – Distribution of the examinees by age.

Table 2 shows general habits of the subjects that could affect the periodontal health of the military population. Out of the total number of subjects, 69 (68.3%) were non-smokers. Smokers smoked cigarettes on the average for 6.38 ± 5.23 years. More than half of them (59.4%) consumed alcohol. Only five of the subjects consumed alcohol more than once a week. They consumed carbonated drinks in large amounts (74.3%), and almost all the subjects (91.1%) consumed sugar. Only a small number (6.9%) of subjects used fast food, while most of them (91.1%) had their meals at home. They usually used snacks several times a day (75.2%).

Table 2
Incidence of the major factors influencing oral health in the studied subjects

Factor influencing oral health	Values
Smoking, n (%)	
no	69 (68.3)
yes	32 (31.7)
How many years of smoking? (x̄ ± SD)	6.38 ± 5.23
Consuming alcohol, n (%)	
no	41 (40.6)
yes	60 (59.4)
more times *per* week	5 (5.0)
more times *per* month	15 (14.8)
rarely	40 (39.6)
Consuming carbonated drink, n (%)	
no	26 (25.7)
yes	75 (74.3)
every day	11 (10.9)
more times *per* week	26 (25.7)
more times *per* month	14 (13.9)
rarely	24 (23.8)
Consuming sugars, n (%)	
no	8 (7.9)
yes	92 (91.1)
missing	1 (1.0)
every day	22 (21.8)
more times *per* week	33 (32.7)
more times *per* month	25 (24.8)
rarely	12 (11.9)
Nutrition, n (%)	
feeding at home	92 (91.1)
fast food	7 (6.9)
missing	2 (2.0)
Snack, n (%)	
onse a day	22 (21.8)
more times *per* day	76 (75.2)
missing	3 (3.0)

x̄ – mean; SD – standard deviation.

In the examined military population, especially in the younger age (21–30 and 31–40 years), more than a half of them (66.3%) brushed their teeth several times a day. Only two (2%) brushed their teeth less than once a day. In addition, most of them (89.1%) brushed their teeth for longer than 1 minute. Only 27.7% used dental floss, mouthwash (25.7%), and interdental brushes (18.8%). There was a significant difference in the use of interdental brushes between the age groups ($p = 0.009$, Table 3). Most of them changed toothbrushes after four (27 patients) or five (28 subjects) months. Half of the examined population confirmed that their gums bleeded when brushing [50 (49.5%)], and 38 (37.6%) of them had tooth sensitivity to certain stimuli. Half of the subjects had a regular dental check-ups, while the other half visited a dentist only when they felt pain in the oral cavity. Indeed, almost all (99.0%) the subjects agreed that oral diseases can affect general health (Table 3).

Table 3

Distribution of examinees according to age and habits/attitudes important for oral health

Habits/attitudes for oral health	Total, n (%)	Age distribution					p^*
		21–30, n	31–40, n	41–50, n	51–60, n	61–70, n	
How long do you brush your teeth?							
longer than 1 minute	90 (89.1)	27	29	16	15	3	0.661
less than 1 minute	11 (10.9)	2	4	1	3	1	
Do you use dental floss?							
no	73 (72.3)	23	22	10	14	4	0.334
yes	28 (27.7)	6	11	7	4	0	
Do you use mouthwash?							
no	75 (74.3)	21	25	13	12	4	0.724
yes	26 (25.7)	8	8	4	6	0	
How often do you brush your teeth?							
once a day	30 (29.7)	10	7	5	5	3	
several times during the day	67 (66.3)	18	24	11	13	1	0.703
every other day	2 (2.0)	1	1	0	0	0	
reare	2 (2.0)	0	1	1	0	0	
Do you use interdental brushes?							
no	82 (81.2)	28	27	13	10	4	0.009
yes	19 (18.8)	1	6	4	8	0	
How often do you change your toothbrush?							
once a month	6 (5.9)	2	1	2	0	1	
two months	18 (17.8)	7	5	1	4	1	
three months	16 (15.8)	6	7	2	0	1	
four months	27 (26.7)	6	7	6	7	1	0.414
five months	28 (27.7)	7	11	4	6	0	
half year	1 (1.0)	0	0	1	0	0	
missing	5 (5.0)	1	2	1	1	0	
Do you feel pain in the teeth to hot, cold and sweet?							
no	61 (60.4)	15	19	12	13	2	
yes	38 (37.6)	13	14	5	5	1	0.652
missing	2 (2.0)	1	0	0	0	1	
How many times a year visit the dentist?							
just when you feel the pain	44 (43.6)	14	16	7	5	2	
periodic reviews	56 (55.4)	15	16	10	13	2	0.606
missing	1 (1.0)	0	1	0	0	0	
Do you agree that the diseases of the oral region affect the overall health?							
no	1 (1.0)	0	0	1	0	0	0.288
yes	100 (99.0)	29	33	16	18	4	

$^*\chi^2$ **test.**

The average index value of soft plaque (DI-S) was 0.73 ± 0.32, where the minimum value was present in the age group of 51–60 years (0.65 ± 0.35), and in the age group of 21–30 years (0.67 ± 0.33), but most prominent value was among the oldest one (0.93 ± 0.32) (Table 4). A similar relationship between the age groups was present in the dental calculus index (CI-S). Dental plaque was the most prevalent in the oldest population (0.70 ± 0.17) and the least frequent in the population aged 51–60 years (0.29 ± 0.37). Since the OHI-S is the sum of the previous two indices, the subjects aged 51–60 years had the best oral hygiene (OHI-S = 0.95 ± 0.65), while the oldest population had the worst oral hygiene (OHI-

S = 1.63 ± 0.42). However, the differences between the three indices in relation to population groups were not statistically significant (Table 4).

When the mean values of sextants and percentage of people with CPITN category in line with the age groups were observed (Tables 5 and 6), only 5.6% of subjects in the group at the age of 51–60 years had a completely healthy periodontium (score 0) which was 1% of all the examined subjects. In other age groups, nobody had a healthy periodontium.

Regarding the other categories (Table 6), the mean values of CPITN (score 1–4) grew linearly in the age group of 21–30 years (0.72 ± 0.19, 1.22 ± 0.38, 2.04 ± 0.35, 2.65 ± 0.61), in

Table 4

Distribution of oral index ($\bar{x} \pm SD$) among the participants in the study depending on their age

Oral indices	Age (years)					Total	p^*
	21–30`	31–40	41–50	51–60	61–70		
DI-S	0.67 ± 0.33	0.77 ± 0.31	0.79 ± 0.30	0.65 ± 0.35	0.93 ± 0.32	0.73 ± 0.32	0.190
CI-S	0.46 ± 0.45	0.45 ± 0.44	0.51 ± 0.47	0.29 ± 0.37	0.70 ± 0.17	0.44 ± 0.43	0.218
OHI-S	1.13 ± 0.65	1.23 ± 0.64	1.31 ± 0.60	0.95 ± 0.65	1.63 ± 0.42	1.18 ± 0.64	0.213

$\bar{x}$ **– mean; SD – standard deviation; DI-S – Soft debris index, according to Greene Vermillion; CI-S – Simplified Calculus Index; OHI-S – simplifed oral hygiene index; * – Kruskal-Wallis analysis.**

Table 5

The average value of the community periodontal index of treatment needs (CPITN)
in the examinees of different age groups

CPITN	Age groups (years)					Total	$p*$
	21–30	31–40	41–50	51–60	61-70		
0	-	-	-	0.00 ± 0.00	-	0.00 ± 0.00	-
1	0.72 ± 0.19	0.67 ± 0.00	0.75 ± 0.35	0.42 ± 0.35	-	0.65 ± 0.26	0.686
2	1.22 ± 0.38	1.32 ± 0.45	1.14 ± 0.55	0.83 ± 0.00	-	1.24 ± 0.42	0.712
3	2.04 ± 0.35	2.18 ± 0.42	2.37 ± 0.29	2.15 ± 0.52	2.12 ± 0.40	2.15 ± 0.41	0.420
4	2.65 ± 0.61	2.62 ± 0.39	2.99 ± 0.49	3.61 ± 0.22	3.50 ± 0.00	2.90 ± 0.56	0.032
Average	1.84 ± 0.71	1.99 ± 0.67	2.18 ± 0.93	2.01 ± 1.12	2.58 ± 0.85	2.00 ± 0.82	0.538

Note: results are given as mean ± standard deviation (SD); CPITN: 0 – healthy; 1 – bleeding observed, directly or by using mouth mirror, after sensing; 2 – calculus felt during probing but all the black area of the visible; 3 – pocket 4 or 5 mm (gingival margin situated on black area of probe); 4 mm – pocket > 6 mm (black area of probe not visible); *Kruskal-Wallis analysis.

Table 6

Distributions of the examinees n (%) according to the community periodontal index of treatment needs (CPITN)
value and age

CPITN	Age (years)					Total	$p*$
	21–30	31–40	41–50	51–60	61–70		
0	-	-	-	1 (5.6)	-	1 (1.0)	
1	3 (10.3)	1 (3.0)	2 (11.8)	2 (11.1)	-	8 (7.9)	
2	6 (20.7)	9 (27.3)	3 (17.6)	1 (5.6)	-	19 (18.8)	0.593
3	15 (51.7)	16 (48.5)	6 (35.3)	11 (61.1)	3 (75.0)	51 (50.5)	
4	5 (17.3)	7 (21.2)	6 (35.3)	3 (16.7)	1 (25.0)	22 (21.8)	
Total	29 (100)	33 (100)	17 (100)	18 (100)	4 (100)	101 (100)	

Note: results are give as number (%) of examinees; CPITN – community periodontal index of treatment needs; 0 – healthy; 1 – bleeding observed, directly or by using mouth mirror, after sensing; 2 – calculus felt during probing but all the black area of the visible; 3 – pocket 4 or 5 mm (gingival margin situated on black area of probe); 4 – pocket > 6 mm (black area of probe not visible); *χ^2-test

the age group of 31–40 years (0.67 ± 0.00, 1.32 ± 0.45, 2.18 ± 0.42, 0.39 ± 2.62) and in the age group of 41–50 years (0.75 ± 0.35, 1.14 ± 0.55, 2.37 ± 0.29, 0.49 ± 2.99). The age group of 51-60 years, except having a sextant with the score of 0 (5.6%), had a gingivitis (11.1%), and calculus (5.6%) only in a small percentage. However, there were more sextants with periodontal pockets of 4 or 5 mm (2.15 ± 0.52), i.e. 61.1% of the subjects, and the periodontal pockets of more than 6 mm (3.61 ± 0.22), or 16.7% of the subjects. The oldest group (61–70 years) did not have sextants with gingivitis and calculus (scores 2 and 3 respectively), but only the presence of periodontal pockets in 75.0%, and 25% of the cases, respectively (Tables 5 and 6).

The only significant difference between the groups was recorded between the mean values of the sextants in the fourth category of CPITN ($p = 0.032$) (Table 5).

Figure 2 shows the distribution of CPITN categories by sextants in relation to the total number of subjects. Thus, the category (score) 3 dominated in the sextant of the both sides of the upper and lower jaw, that is, most patients had periodontal pockets of 4 mm and 5 mm. On the upper front teeth, gingivitis and calculus were most frequent, while calculus (score 2) was the most prevalent on the lower front teeth. In total, the most common CPITN category had periodontal pockets of 4 mm and 5 mm (score 3).
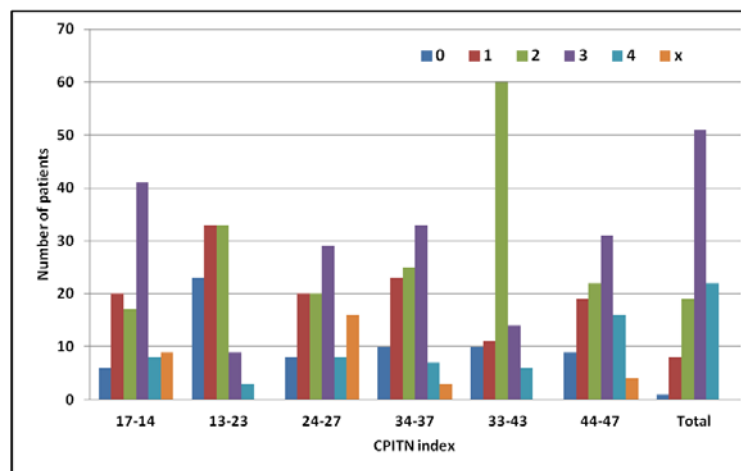
Fig. 2 – Distribution of the community periodontal index of treatment needs (CPITN) by sextants in relation to the total number of examinees
0 – healthy; 1 – bleeding observed, directly or by using mouth mirror, after sensing; 2 – calculus felt during probing but all the black area of the visible; 3 – pocket 4 or 5 mm (gingival margin situated on black area of probe); 4 – pocket > 6 mm (black area of probe not visible); x – missing teeth, consequently in those sextant did not have CPITN.

VOJNOSANITETSKI PREGLED

Discussion

The presence of risk factors in military population can significantly affect the frequency and severity of periodontal disease. This study shows that among study subjects most of them have bad habits such as the use of alcohol (59.4%), carbonated drinks (74.3%) and sugar (91.1%). However, there is a small number of non-smokers (32, 31.7%) compared to the smokers (69, 68.3%), who have approximately smoked for 6.38 ± 5.23 years. This is in accordance with the study conducted among the U.S. Army personnel, where a third of the examinees were smokers [15]. Other studies show that the incidence of smoking cigarettes is significantly higher (70%) among Lithuanian Army recruits [16]. This study shows that there is a clear relationship only between the soft debris index and the aforementioned bad habits ($r = 0.200$, $p = 0.046$).

According to the American Dental Association (ADA), the use of toothbrush and toothpaste, as well as a dental floss, is a fundamental aspect in maintaining oral hygiene [17]. Our study showed that more than a half (66.3%) of the subjects brushed their teeth more than once a day, while one-third (29.7%) brushed their teeth once a day. Almost all (89.1%) reported that they brushed their teeth for more than one minute. However, most of them did not use a dental floss (72.3%), mouthwash (74.3%), or interdental brushes (81.2%). This may be related to the wrong habits of oral hygiene, obligations at work, military activities in field conditions, fast-paced lifestyle and dietary habits.

Regardless of the fact that almost all (99%) agreed that the oral diseases affect the general health, only half (55.4%) of the subjects comes to the regular annual dental check-ups, while the other half (43.6 %) comes only when feeling pain in the oral cavity. Similar results were found in the Israel Army, where 55% of the population visited the dentist once in a year, 22% in two years and 20% in more than two years [18]. Much higher level of responsibility for oral health was present among the military population of high-income countries such as in the German Army [19].

When we looked at the values of oral hygiene (OHI-S), the oldest group of subjects aged 61–70 years (OHI-S = 1.63 ± 0.42) showed relatively poor hygiene in relation to the population aged 51–60 years (OHI-S = 0.95 ± 0.65). In addition, it was the same ratio of indices of soft layers (S-DI = $0.93 \pm 0.32 : 0.65 \pm 0.35$, respectively), and dental calculus (CI-S = $0.70 \pm 0.17 : 0.29 \pm 0.37$, respectively) of the two population groups. Apparently, the oldest study group was the least motivated, while the population aged 51–60 years showed the highest level of oral hygiene compared to other tested groups.

Among Croatian recruits, about 30% of the examinees had various degrees of gingival inflammation, whereas gingivitis was diagnosed in more that 80% among military professionals [2].

If one takes into account that half (49.5%) of our tested military population had bleeding gums when brushing their teeth, we can conclude that the technique of maintaining oral hygiene of large number of the subjects was rather inappropriate, although the adequate training on oral hygiene mainte- nance was continuously provided as well as the educational programs on proper brushing technique and use of additional means for maintaining oral hygiene. In our study, the mean CPITN increased from the youngest group (1.84 ± 0.71) to the oldest group of subjects (2.58 ± 0.85), and the average value of CPITN in the study population was 2.00 ± 0.82.

The subjects with the perfectly healthy periodontium (score 0) belonged to the age group of 51–60 years and represented a significant minority (5.6%). In the study of the Israel military personnel, the mean value of subjects with completely healthy periodontal tissues was 1.19% [8]. The call-up soldiers group (40.95%) had a significantly higher percentage of healthy periodontal status, as well as cadets group (57.95%) in the Italian examined population [20], and military personnel in Denmark (53%) [21]. This small number of subjects in our study with a fully healthy periodontium shows that the tested population in all categories has inadequate habits in maintaining oral hygiene. Additionally, the reason of the low percentage could derive from rare visits to dental clinics, although dental care services are easily accessible to them. For this reason, it would be necessary to pay special attention to this population in terms of a continuous preventive oral hygiene program.

When it comes to gingival bleeding (score 1), its frequency among the groups was almost the same: 21–30 years (10.3%), 41–50 years (11.8%) and 51–60 years (11.1%), while it was the least prominent in the group aged 31–40 years (3%). The total mean value of sextants with gingivitis in all the groups was 0.65 ± 0.26 and ranged from 0.42 ± 0.35 (51–60 years) to 0.75 ± 12.35 (41–50 years). These values were smaller compared to other studies [13]. All the age, groups had a lower mean value of the sextants (score 1) compared to sextants with calculus (score 2) and the presence of periodontal pockets (scores 3 and 4) demonstrating that the total population showed a trend in the development of periodontal disease.

The presence of calculus (score 2) was observed in 18.8% of the examined population in our study and it was the least present in the population aged 51–60 years (5.6%), and at the same time the mean value of sextant in the aforementioned age group was the lowest (0.83 ± 0.00). Other age groups had similar mean values of the presence of calculus. However, the percentage of calculus in our population was significantly lower than in the study by Sandoval and Puy [13]. In addition, the presence of calculus and gingival bleeding in the study by Katz et al. [8] showed that the younger generation had higher scores of these sextants, while older subjects had deeper periodontal pockets, which was not in accordance with our results.

The frequency of periodontal pockets of 4–5 mm (score 3) in the total population in our study was 50.5%, and it was also the most common sextant in our military population in relation to age, while 21.8% of the subjects had periodontal pockets greater than 6 mm (score 4), that is, a total of 72.3% of the military population had periodontal pockets of any kind.

When the presence of periodontal pockets of 4–5 mm by the age groups was observed, this score was dominant in each group, except in the age group of 41–50 (Table 6).

Our results, when compared to other countries, showed the incidence of patients with periodontal pockets of any

type to be significantly lower: 2.77% among Italian call-up soldiers and 0.35% among the cadets with periodontal pockets of 4–5 mm [20], 8% among the younger Danish military personnel [21], 10% among the Spanish military personnel [13], 14% among the American civilian population of the mean age of 30 years [22], and 19.1% in the noncommissioned personnel of the Israel Defense Force [23].

Only the values of shallow periodontal pockets in the Israel permanent force military population were similar to ours, and they were 49.9% [8], as well as in some previous reports [24].

Compared to the age groups, the mean values of sextants increased linearly, indicating that the younger population (21–30 and 31–40 years) also had a periodontal disease as well as the older age groups. Such a large percentage of periodontal pockets in the youngest examined group is a great concern. For more detailed conclusions and analysis of periodontal health, a comprehensive examination of the military population in our country is required. Based on our results, such program is already initiated.

This is the first study conducted within military population in Serbia, with the aim to assess the state of periodontal disease, and assist in the planning of oral health. From the results obtained during this study, we believe that our subjects need a proper preparation and implementation of prevention programs, especially including a relevant training in oral hygiene and education based on the prevention of the occurence and development of periodontal disease. This program should in some parts include clearly defined guidelines regarding the information related to prognosis and treatment of periodontal disease, which would be in the domain of individual optimum. The implemented program should promote healthy lifestyle, proper nutrition, cessation of smoking and use of alcohol and carbonated beverages, as well as healthy physical activity. This would further affect the improvement of psychophysical status required for certain military activities. Also, the treatment needs for the whole military population in our study suggest that, in adition to guidelines for maintaining oral health, the removal of all hard and soft tooth deposits, as well as removal of all iatrogenic factors are needed. In the worst case scenario periodontal scalling, root planing, and surgical treatment are necessary.

König et al. [6] find that more comparable and representative data are still needed to give us a clearer picture of the presence of periodontal disease in Europe, and the future challenges are to evaluate the reasons behind the differences in periodontal health in order to develop optimal prevention and treatment strategies.

Conclusion

In order to develop an oral health improvement strategy in the Serbian military population, it is necessary to collect more representative data that could later be integrated into the WHO database. Although this study was the first one in this field, a relatively small sample of the population was examined. The continuation of the study and new research should confirm or disprove these findings related to periodontal and oral health. Epidemiological studies can provide necessary information on the prevalence and incidence of periodontal disease in certain age and sex groups, indicate the possibility of prevention, appropriate treatment and reduce the incidence of periodontal disease by encouraging preventive measures through the increased activity of dental services in the field of military personnel education. Additional studies are needed because the very awareness of the subjects of the importance of oral hygiene is not sufficient, and it was not correlated well with the state of periodontal disease in the examined population.

Acknowledgements

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Etiology and mechanisms of ulnar and median forearm nerve injuries

Etiologija i mehanizmi povreda lakatnog i središnjeg nerva podlaktice

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Abstract

Bacgraund/Aim. Most often injuries of brachial plexus and its branches disable the injured from using their arms and/or hands. The aim of this study was to investigate the etiology and mechanisms of median and ulnar forearm nerves injuries. **Methods.** This retrospective cohort study included 99 patients surgically treated in the Clinic of Neurosurgery, Clinical Center of Serbia, from January 1st, 2000 to December 31st, 2010. All data are obtained from the patients' histories. **Results.** The majority of the injured patients were male, 81 (81.8%), while only 18 (18.2%) were females, both mainly with nerve injuries of the distal forearm – 75 (75.6%). Two injury mechanisms were present, transection in 85 patients and traction and contusion in 14 of the patients. The most frequent etiological factor of nerve injuries was cutting, in 61 of the patients. Nerve injuries are often associated with other injuries. In the studied patients there were 22 vascular injuries, 33 muscle and tendon injuries and 20 bone fractures. **Conclusion.** The majority of those patients with peripheral nerve injuries are represented in the working age population, which is a major socioeconomic problem. In our study 66 out of 99 patients were between 17 and 40 years old, in the most productive age. The fact that the majority of patients had nerve injuries of the distal forearm and that they are operated within the first 6 months after injury, promises them good functional prognosis.

Key words:
peripheral nerve injuries; forearm injury; diagnosis; median nerve; ulnar nerve; neurosurgical procedures.

Apstrakt

Uvod/Cilj. Povrede brahijalnog pleksusa i njegovih grana najčešće onemogućavaju povređene da koriste ruke. Cilj rada bio je da se ispitaju etiologija i mehanizmi povreda središnjeg i lakatnog nerva podlaktice. **Metode.** U ovu retrospektivnu kohortnu studiju bilo je uključeno 99 osoba operisanih na Klinici za neurohirurgiju Kliničkog centra Srbije u periodu od 1. januara 2000. do 31. decembra 2010. Svi podaci dobijeni su iz istorija bolesti povređenih. **Rezultati.** Većina povređenih bili su muškarci, 81 (81,1%), dok su samo 18 (18,2%) bile žene. Najveći broj povređenih imao je povrede nerava distalnog dela podlaktice – 75 (75.6%). Bila su zastupljena dva mehanizma povrede, transekcija kod 85 povređenih, a trakcija i kontuzija kod 14 povređenih. Najčešći etiološki faktor povrede nerava bila je posekotina, kod 61 povređenih. Povrede nerava često su bile udružene sa drugim povredama. U našem radu bile su 22 vaskularne povrede, 33 povrede mišića i tetiva i 20 fraktura kostiju. **Zaključak.** Većina pacijenata sa povredama perifernih nerava predstavljaju radno sposobnu populaciju, što predstavlja veliki socioekonomski problem. U našoj studiji 66 od 99 povređenih bili su stari od 17 do 40 godina, u svojim najproduktivnijim godinama. Činjenica da je većina povređenih imala povredu nerava distalnog dela podlaktice i da su operisani u prvih 6 meseci nakon povrede daje dobru prognozu njihove funkcionalne sposobnosti.

Ključne reči:
živci, periferni, povrede; podlaktica, povrede; dijagnoza; n. medianus; n. ulnaris; neurohirurške procedure.

Introduction

The forearm and hand represent complex functional unit of the joints, muscles, tendons, nerves, blood vessels and skin. Proper functioning and good condition of all of these elements is necessary to forearm and hand function to its fullest potential.

Most often injuries of brachial plexus and its branches disable patients from using their arm and/or hand. The hand is, along with the brain, the most important organ for the implementation of the tasks of adaptation, research, observation, perception and manipulation, unique to humans [1, 2]. The loss of its function can be a daunting experience, sometimes with serious economic and

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psychological consequences, and not so rarely with consequences of losing the job.

There are numerous ways to classify nerve injuries. However, the most used is classification by Seddon and Sunderland. In 1943 Seddon published his classification, and separated injuries into three categories — neuropraxia, axonotmesis, and neurotmesis – largely based on the scale of injury from microscopic to macroscopic [3]. In 1951, Sunderland [4] expanded upon this idea, subdividing neurotmesis into three additional grades. The Seddon classification is useful to understand the anatomic basis for injury, while the Sunderland classification adds information useful for prognosis and treatment strategies.

Sunderland I degree (neuropraxia)

Macroscopically nerve is intact, but histopathologically there may be segmental demyelination with continuity of the axon. Tinel's sign is negative, because there is no Wallerian degeneration. Nerve conduction is slower but electromioneurographic (EMNG) findings are normal. Spontaneous recovery is complete, and it usually occurs in few minutes to few weeks, three months at the longest [3–6].

Sunderland II degree (axonotmesis)

The axon and myelin sheet are damaged. There is Wallerian degeneration distally and regeneration. Tinel's sign is therefore positive, and it progresses distally. Endoneurium remains intact. Neurological exam shows total motor and sensory deficit, and EMNG registers fibrillations 2–3 weeks after injury, and after that denervation. Complete recovery can be expected [3–6].

Sunderland III degree

There is an injury to the axon, myelin and endoneurium but not the perineurium. In this type of injury the axon regenerates through scar changes of endoneurium tissue, which causes loss of some axons. Recovery is variable and incomplete, and it is accompanied by a certain degree of mismatch [3–5].

Sunderland IV degree

Continuity of the nerve in this type of nerve injuries is maintained by the epineurium, while there is an injury of axon, myelin, endoneurium and perineurium. Wallerian degeneration occurs distally from the injury. Spontaneous recovery is not possible [3–5]. Surgery is usually performed 3 months after injury (period in which previous three degrees of injury would recover).

Sunderland V degree (neurotmesis)

There is a complete transection of the nerve. Usual cause of injury is laceration, spontaneous recovery is not possible [3–6].

In the year 1988 Mackinon and Dellon added VI degree of injury to this classification. It is the combination of previous types of injuries, and it is characterized by neuroma in continuity. In accordance with this, the degree of recovery is different, and it can be complete (I and II degree), partial (III degree) or absent (IV and V degree) [5, 7].

Peripheral nerve injuries result in significant changes of proximal and distal nerve segments. Those changes are usually classified as morphological, biochemical and functional [7]. There are different mechanisms of peripheral nerve injuries [7]: Laceration (transection); Stretching (traction) and contusion; Ischemia and compression; Electric, thermal and radiation injury; Injection injury.

Approximately 30% of peripheral nerve injuries are lacerations. Traction and contusion injuries are also quite frequent and they usually occur combined with fractures and joint dislocations [7].

This paper focuses on ulnar and median nerve injuries. Traumatic isolated ulnar nerve injuries result in functional loss of ring and little finger flexion, thumb adduction and also interosseal muscles palsy. During examination Froment's sign can be seen.

Isolated median nerve injuries result in the loss of pronation of forearm, flexion of the wrist, index finger, middle finger and distal phalanx of the thumb; and also abduction and opposition of the thumb. Hypoesthesia of the first three fingers can be seen. During examination, thenar muscles atrophy can be seen.

The level of forearm nerve injuries can roughly be divided into high or low, referring to the distance of the lesion to the sensory and motor end organs. Surgical repair of high lesions generally have poorer outcome than low lesions [8, 9]. In high lesions, axons have to bridge a larger distance to the end organ than in lower lesion. Factors that influence outcome of nerve injury repair and healing are described in many studies [10–17]. They are often divided into intrinsic and extrinsic factors. Intrinsic factors can not be influenced by medical treatment, and they include age of patient [13], level and severity of injury [14, 16], nerve tissue loss [13], associated injuries [14, 15] and nerve type [14]. Opposite to them, extrinsic factors are dependent on the quality of treatment: type of repair [10], surgical technique [12], use of microsurgical equipment [17], timing of the surgery [14] and postoperative protocol [11].

The aim of this study was to present etiology and mechanisms of injuries of median and ulnar nerve of the forearm in 99 patients treated in the Clinic of Neurosurgery, Clinical Center of Serbia, from January 1st, 2000 to December 31st, 2010.

Methods

This retrospective cohort study included a total of 99 subjects, who were diagnosed and treated of ulnar and median nerve injuries of the forearm in the Clinic of Neurosurgery, Clinical Center of Serbia from January 1st, 2000 to December 31st, 2010. This study excluded patients with injuries that occurred as result of nerve entrapments, such as cubital and carpal tunnel syndrome, Guyon's canal syndrome or pronator teres syndrome. All the data were obtained from the patients histories. The patients were divided into three groups, dependent on the injured nerve: ulnar nerve group (U group), median nerve group (M group) and median-ulnar nerve group (MU group).

Statistical analysis was performed using statistical package PASW 18. For a description of the parameters of interest we used the methods of descriptive statistics: measures of central tendency (mean value), range, percentages and tabulation.

The study was approved by the relevant Ethics Committee, and it presents a part of the doctoral thesis project.

Results

Demographic characteristics of the patients, the side of injured arm, level, mechanism and etiology of injuries, associated injuries and post-injury period to surgery are shown in Table 1. The majority of injured patients from our series were male – 81 (81.8%), while only 18 (18.2%) were females. Male patients were the majority in all the three groups made according to the injured nerve. Age of patients was comparable in all the three groups, including both the mean age and the range of patients' age, although the widest range was in the U group, because of one 8-year-old patient. In our study, there were 25 ulnar nerve injuries of the left forearm and 21 ulnar nerve injuries of the right forearm. The median nerve was injured almost twice as often in the right than in the left forearm, 19 to 11, respectively. Injuries of both median and ulnar nerves occurred in the left forearm in 15 patients and in the right forearm in 8 patients. In total, occurrence of injuries was comparable between the left and the right forearm, with a slightly higher number of injuries in the left forearm. The majority of the patients had nerve injuries of the distal forearm – 75 (75.6%), of who in 71 cases the mechanism of injury was transection, while in just 4 cases mechanism was traction and contusion. In 24 (24.2%) patients with proximal forearm nerve injuries, mechanism of injury in 14 of them was transection, while in other 10 patients mechanism of injury was traction and contusion (Tables 1 and 2).

Table 1

Demographic data, the side of the injured arm, level, mechanism, associated injuries, and the period between injury and surgery of the studied patients

Parameters	Groups of patients			
	M	U	MU	Total
Number of patients, n (%)	30 (30.3)	46 (46.5)	23 (23.2)	99
Gender, n				
male	29	34	18	81
female	1	12	5	18
Age (years), mean (range)	33 (10–55)	33 (8–56)	36 (17–53)	33 (8–56)
Forearm affected, n				
left	11	25	15	51
right	19	21	8	48
Level of injury, n				
proximal forearm	7	12	5	24
distal forearm	23	34	18	75
Mechanism of injury, n				
transection	28	35	22	85
traction and contusion	2	11	1	14
Etiology of injury, n				
cutting by knife, axe, glass or ceramic	19	25	17	61
injury by circular saw, motor saw or grinder	3	5	2	10
fall	1	9	0	10
gunshot injury	2	2	2	6
car accident	2	4	1	7
unknown	3	1	1	5
Associated injuries, n				
yes	12	18	14	44
no	18	28	9	55
Time between injury and surgery, n				
3 weeks–3 months	12	19	6	37
3 months–6 months	9	18	11	38
6 months–12 months	9	8	5	22
more than 12 months	0	1	1	2

M – median nerve; U – ulnar nerve; MU – median-ulnar nerve.

Table 2

Mechanisms and the etiology of nerve injuries by the level of injery and by the gender

Variable	Level of injury		Gender		Total
	proximal	distal	male	female	
Mechanism of injury, n					
transection	14	71	72	13	85
traction and contusion	10	4	9	5	14
Etiology of injury, n					
cutting by knife, axe, glass or ceramic	10	51	49	12	61
injury by circular saw, motor saw or grinder	0	10	10	0	10
fall	5	5	7	3	10
gunshot injury	5	1	5	1	6
car accident	4	3	7	0	7
unknown	0	5	3	2	5

The most frequent cause of nerve injuries is cutting (by knife, axe, glass or ceramic), and it is four times more frequent in male than in female subjects. This type of injury is dominant in all three groups made according to the injured nerve. Also, it is five times more frequent in the distal than in the proximal region of the forearm. In our study sample there were 10 patients injured by circular saw, motor saw or grinder, and all of them were male with injuries of distal forearm. Gunshot injury was the etiological factor in 6 patients (Tables 1 and 2).

The most frequent type of injury in our study was transection. Out of 85 patients injured in this way, 34 had associated injuries. Although the number of patients in whom mechanism of injury was traction and contusion was significantly lower, out of 14 patients injured in this way, 9 had associated injuries. It should be noted that in our study every patient with gunshot injury had associated injuries (Table 3).

The most common associated vascular injury in the MU group of patients was injury of ulnar artery – in 7 of the cases, while the total number of associated vascular injuries in this group was 16. However, the highest number of bone fractures and muscle and tendon injuries was in the U group. The most common was fracture of the ulna and injury of *flexor digitorum superficialis* muscle and *flexor carpi ulnaris* muscle. The most frequently injured muscle in all the three groups was *flexor digitorum superficialis* muscle (Table 4).

Table 3

Mechanism and cause of nerve injuries according to associated injuries

Variable	Associated injuries (yes/no), n			
	group M	group U	group MU	Total
Mechanism of injury				
transection (n = 85)	10 / 18	11 / 24	13 / 9	34 / 51
traction and contusion (n = 14)	1 / 1	7 / 4	1 / 0	9 / 5
Etiology of injury				
cutting by knife, axe, glass or ceramic (n = 61)	7 / 12	6 / 19	9 / 8	22 / 39
injury by circular saw, motor saw or grinder (n = 10)	1 / 2	3 / 2	1 / 1	5 / 5
fall (n = 10)	0 / 1	4 / 5	0 / 0	4 / 6
gunshot injury (n = 6)	2 / 0	2 / 0	2 / 0	6 / 0
car accident (n = 7)	1 / 1	3 / 1	1 / 0	5 / 2
Unknown (n = 5)	0 / 3	0 / 1	1 / 0	1 / 4
Total	11 / 19	18 / 28	14 / 9	43 / 56

M – median nerve; U – ulnar nerve; MU – median-ulnar nerve.

Table 4

Distribution of associated injuries *per* nerve injuries

Types of associated injuries	Associated injuries, n			
	group M (n = 36)	group U (n = 46)	group MU (n = 23)	Total
Vascular injury				
brachial artery	2		2	4
ulnar artery	1	2	7	10
radial artery			2	2
ulnar vein			2	2
radial vein			1	1
cubital artery	1			1
interosseal artery			1	1
interosseal vein			1	1
Total vascular injuries	4	2	16	22
Bone fractures				
radius	1	4	2	7
ulna	1	5	1	7
humerus		2	1	3
costae*		1 (I–VII)	1 (I–V)	2
clavicula			1	1
Total bone fractures	2	12	6	20
Muscles and tendons				
abductor pollicis longus muscle	1			1
abductor pollicis brevis muscle	1			1
opponens pollicis muscle	1			1
flexor digitorum superficialis muscle	4	4	5	13
flexor digitorum profundus muscle	1	1	2	4
flexor carpi ulnaris muscle		4	4	8
flexor carpi radialis muscle	1			1
flexor pollicis longus muscle	1			1
palmaris longus muscle		1		1
abductor digiti minimi muscle		1		1
flexor digiti minimi brevis muscle		1		1

M – median nerve; U – ulnar nerve; MU – median-ulnar nerve.
*numbers in front the brackets indicate the number of patients with fractures of the ribs, while the number in brackets indicates broken ribs. The sum of serial rib fracture in one person is counted as one associated injury.

Figure 1 represents the distribution of frequency of the injured patients according to their age. It should be noticed that except for the two children of 8 and 10 years of age, all the injured patients from our study represent the population of working age and that the majority of those patients were 40 years old or younger.
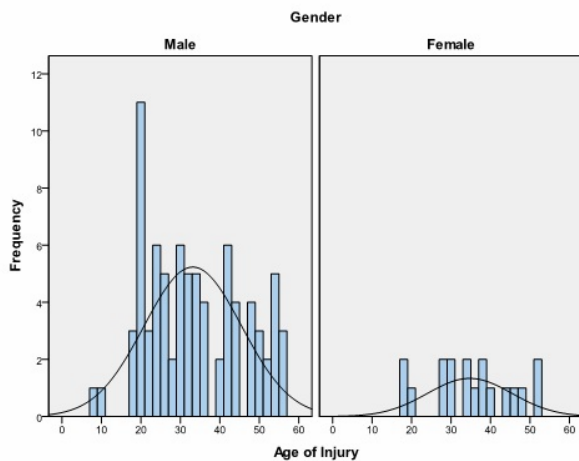
Fig. 1 – Age distribution at the time of getting injured.

Timing of surgery in our population is shown in Table 5. The majority of patients with transection injuries were surgically treated during the first 6 months of the injury, whereas the majority of the patients with traction and contusion injuries were treated between 6 months and one year after the injury.

bone fractures. From 22 vascular injuries, 16 was in MU group, the most common vascular injury was ulnar artery injury – 10 cases, of which 7 are in the MU group. The highest number of associated injuries were injuries of muscles and tendons, 33 of them. The most common were injuries of *flexor digitorum superficialis* muscle – in 13 cases, and they were dominant in all the three groups of patients.

The majority of patients from this study were male, 81 (81.8%). All mechanisms and causes of injuries in our study were predominant in males. If we take in observation that male population more often use knifes, axes, sharp metals, saws, chainsaws and weapons it can give us a possible explanation for a high male sex predomination. There is no significant difference between our results and the results from Galanakos et al. [18] and Ahmed et al. [19], who reported 72% and 89.19% injured males in their studies, respectively. The range of age in our study was from 8 to 56 years, and also except for the two patients who were 8 and 10 years old, all the others were in the working-age population. Our results are also comparable with the results of Ahmed et al. [19] who had reported the range of patients from 25 to 50, and Galanakos et al. [18] who had the range from 15 to 62. In our study we did not have data about professions of injured patients, and their return to work. However, as it can be seen from studies of other authors [20, 21], injuries of forearm nerves can lead to a delayed return to work, reduced productivity, lower functional abilities, loss of job and they can have unwanted social consequences. All of this provides the economic and social importance to this problem, since, most of the injured

Table 5

Distribution of patients according to mechanisms and the etiology of injury and timing of surgery

Parameter	Surgically treated patients (number)			
	timing of surgery			
	3 weeks to 3 months	3–6 months	6–12 months	more than 12 months
Mechanism of injury				
transection	34	34	16	1
traction and contusion	3	4	6	1
Etiology of injury				
cutting by knife, axe, glass or ceramic	30	19	11	1
injury by circular saw, motor saw or grinder	1	7	2	0
fall	2	5	2	1
gunshot injury	2	2	2	0
car accident	1	2	4	0
unknown	1	3	1	0

Discussion

The emphasis of this study was on etiology and mechanisms of forearm median and ulnar nerve injuries. These injuries are the most common peripheral nerve injuries. As it was also shown in some previous studies [18] they are often associated with injuries of surrounding structures, which this already difficult problem makes even harder. Associated vascular injuries compromise wound healing, and also, these injuries have direct effect on peripheral nerve regeneration.

Associated injuries of forearm nerves in our sample are: 22 vascular injuries, 33 muscle and tendon injuries and 20

are working-age people. If we take into observation that 66 out of 99 of our population were the patients in most productive age, between 17 and 40 years, it gives even bigger socioeconomic impact on this problem. These results are consistent with the results of peripheral nerve injuries of other authors cited by Kouyoumdjian [22].

Out of 99 patients in our study, 75 of them had nerve injuries of the distal part of the forearm. Nerve injuries of distal forearm, have better prediction for motor and sensory recovery than injuries of proximal parts of forearm [8, 9, 14, 16]. The reason for better functional recovery in distal nerve injuries is that axons have to bridge a shorter distance to the

organ in comparing to high or proximal nerve injuries. Besides that, proximal nerve injuries of forearm affect bigger number of muscles which causes lower functional abilities of the hand of injured patient. In our study two causes of nerve injuries that led mostly to injuries of the proximal forearm region are gunshot injuries and car accidences. In the majority of cases, these etiological factors are associated with other injuries. All 6 patients with gunshot injury had associated injuries, while out of 7 patients injured in a car accident, 5 had associated injuries. The reason that most of injured patients in our sample had injuries of the distal forearm and that 56 out of 99 patients did not have associated injuries gives good functional prognosis to the majority of them.

The majority of patients with transection injuries were surgically treated within the first 6 months of the injury, whereas the majority of the patients with traction and contusion injuries were treated within 6 months and one year after the injury, as shown in Table 5. Opinions about timing of the surgery are divided among experts in peripheral nerve surgery. Some experts believe that patients with clear nerve transection injuries should be operated urgent [23]. Others, however, believe that it is better to wait 3 weeks, when the process of Wallerian degeneration is over. The majority of surgeons agree that nerve reparation procedure should be executed within the first 6 months after injury, at the latest within a year. After that period of time, results of surgical treatment are poorer. However, in the last years, more and more experts of peripheral nerve surgery advise additional examination for late referrals. The claim is that if there are fibrillations present in the muscle, surgical treatment is indicated even one or more years after injury, and the results are satisfactory [24].

The etiology and mechanism of injury are of the most significant factors in making decision on treatment modality. Timing of the surgery is determined by it, as previously explained. Also, the choice of treatment depends on these factors. In transection injuries, and especially in the cases where injury of the nerve is evident from the moment of injury, surgery will be performed earlier. With this in mind, nerve tissue will not be contracted, so direct suture of the nerve usually can be accomplished. And with this, the chances for full recovery are high. However, in traction and contusion injuries, although continuity of the nerve is macroscopically intact, larger portion of the nerve is affected. Surgery is usually performed between three and six months after injury, and in this period nerve stumps are retracted. During surgery, a particular portion of the nerve is found "empty", so together with retraction of the nerve stumps, and resection of the damaged nerve, a large defect is present. There is no possibility for direct suture, so nerve grafting or neurotization must be performed. Results of these types of treatment are usually poorer [3].

Conclusion

The etiology and mechanism of injury are of the most significant factors in choosing treatment. Traumatic injury of peripheral nerves is a worldwide problem and can result in a significant disability. Median and ulnar nerves are the two most commonly injured nerves in upper extremity, which occur most in the working age population. In our sample there were two mechanisms of injuries of forearm nerves, transection, and traction and contusion. The dominant cause of injury was a transection with a knife, axe, glass or ceramic. The most common associated injury was injury of the ulnar artery, injury of the *flexor digitorum superficialis* muscle and fracture of the ulna.

The fact that the majority of patients had nerve injuries of the distal forearm and that they are operated within the first months after the injury, gives them good functional prognosis.

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Quality of life of the elderly in urban and rural areas in Serbia

Kvalitet života starih u urbanoj i ruralnoj sredini u Srbiji

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Abstract

Background/Aim. The number of elderly people in the world is growing, in Serbia as well. Serbia is already among the top ten countries with the oldest population, it is the fact. Aging influences the quality of life in different ways. The aim of this study was to assess the health-related quality of life of the elderly in urban and rural areas in Serbia. **Methods.** The study included 100 elderly people aged 65 years and above in urban and rural areas in Serbia. The next questionnaires were used: a sociodemographic questionnaire and a Serbian version of standardized European Euro-QoL questionnaire (EQ-5D-3L), as a basic index for the assessment and description of the quality of life. **Results.** In the structure of the respondents, according to the achieved social contacts ($p = 0.012$), the life of those with family members ($p = 0.009$), and health status ($p = 0.000$), in relation to the place of residence there was a statistically significant difference. There was a significant difference ($p = 0.040$), predominantly poor score for anxiety/depression within the rural population. The average value of quality of life in urban and rural areas was not statistically significant ($p = 0.720$). For those living in rural areas there was a statistically significant positive correlation between anxiety/depression and age, wealth status, marital status, living with family members and achieving social contacts, while a negative correlation was observed between anxiety/depression and education. **Conclusion.** On the basis of the data of our study, we can say that the presence of anxiety/depression among older people is greater in rural than in urban areas. The results of this study show that the perception of anxiety/depression among older in rural areas is bigger with the age and poverty increasing, the loss of a spouse, life without family members, lack of achievement of social contacts and lower education.

Key words:
quality of life; aged; residence characteristics; questionnaires; risk factors.

Apstrakt

Uvod/Cilj. Broj starih osoba u svetu i Srbiji raste, a činjenica je i da se Srbija već nalazi među deset zemalja sveta sa najstarijim stanovništvom. Proces starenja na različite načine utiče na kvalitet života. Cilj rada bio je da se proceni kvalitet života u vezi sa zdravljem starih u urbanoj i ruralnoj sredini u Srbiji. **Metode.** Istraživanje je obuhvatilo 100 starih osoba od 65 godina i više, u urbanoj i ruralnoj sredini. Korišćeni upitnici bili su sociodemografski upitnik i srpska verzija standardizovanog evropskog upitnika Euro-QoL (EQ-5D-3L), kao bazični indeks za procenu i opis kvaliteta života. **Rezultati.** U strukturi ispitanika prema ostvarivanju socijalnih kontakata ($p = 0,012$), životu ispitanika sa članovima porodice ($p = 0,009$) i zdravstvenom stanju ($p = 0,000$), u odnosu na mesto stanovanja, utvrđena je statistički značajna razlika. Nađena je značajna razlika ($p = 0,040$), sa dominacijom loših ocena, za usamljenost unutar ruralne populacije. Kod ispitanika koji žive u ruralnoj sredini utvrđena je statistički značajna pozitivna povezanost između usamljenosti i godina života, materijalnog stanja, bračnog statusa, života sa članovima domaćinstva i ostvarivanja socijalnih kontakata, dok je negativna povezanost uočena između usamljenosti i obrazovanja. **Zaključak.** Na osnovu podataka ove studije možemo reći da su usamljenije stare osobe u ruralnoj sredini. Rezultati ovog istraživanja pokazuju da se percepcija anksioznosti/depresije kod starih u ruralnoj sredini povećava porastom godina starosti i siromaštva, gubitkom bračnog druga, životom bez članova porodice, neostvarivanjem socijalnih kontakata i nižim stepenom obrazovanja.

Ključne reči:
kvalitet života; stare osobe; stanovanje; upitnici; faktori rizika.

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Introduction

The World Health Organization (WHO) defines quality of life as the perception to individuals about their own position in life in the context of culture and value systems in which they live, and according to their goals, expectations, standards and interests. It is a broad concept which consists of an individual's physical health, psychological status, material independence, social relationships and their relationships with important external characteristics [1].

When the concept of quality of life relates to health, i.e. to monitor certain medical interventions, often uses the term "health-related quality of life" (HRQoL). From the perspective of health (or disease), quality of life refers to the social, emotional and physical well-being of individuals. Areas that contribute to the overall quality of life are natural and work ability, psychological status, social contact and somatic feeling [2].

Basically the person, regardless of the ages, status, education, religion or race, lies a unique desire to live out the life in satisfaction. Terms related to pleasure, prosperity, fulfillment, and happiness are closely related to the notion of quality of life (QL) [3]. Health is one of the most important prerequisites for quality of life, but not the only one. Information on quality of life can only be obtained from a person who only has access to their feelings and thoughts [4].

Demographic research in the world and in our country points the rapid growth in the number of elderly in the total population. In addition to the increase in the elderly population this period is characterized by the extension of life expectancy, which indicates the need to develop a comprehensive and well-organized social care for the elderly. Old people meet their needs, both within their families and nearest community, and in the institutions of health and social care. Behavior of family members and the community has an important role in adaptation to aging [5].

Most researchers agree with the statement that in old age there is no major change in personality traits, those that were present in the younger years are reflected, becoming strengthened and more prominent. When we talk about the personality traits of the elderly, it is an interesting question whether there are personality traits that allow the elderly a better adaptation to the aging process and old age than others. In addition, old age is characterized by the presence of one or more diseases. The old are often alienated, lonely, abandoned by their primary group. Lack of social support, change of residence, the loss of a close person, the process of retirement and so on occur as the crisis events [6].

In the definition of the health of the WHO is also mental health [6, 7]. Anxiety/depression is a disease of modern times. Anxiety is a feeling of restlessness, uneasiness, fear, and depression is a mood disorder in which the dominant feelings are low mood, sadness, apathy, indifference, social withdrawal, anxiety, and feelings of guilt and lower values, as well.

Contemporary arguments suggest that aging is not just a series of life crises, but growing through new lifetime opportunities and challenges. Successful and active aging involves harmonizing its capabilities with the capabilities satisfaction with life. Life satisfaction refers to the evaluation of their own lives, and is the cognitive component of subjective well-being. By self-esteeming, an individual maintains (non) acceptance of her/himself, which indicates the level of an individual's belief in his own abilities, importance, performance, and value [8].

The tendency of society should be focused on non-institutional care of the elderly because they feel most comfortably in their surroundings and their home, so that they could be more active. Unlike depressed people who are dissatisfied with themselves, lonely people are unhappy with relationships which they do (not) have with others. The increased care for the elderly in the local community can be provided in the clubs for their socializing, with a tendency that these clubs will grow into day care centers for the elderly. Prevention and mitigation of social isolation and loneliness among older people is important for practice, and for planning social interventions [9, 10].

The aim of this study was to assess the health-related quality of life of the elderly in urban and rural areas.

Methods

The study was conducted at the Health Center Jagodina in the period from September 15, 2013 to February 2, 2014 year on a sample of 100 respondents, 45 men and 55 women, where 50 patients were from urban and 50 from rural areas. Age of the patients ranged from 65 to 87 years (average age 71 ± 5 years).

The next socioeconomic characteristics were obtained by a questionnaire and were analyzed: gender, age, education, financial status, marital status, living with family members, the achievement of social contacts and health status.

As an instrument for assessing quality of life, the Serbian version of the standardized European EuroQoL questionnaire (EQ-5D-3L) [11, 12] was applied, as a generic instrument for the assessment and description of the quality of life of the elderly. The EQ-5D-3L descriptive system comprises the following 5 dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has 3 levels (no problems, some problems, extreme problems) as a categorical answer, based on personal health assessments, and responses were used to calculate the index. The results were analyzed by using the SPSS statistical software (SPSS version 21 IBM). All variables were analyzed by using the basic parameters of descriptive statistics.

Results

Table 1 shows the frequency of the socio-demographic characteristics of respondents (50 respondents from urban and 50 respondents from rural areas). The results showed that there was no statistically significant difference in sex in relation to place of residence ($p = 0.841$). The average age of respondents in urban areas was 77.44 ± 7.21 years. The respondents in rural areas were a little bit younger; their average age was 76.78 ± 6.74 years. The average age of respondents by place of residence was not statistically significant ($p = 0.636$). The respondents from urban and rural area did not differ in the level of education ($p = 0.266$), wealth status

Table 1
The frequency of socio-demographic characteristics of the studied elderly

Socio-demographic characteristics	Total, n (%)	Urban, n (%)	Rural, n (%)	p (χ^2-test)
Sex	100 (100)	50 (100)	50 (100)	0.841
male	45 (45)	22 (44)	23 (46)	
female	55 (55)	28 (56)	27 (54)	
Age (years), mean ± SD		77.44 ±7.21	76.78 ± 6.74	
Age (years)	100 (100)	50 (100)	50 (100)	0.636
65–74	39 (39)	18 (36)	21 (42)	
75–84	45 (45)	23 (46)	22 (44)	
> 84	16 (16)	9 (18)	7 (14)	
Education	100 (100)	50 (100)	50 (100)	0.266
without education	34 (34)	15 (30)	19 (38)	
elementary	41 (41)	19 (38)	22 (44)	
secondary and university	25 (25)	16 (32)	9 (18)	
Wealth status	100 (100)	50 (100)	50 (100)	0.194
the poorest	18 (18)	7 (14)	11 (22)	
poor	31 (31)	13 (26)	18 (36)	
average and rich	51 (51)	30 (60)	21 (42)	
Marital status	100 (100)	50 (100)	50 (100)	0.578
married	37 (37)	21 (42)	16 (32)	
never married	18 (36)	8 (16)	10 (20)	
divorced/widowed	45 (45)	21 (42)	24 (48)	
Family members	100 (100)	50 (100)	50 (100)	0.009[*]
living with	53 (53)	33 (66)	20 (40)	
living without	47 (47)	17 (34)	30 (60)	
Achievement of social contacts	100 (100)	50 (100)	50 (100)	0.012[*]
realized	64 (64)	38 (76)	26 (52)	
unrealized	36 (36)	12 (24)	24 (48)	
Health status	100 (100)	50 (100)	50 (100)	0.000[*]
1 disease	35 (35)	24 (48)	11(22)	
2 diseases	43 (43)	26 (52)	17 (34)	
3 or more diseases	22 (22)	0 (0)	22 (44)	

[*]**statistically significant difference.**

($p = 0.194$) and marital status ($p = 0.578$). There was statistically significant difference in the life of those with family members ($p = 0.009$), achieving social contacts ($p = 0.012$) and health status ($p = 0.000$) in relation to place of residence.

Table 2 shows the distribution of respondents according to the predictors of subjective assessment of quality of life (mobility, self-care, usual activities, pain/discomfort and anxiety/depression) in relation to place of residence. The results indicate that there was a statistically significant difference in anxiety/depression in relation to place of residence ($p = 0.04$), predominantly poor score of anxiety/depression within the rural population.

In order to determine the influence of socio-demo-graphic characteristics of respondents on anxiety /depression, as one of

Table 2
Distribution of respondents according to the predictors of subjective assessment of quality of life in relation to place of residence

EQ-5D-3L dimensions	Total, n (%)	Urban, n (%)	Rural, n (%)	p (χ^2-test)
Mobility	100 (100)	50 (100)	50 (100)	0.194
1.00	51 (51)	30 (60)	21 (42)	
2.00	31 (31)	13 (26)	18 (36)	
3.00	18 (36)	7 (14)	11 (22)	
Self care	100 (100)	50 (100)	50 (100)	0.266
1.00	25 (25)	16 (32)	9 (18)	
2.00	41 (41)	19 (38)	22 (44)	
3.00	34 (34)	15 (30)	19 (38)	
Usual Activities	100 (100)	50 (100)	50 (100)	0.474
1.00	41 (41)	21 (42)	20 (40)	
2.00	34 (34)	19 (38)	15 (30)	
3.00	25 (25)	10 (20)	15 (30)	
Pain/Discomfort	100 (100)	50 (100)	50 (100)	0.079
1.00	16(16)	12 (24)	4 (8)	
2.00	37(37)	18 (36)	19 (38)	
3.00	47(47)	20 (40)	27 (54)	
Anxiety/Depression	100 (100)	50 (100)	50 (100)	0.004[*]
1.00	50 (50)	33 (66)	17 (34)	
2.00	28 (28)	8 (16)	20 (40)	
3.00	22 (22)	9 (18)	13 (26)	

[*]**a statistically significant difference; EQ-5D-3L – European Quality of Life Questionnaire – Serbian version.**

the predictors of subjective assessment of quality of life, the correlation analysis was done. The values of Pearson's correlation coefficient (r) for all subjects together and separately by place of residence are shown in Table 3. There was a statistically significant positive correlation between anxiety/depression and the following socio-demographic characteristics: place of residence, age, marital status, living with family members and achievement of social contacts while statistically significant negative correlation was observed for the level of education and financial status of participants. When it comes to those living in urban areas a statistically significant positive correlation was observed between anxiety/depression and age and marital status, and there was negative correlation between anxiety/depression and financial situation. For those living in rural areas there was a statistically significant positive correlation between anxiety/depression and age, marital status, living with family members and achieving social contacts, while negative correlation was observed between anxiety/depression and education (Table 4).

group of people will decrease by 2.565 if moving from the group with moderate depression to the group with no depression. The relative log odds of being in a group of living with family members *vs* the group not living with family members will decrease by 2.565 if moving from the group with moderate depression to the group with no depression. The relative log odds of being in the group of living with family members *vs* the group not living with family members will decrease by 2.383 if moving from the group with extreme depression to the group with no depression. The relative log odds of being in a group not achieving social contacts *vs* the group not achieves social contacts will decrease by 2.383 if moving from the group with extreme depression to the group with no depression.

Discussion

The aim of this study was to assess the health-related quality of life of the elderly in urban and rural areas. The

Table 3

Correlation of socio-demographic characteristics with anxiety/depression showed by Pearson's correlation coefficient (r)

Socio-demographic characteristics	Anxiety/depression					
	total		urban		rural	
	r	p	r	p	r	p
Place of residence	0.250*	0.012				
Sex	0.110	0.274	0.178	0.217	0.060	0.677
Age (years)	0.600**	0.000	0.548**	0.000	0.725**	0.000
Education	-0.352**	0.000	-0.277	0.051	-0.389**	0.005
Wealth status	-0.406**	0.000	-0.598**	0.000	-0.174	0.226
Marital status	0.640**	0.000	0.615**	0.000	0.668**	0.000
Living with family members	0.354**	0.000	0.171	0.235	0.445**	0.001
Achievement of social contacts	0.366**	0.000	0.226	0.115	0.412**	0.003
Health status	0.140	0.164	0.076	0.600	-0.004	0.978

r – Pearson's correlation coefficient; *p* – statistical significance: * *p* < 0.05, ** *p* < 0.01.

The results of multinominal logistic regression analysis showed that the following socio-demographic characteristics of respondents were associated with anxiety /depression of elderly in rural area: age, education, wealth status, marital status, living with family members and achievement of social contacts. A one-unit increase in the variable age was associated with 0.242 increases in the relative log odds of being in moderate depression *vs* no depression. A one-unit increase in the variable age was associated with 0.566 increases in the relative log odds of being in extreme depression *vs* no depression. The relative log odds of being in a group with elementary school *vs* a group with secondary school and university will increase by 3.450 if moving from the group with moderate depression to the group with no depression. The relative log odds of being in a group without education *vs* a group with secondary school and university will increase by 20.922 if moving from a group with extreme depression to the group with no depression. The relative log odds of being in a group of poor people *vs* the average wealth people will increase by 2.457 if moving from the group with moderate depression to the group with no depression. The relative log odds of being in a group of married people *vs* divorced/widowed

analysis of respondent's subjective perception of mobility, self-care, usual activities, pain/discomfort and anxiety/ depression, was conducted. In this study, respondents mostly estimated to have some problems with walking about or are confined to bed; to have some problems with self-care and performing usual activities and with not to be able to take care of themselves, to perform daily activities; they told to feel moderate and extreme pain and to be moderately or extremely anxious/depressed. Apart from the frequency of bad scores in the total sample, there was a significant statistical difference in anxiety/depression in relation to place of residence. Specifically, the older people in rural areas showed the anxiety/depression to a greater extent. The studies on the differences in people's depression in urban and rural areas are contradictory. One study confirms a higher prevalence of depression among patients in rural environment [13]. The increased prevalence of depressing mood of older people in rural areas may be influenced by socio-demographic characteristics of the population, the decline of psychophysical capacities as the result of ageing, the loss of a beloved person, the drain of young people in the cities, inadequate networks of social support; all this results in an un

Table 4

Logistic regression model of the correlation of socio-demographic characteristics with anxiety/depression of elderly in rural area

Socio-demographic characteristics	Moderately anxious/depressed				Extremely anxious/depressed			
	B	p	Exp(B)	95% CI	B	p	Exp(B)	95% CI
Sex								
male	-1.204	0.083	0.3	0.077–1.168	-0.203	0.785	0.817	0.19–3.505
female‖	-	-	-	-	-	-	-	-
Age								
age (years)	0.242	0.005*	1.274	1.078–1.506	0.566	0.000*	1.762	1.319–2.352
Education								
without education	-18.27	0.998	1.16E-08	0.000	20.922	0.000*	1.22E+09	1.4E+08–1.06E+10
elementary	3.45	0.002*	31.5	3.685–269.24	20.603	-	8.87E+08	9E+08–8.87E+08
secondary and university‖	-	-	-	-	-	-	-	-
Wealth status								
the poorest	-20.04	-	1.99E-09	1.99E-09–1.99E-09	0.875	0.284	2.400	0.484–11.891
poor	2.457	0.007*	11.667	1.94–70.178	0.693	0.543	2.000	0.214–18.687
average and rich‖	-	-	-	-	-	-	-	-
Marital status								
married	-2.565	0.006*	0.077	0.013–0.471	-23.1	-	9.34E-11	9.34E-11–9.34E-11
never married	0.981	0.434	2.667	0.229–31.069	-1.386	0.373	0.250	0.012–5.262
divorced/widowed‖	-	-	-	-	-	-	-	-
Family members								
living with	-2.565	0.001*	0.077	0.016–0.369	-2.383	0.006*	0.092	0.017–0.510
living without‖	-	-	-	-	-	-	-	-
Achievement of social contacts								
realized	-1.179	0.104	0.308	0.074–1.276	-2.383	0.006*	0.092	0.017–0.510
unrealized‖	-	-	-	-	-	-	-	-
Health status								
1 disease	0.644	0.472	1.905	0.33–11.009	0.134	0.89	1.143	0.172–7.601
2 diseases	0.421	0.573	1.524	0.352–6.601	-0.56	0.523	0.571	0.103–3.183
3 or more diseases‖	-	-	-	-	-	-	-	-

‖ Reference category; B – coefficient of regression; Exp (B) – odds ratio; 95% CI – confidence interval; * – Statistically significant difference.

favourable social situation for older people in rural areas and they express feelings of loneliness, uncertainty and concern [4, 6].

Numerous studies have confirmed a connection between depressing mood of older people and socio-demographic characteristics [14]. The overall results of this analysis conducted among the respondents in rural areas, indicate a statistically significant positive correlation between anxiety/depression and age, marital status, living with family members and achieving social contacts, while a negative correlation was observed between anxiety/depression and education.

Our findings showed that older respondents had a greater sense of anxiety and depression. Some of the earlier studies have shown that depression increases more frequently among the oldest old than among younger old people [15]; depression is related to ageing and it is one of the most common mental disorders among older people [16]. This study indicate that older married people have the slightest feeling of depression, while divorced people, and widowed people expressed major depressing moods. In fact, the absence of a spouse makes the quality of life significantly worse. It's likely that older people will often experience the death of a spouse or a family member dealing with grief and mourning after his/her death. The loss of a spouse or beloved one can cause pathological responses of older people, as an introduction to a depressing mood. In old age, loss of a spouse is one of the biggest stresses in life. It is obvious that the results of this study suggest that old people living in single households have greater feelings of depression than old people living with family members. The lack of the usual family support makes the feeling of depression, loneliness and abandonment bigger [17]. The survey conducted by Buber and Engelhardt [18] confirms that the proximity of children and frequency of contacts between older parents and children are positively associated with depressing mood of older people because people without children expressed more symptoms of depression. According to a Coward's and Cutler's research, there has been a reduction in the number of older people living with their children worldwide. This decrease is found in all countries where there is a migration from rural to urban areas [19]. In our environment, life in rural areas is based on traditional resources – self-help and family support [20]. Migration from the countryside to the cities led to the gradual disappearance of traditional support for older people from their neighbours and relatives [10]. According to this study, the respondents who achieve social contacts feel less depressed than the respondents who do not achieve social contacts. Results of previous studies show that poor social support and under-developed network of social contacts can lead a person to social isolation. Facing the unfulfilled life activities and the decline of self-esteem, and not putting enough effort into life renovation and reorganization, lead the individual to a higher or lower degree of social isolation [10, 21]. Feelings of social isolation, abandonment and loneliness may also be the signs of depression [22]. Taking into account that the achievement of social contacts (more visible in urban areas according to the results of the experiment) carries less risk of depression increase, it is not surprising that the rate of depression in rural areas is significantly higher than in urban ones. The results showed that respondents with no formal education feel more depressed than those with primary education, while respondents with secondary and higher education level feel least depressed. Several previous studies show that respondents with lower education have a weaker sense of mental and physical health, and that certain psychosocial interventions have a greater effect on individuals who have less psychosocial resources, less optimism, or a lower level of education [7]. A "Study of Living Standards", implemented by the Republic Institute for Statistics of Serbia [23] and a study conducted by Sataric et al. [24], present the results that older people without school, with incomplete primary education or with primary school in a rural area have the highest poverty rate and negative feelings such as abandonment, helplessness, loneliness. Our research show that poor people feel more depressed than average wealth people. Other studies confirm that worring about money is a significient predictor of depression of elderly people [25].

Conclusion

On the basis of the results of our study, we can conclude that the presence of anxiety/depression among older people is greater in rural than in urban areas. The perception of anxiety/depression among older people in rural areas increases with increasing of age and poorness, lossing of a spouse, living without family members, lacking of achievement of social contacts and lower education. Anxiety/depression influences the quality of life of older people, so early detection of depression, and the assessment of correlation with socio-demographic characteristics are very important for improving the quality of life of older people in urban, and especially, in rural areas.

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ORIGINAL ARTICLE

Adverse drug reactions in hospitalized cardiac patients: characteristics and risk factors

Neželjena dejstva lekova kod hospitalizovanih kardioloških bolesnika – karakteristike i faktori rizika

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Abstract

Background/Aim: Adverse drug reactions (ADRs) appear more frequently than actually reported and registered. The main goal of our work was to analyze risk factors, incidence and characteristics of ADRs in hospitalized cardiac patients. **Methods.** This prospective study included 200 patients, hospitalized at Cardiology Center of the Clinical Centre of Montenegro. ADRs were collected using specially designed questionnaire, based on the list of symptoms and signs that could point out to potential ADRs. Data from medical charts of patients, lab tests and other available parameters were observed and combined with the data from questionnaire. Severity of ADRs were assessed as serious or nonserious according to the World Health Organization criteria. Causality was assessed using the Naranjo probability scale. **Results.** A total of 34% of all the patients experienced at least one ADR. The most common ADRs occurred as nervous system disorders, less frequent were cardiovascular disorders, while the immune system disorders were the rarest. Sixteen percent of all ADRs were characterized as serious, most often caused by carvedilol and amiodarone. The majority of patients (97.3%) recovered without consequences. The multivariate analysis showed independent significant associations between ADRs and age, gender, comorbidities and polypragmasia. **Conclusion.** ADRs represent a significant issue in hospitalized cardiac patients population. The most significant predictors for ADRs in observed population were age, comorbidity, number of medications used during hospitalization and patients' gender. Preventive measures such as pharmacotherapy rationalization and continual education of health care professionals could reduce the frequency of ADRs appearance in patients with detected risk factors.

Key words:
drug toxicity; heart diseases; hospitalisation; risk factors.

Apstrakt

Uvod/Cilj. Neželjene reakcije na lekove (NRL) javljaju se mnogo češće nego što se registruju i prijavljuju. Glavni cilj rada bio je analiza rizičnih faktora, učestalosti pojavljivanja i karakteristika NRL kod hospitalizovanih kardioloških bolesnika. **Metode.** Sprovedena je prospektivna studija, u koju je bilo uključeno 200 hospitalizovanih bolesnika u Centru za kardiologiju Kliničkog centra Crne Gore. NRL su prikupljane korišćenjem specijalno urađenog upitnika, baziranog na listi simptoma i znakova koji bi mogli ukazati na eventalne NRL. Iz istorija bolesti prikupljani su podaci o laboratorijskim nalazima i drugim relevantnim parametrima, koji su kombinovani sa podacima iz upitnika. Klasifikacija NRL je izvršena po kriterijumima Svetske zdravstvene organizacije, a uzročno-posledična povezanost korišćenjem Naranjo skale. **Rezultati.** Ukupno 34% bolesnika ispoljilo je bar jednu NRL. Najčešće NRL su se ispoljile kao poremećaj u centralnom nervnom sistemu, zatim kao kardiovaskularni poremećaji, dok su najređe bili zastupljeni poremećaji imunog sistema. Ozbiljne NRL su činile 16% od svih otkrivenih NRL, najčešće prouzrokovane korišćenjem lekova karvedilol i amjodaron. Većina bolesnika (97,3%) oporavila se bez posledica. Multivarijantna analiza je ukazala na postojanje nezavisne povezanosti između pojavljivanja NRL i starosti bolesnika, pola, pridruženih bolesti kao i pol-

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ipragmazije. **Zaključak.** Pojava NRL predstavlja veliki problem u populaciji hospitalizovanih kardioloških bolesnika. Najznačajniji prediktori za njihov nastanak su starost bolesnika, pridružene bolesti, polipragmazija i pol bolesnika. Uvođenjem preventivnih mera, kao što su racionalizacija farmakoterapije i dodatne mere obuke zdravstvenih radnika, mogla bi se sniziti

učestalost pojavljivanja NRL kod bolesnika sa faktorima rizika.

Ključne reči:
lekovi, toksičnost; srce, bolesti; hospitalizacija; faktori rizika.

Introduction

Adverse drug reactions (ADRs) appear more frequently than actually reported and registered. Adverse drug reactions are common causes of morbidity and mortality within the hospital setting. The hospital environment, with its clearly defined patient population, is an ideal setting to identify potential adverse drug reaction signals [1].

According to the literature information, it has been estimated that 10–30% of hospitalized patients experience ADRs [2–5] and 0.3–10% of all hospital admissions are actually the results of ADRs [6–8]. In hospital environment, 3% of all fatal outcomes are caused by ADRs [9]. ADRs also cause prolongation of the hospitalization period and increase of hospital costs [5].

It is estimated that ADRs could have been prevented in about 50% of cases [8–11].

Varieties in frequency of occurrence of ADRs during hospitalization among different studies could be explained by different methods of investigation. While in some studies only spontaneously reported ADRs were recorded, in others, ADRs were recorded by using intensive monitoring systems [6, 12]. Furthermore, there are significant differences between stimulated *versus* non-stimulated reporting systems, as well as between manual and electronic active monitoring systems [12]. Prospective collection of ADRs, in contrast to retrospective data collection (which rely on chart review), has many advantages, mostly due to, most often, daily visits by a trained health care professionals on selected departments, over a restricted time period, in order to obtain records of all patients and suspected events [13–15].

Furthermore, earlier studies have emphasized that adverse drug events (ADEs) could often be prevented if physicians had had possible risk factors in mind [16–19]. Risk factors for ADEs include patient characteristics, drug-drug interactions, inappropriate number or dose of drugs and poor compliance [20].

Cardiovascular diseases are still the leading cause of morbidity and mortality worldwide. It is estimated that cardiovascular medications are one of the most common class of drugs associated with medication errors and ADRs [21]. The ADE prevention study group showed that odds ratio (OR) of severe ADEs with cardiovascular medication was 2.4 times greater than with other medications [22].

National ADR reporting system in Montenegro is organized by the Pharmacovigilance Department of the Agency for Medicines and Medical Devices of Montenegro. However, data show that the number of reports coming from health care professionals is quite low [23].

The main goal of this study was to analyze ADRs, as well as the potential risk factors for their appearance in pati-

ents hospitalized in the Cardiology Center of the Clinical Center of Montenegro. In order to prevent the occurrence of ADRs, it is necessary to provide proposals and measures for establishing ADR monitoring system in hospital environment.

Methods

Study design and patients selection

This prospective study included 200 patients hospitalized in the Cardiology Center of the Clinical Center of Montenegro in a 6-month period (April 1–October 1 2013).

Before the interview, the patients received an information sheet and gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Clinical Center of Montenegro.

Inclusion criteria were: adult patients, older than 18 years, of both gender admitted to Cardiology Center, hospitalized for three or more days, conscious, oriented and capable to understand questions and provide clear and comprehensible answers.

Exclusion criteria were: patients younger than 18 years, those with dementia or other causes of disorientation, with severe illness (e.g. cardiogenic shock, pulmonary oedema, etc.), short period of hospitalization (less than 3 days) and patient's refusal to participate in the trial.

Definition and classification of adverse drug reactions (ADRs)

Definition of ADRs according to the World Health Organization (WHO) was used in this research [24].

ADRs were characterized by using Rawlins and Thompson classification [25]. Each ADR severity was assessed in accordance with the WHO criteria [24]. The causality relationship between the drug and the effect was established by using Naranjo's ADR probability scale [26]. ADRs were classified by criteria suggested by Meyboom et al. [27] as type A ("drug actions"), type B ("patients reactions") and type C ("statistical ").

In addition, the level of intervention was attributed, using a 4-level scale (level 1 – no change in the treatment; level 2 – dose adjustment or drug stop, no additional treatment required; level 3 – dose adjustment or drug stop, additional treatment required, and level 4 – transfer to intensive care unit). Each ADR was also classified according to the system-organ class.

Patient interview

A special questionnaire was designed to register patient data, disease state(s), reason(s) for hospitalization and use of medi-

cation in the hospital. The interviewers completed this part of the questionnaire before interviewing the patient, in order to have the drug use in mind when interviewing the patients.

There were three approaches of gathering information from patients, regarding ADRs. At the beginning, patients were asked a standard open question, i.e. whether they experienced an ADR. In case of a positive answer, such ADRs were noted. Afterwards, patients were asked questions regarding complaints concerning the different organ systems, which helped them to recall experienced ADRs. Finally, the patients were asked about specific ADRs, mentioned in summary of product characteristics, in relation to drugs administered during hospitalization.

For reports based on the patient interview, interviewer and the treating physician discussed the causality of ADRs.

Data from patients' history, referring possible ADRs, complemented by data from the questionnaire, were imported together in the electronic database.

Statistical analysis

Statistical data analysis was performed using IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). Results were presented as frequency, percent and mean ± standard deviation (SD). For parametric data, independent samples, *t*-test was used to test differences between the groups. Mann-Whitney U test was used for obtaining a significance between ordinal data. χ^2 test or Fisher's exact test was used to test the differences between nominal data (frequencies). The association between potential risk factors and ADRs was evaluated using binary logistic regression, expressing the strength of association by crude and adjusted odds ratio (OR) with 95% confidence intervals (CI). A *p* value of < 0.05 was considered significant.

Results

Inclusion criteria were met by 200 patients, whose general characteristics are presented in Table 1.

The average age of all the patients was 60.5 ± 10.0 years. Significantly more ADRs occurred in the elderly.

A significance was also obtained in the frequency of ADRs between the male and the female patients (significantly higher in females), also in the patients with comorbidities. No significant differences in ADRs occurence were observed among different patients occupations, as well as concerning education level. The presence of risk factors for cardiovascular diseases has not affected significantly ADRs manifestation.

The most commonly used medicines among our patients were acetylsalicylic acid, clopidogrel, pantoprazole, simvastatin and ramipril (Table 2).

Table 1

Demographic data of the tested cardiac patients

Characteristic	Patients without ADRs (n = 132)	Patients with ADRs (n = 68)	p
Age (years), n (%)			
≤ 65	95 (70.9)	39 (29.1)	0.037*
> 65	37 (56.1)	27 (43.9)	
Sex, n (%)			
male	100 (72.5)	38 (27.5)	0.004*
female	32 (51.6)	30 (48.4)	
Occupation, n (%)			
employed	24 (70.6)	10 (29.4)	0.643
unemployed	46 (62.2)	28 (37.8)	
retiree	62 (67.4)	30 (32.6)	
Education level, n (%)			
elementary	7 (63.6)	4 (36.4)	
college	87 (66.9)	43 (33.1)	0.871
undergraduate	19 (59.4)	13 (40.6)	
graduate	18 (69.2)	8 (30.8)	
Comorbid condition, n (%)	32 (24.2)	36 (52.9)	< 0.001*
Risk factors for CVD*, n (%)	124 (93.9)	63 (92.6)	0.766
Number of drugs, x̄ ± SD	7.1 ± 2.6	8.9 ± 2.6	< 0.001*
Duration of hospitalization (days), x̄ ± SD	6.4 ± 3.9	8.6 ± 6.6	0.016*

CVD – cardiovascular disease; ADRs – adverse drug reactions; *statistically significant difference.

Table 2

The most commonly used medicines and therapeutic drug groups

10 most commonly used medicines		10 most commonly used therapeutic drug groups	
Name of the medicine	n (%)	Name of the therapeutic drug group	n (%)
Acetylsalicylic acid	168 (11.2)	Antiplatelet drugs	281 (18.7)
Clopidogrel	112 (7.4)	ACE inhibitors	179 (11.9)
Pantoprazole	108 (7.2)	Beta blockers	143 (9.5)
Simvastatin	87 (5.8)	Diuretics	140 (9.3)
Ramipril	69 (4.6)	Statins	138 (9.2)
Metoprolol	66 (4.4)	Proton-pump inhibitors	110 (7.3)
Enoxaparin	54 (3.6)	Nitrates	86 (5.7)
Furosemide	47 (3.1)	Anticoagulants	69 (4.6)
Hydroclorothiazide	46 (3.1)	Antidiabetics	56 (3.7)
Atorvastatin	43 (2.9)	Calcium channel blockers	43 (2.9)
Total	1.505 (100.0)	Total	1.505 (100.0)

A total of 34% of all the patients experienced one of the ADRs, but 7 of them experienced two ADRs at once.

The most frequent ADRs were caused by isosorbide mononitrate in 10.7%, by carvedilol in 8.0%, by metoprolol in 8.0% and by simvastatin and enoxaparin in 6.7% of patients. The characteristics of detected ADRs are presented in Table 3.

Table 3
Characteristics of the detected adverse drug reactions (ADRs)

Characteristics of ADRs	n (%)
Type	
A	64 (85.3)
B	4 (5.3)
C	7 (9.3)
Causality	
certain	8 (10.7)
probable	36 (48.0)
possible	31 (41.3)
Level of intervention	
1 (no change in dose)	29 (38.7)
2 (dose changed or drug stopped)	35 (46.7)
3 (drug stopped + additional therapy)	6 (8.0)
4 (transfer to intensive care unit)	5 (6.7)
Severity	
serious ADR	12 (16.0)
non serious ADR	63 (84.0)
Outcome	
recovery without consequences	73 (97.3)
recovery with consequences	2 (2.7)
ADR reported by	
a patient	30 (40.0)
the treating physician	29 (38.7)
the interviewer	16 (21.3)

According to Naranjo algorithm, causality was most commonly determined as probable. Certain ADRs were most commonly presented in patients who had taken isosorbid-mononitrate (flushing, headache), probable ADRs appeared with taking enoxaparin (injection site reactions) and possible ADRs were caused by metoprolol (bradycardia), carvedilol (bradycardia) and simvastatin (abdominal pain, constipation).

In almost 50% of all the patients with detected ADRs, dose change or discontinuation of the therapy had to be carried out.

A total of ADRs (16% of all of them) were classified as serious.

Serious ADRs were mostly caused by carvedilol (bradycardia that required additional therapy) and amiodarone (thyroid gland disorders, impaired vision). The majority of serious ADRs (9 of them) were recognized by treating physicians.

A great proportion of the patients recovered with no fur-ther consequences, but two patients had further complications.

ADRs most frequently affected the central nervous system (27%), than cardiovascular system (18%), gastrointestinal system (13%) and skin and subcutaneous tissue (12%).

Most common manifestations of ADRs were headache (16%), administration site reactions (10%), bradycardia (9%), dizziness (6%) and stomach ache (5%). The logistic regression analysis in which ADR was dependant variable was performed (Table 4).

Multivariate analysis, using binary logistic regression analysis with adjustment for the risk factors, is summarized in Table 4. There were several independent significant associations between ADR and age, gender and comorbidities (Adjusted OR > 2). We observed no significant co-linearity among potential risk factors. Interactions of all predictors in the model were examined, but we did not find any statistical significance among them.

Discussion

In the present study, ADRs occurred in 34% of the interviewed patients, and 16% of them were classified as serious ADRs.

The reported incidence of ADRs was higher than those reported in other studies, estimating that ADRs were present in 10–30% of hospitalized patients [27–38]. In the meta-analysis of Lazarou et al. [37], an incidence of 10.9% was found for patients experiencing an ADR during their hospitalization, among them serious ADRs amounted to 6.7%.

There could be several explanations for higher frequency of ADRs found in our study. Lazarou et al. [37] included only "definite" and "probable" ADRs, while in our analysis, we comprehended occurrence of "possible" ADRs [39, 40]. Furthermore, in our study, the patient interview was intensive, since the patients were also asked about ADRs related to their medication therapy.

In addition, hospitalized cardiology patients are often elderly with underlying comorbidities that impair the pharmacokinetics of drugs. These elderly patients are more likely to experience ADRs. Clearly, hospitalized patients are exposed to multiple risk factors predisposing them to ADRs [40, 41]. Predisposing factors like age, gender, comorbidity, number of drugs taken, and duration of hospitalization, have been reported as significant risk factors for the development of ADRs [42, 43].

It is shown that age is an important risk factor for ADRs. The incidence of ADRs is significantly higher in elderly, which is understandable since pharmacodynamics

Table 4
Logistic regression analisys [advanse drug reactions (ADRs) as dependent variable]

Independent variables	Univariate logistic regression		Multivariate logistic regression	
	crude OR (95% CI)	p	adjusted OR (95% CI)	p
Age (≤ 65 years old)	1.91 (1.03–3.52)	0.039*	2.29 (1.14–4.63)	0.020*
Gender	2.47 (1.32–4.60)	0.004*	2.04 (1.01–4.11)	0.047*
Co-morbidity	3.52 (1.89–6.54)	< 0.001*	3.81 (1.89–7.64)	< 0.001*
No of medications used during hospitalization	1.29 (1.14–1.46)	< 0.001*	1.29 (1.12–1.47)	< 0.001*
Duration of hospitalization	1.09 (1.02–1.15)	0.008*	1.07 (0.99–1.14)	0.073

*Statistically significant potential risk factors; OR – odds ratio; CI – confidence interval.

and pharmacokinetics change with age. In addition, homeostatic mechanisms become more and more impaired, which contributes to the increased occurrence of ADRs, along with the effect of coexisting disease. Increased consumption of medicines is another contributing factor for increased incidence of ADRs [44].

A study of Carbonin et al. [45] on 9,000 Italian patients, mainly older than 60 years, show that frequency of ADRs occurrence increases from 1.2% of patients medicated with one drug to 10% of patients comedicated with 9 drugs and 50% of patients with more than 10 drugs.

The presence and frequency of ADRs in Canadian patients, older than 50 years, were observed in a study of Grymonpre et al. [46] showing the increase of ADRs frequency from 5% of patients on therapy with 2 drugs, to more than 20% of patients co-medicated with 5 and more drugs.

Earlier studies have also reported a higher incidence of ADRs in females [34, 47].

This could be explained by the gender differences in the rate of drug metabolism, since they are significant even after correction made for lean body mass and body surface area [48]. In this context, higher occurrence of ADRs in women could be the consequence of lower body weight and glomerular filtration rate, as well as higher percentage of body fat in comparison with men [49].

In our study, causality was assessed as "certain" in 8% of cases, which does not differ from other available literature data [50–52], where the most "certain" ADRs were below 10% of cases. The majority of ADRs were assessed as probable and possible.

Frequency of serious ADRs in our study was lower (16%) comparing with some other research data. In a study performed by French Pharmacovigilance Center [53], serious ADRs occurred in 33% of cases, and Somers et al. [52] reached even 38%. Some other researchers [6, 34, 37] reported even lower frequency of serious ADRs, possibly as the consequence of differences in methodology and population of patients among performed studies.

Among ADRs registered using intensive monitoring system, the most frequent manifestations were observed as nervous system disorders, followed by cardiovascular and gastrointestinal disorders, which is consistent with literature data [54], especially when safety profile of cardiology patient therapy was considered.

Of all medicines, nitrates and beta-blockers caused the most ADRs. In similar research, performed by Sharminder et al. [55], nitrates and diuretics caused the majority of ADRs. In other study of Zaidenstein et al. [30], that included only cardiology patients, the main causes of ADRs were fibrinolytics, anticoagulation drugs and beta-blockers.

The occurence frequencies of ADRs type A, B and C in our study fully comply to data obtained from other authors [34, 52]. The higher incidence of type A ADRs compared to type B and type C suggests that numerous ADRs could be avoided.

Conclusion

Our results show that ADRs represent a significant issue in the population of hospitalized cardiac patients. The most significant predictors for ADRs occurred in the observed population are age, comorbidity, number of medications used during hospitalization and gender. It is necessary to implement preventive measures, recommended for all hospitalized cardiology patients in order to minimize the frequency of ADRs, as well as for better control of its detection. There is a necessity for urgent pharmacotherapy rationalization, in order to reduce the risk for ADRs. Therefore, additional educational efforts assigned for health care professionals should be made in order to raise consciousness regarding ADRs importance and risk factors contributing to their occurrence.

The importance of this research lies in the fact that this is the first ADRs monitoring in hospitalized cardiology patients in Montenegro, conducted in accordance with internationally accepted methodology, which may help increasing awareness to ADRs and conducting of further pharmacovigilance studies.

Acknowledgements

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ORIGINAL ARTICLE

Impact of Ki-67 and E-cadherin expression on lymphovascular invasion in upper urinary tract urothelial carcinoma

Uticaj ekspresije Ki-67 i E-kaderina na limfovaskalarnu invaziju kod urotelnog karcinoma gornjeg urotrakta

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Abstract

Bacground/Aim. Upper urinary tract urothelial carcinoma (UUT-UC) constitutes 5% of malignant neoplasms arising from transitional epithelium, but is more invasive than bladder cancer. Lzmphovascular invasion (LVI) is associated with biologically aggressive carcinoma and with occult metastases. The aim of this study was to investigate the correlation between LVI and immunohistochemical expression of two frequently routinely applied immunohistochemical biomarkers, Ki-67 and E-cadherin, in UUT-UC. **Methods.** The specimens from 106 patients with UUT-UC who had undergone nephroureterectomy were analyzed for pathologic parameters and LVI, while Ki-67 and E-cadherin expression were assessed by immunohistochemistry. **Results.** Ki-67 was overexpressed in 38% of the cases, while 45% of tumors demonstrated aberrant E-cadherin staining. The presence of LVI was significantly associated with tumor stage, grade, non-papillary growth, nodular invasion pattern, high Ki-67 labeling index and altered E-cadherin expression. Analyzing logistic regression models, we have shown that tumor properties such as stage, grade, growth and invasion pattern ($p < 0.001$), as well as the expression of Ki-67 and E-cadherin ($p < 0.001$) significantly predicted the presence of LVI. In the first model, only solid tumor architecture ($p < 0.05$) and nodular invasion pattern ($p < 0.05$) were significant predictors of LVI. In the second model, Ki-67 expression was found to improve the prediction of LVI ($p < 0.05$). **Conslusion.** Our results suggest that Ki-67 overexpression is an independent predictor of LVI in UUT-UC, indicating the progression of the disease. E-cadherin staining adds no valuable information to LVI probability assessment. This emphasizes the importance of Ki-67 staining of UUT-UC sections in routine pathological practice. Patients with Ki-67 overexpression, especially in solid tumors with nodular invasion, should be monitored more closely after surgery.

Key words:
urologic neoplasms; lymphatic metastasis; ki-67 antigen; cadherins; immunohistochemistry; predictive value of tests.

Apstrakt

Uvod/Cilj. Urotelni karcinom gornjeg dela urinarnog trakta (UUT-UC) čini 5% malignih neoplazmi koje potiču iz tranzicionalnog epitela, ali je invazivniji nego karcinom mokraćne bešike. Limfovaskularna invazija (LVI) je pokazatelj biološki agresivnog karcinoma, kao i okultnih metastaza. Cilj istraživanja bilo je ispitivanje povezanosti LVI i imunohistohemijske ekspresije dva često rutinski primenjivana biomarkera, Ki-67 i E-kaderina, u UUT-UC. **Metode.** Patohistološka analiza i određivanje prisustva LVI urađeni su na uzorcima UUT-UC dobijenih od 106 bolesnika podvrgnutih nefroureterektomiji. Ekspresija Ki-67 i E-kaderina procenjivana je imunohistohemijskom metodom. **Rezultati.** Prekomerna ekspresija Ki-67 zabeležena je kod 38% bolesnika, dok je 45% tumora pokazalo izmenjenu ekspresiju E-kaderina. Prisustvo LVI bilo je značajno udruženo sa stadijumom, gradusom tumora, nepapilarnim načinom rasta, nodularnim tipom invazije, visokim Ki-67 proliferativnim indeksom i aberantnom ekspresijom E-kaderina. Analizom logističkih modela regresije utvrđeno je da karakteristike tumora poput stadijuma, gradusa, načina rasta i invazije ($p < 0,001$), kao i ekspresija Ki-67 i E-kaderina ($p < 0,001$), značajno predviđaju prisustvo LVI. U prvom modelu samo solidna arhitektura tumora i nodularni način invazije ($p < 0,05$) predstavljali su značajne prediktore LVI. Drugi model utvrdio je da povećana ekspresija Ki-67 povećava verovatnoću za LVI ($p < 0.05$). **Zaključak.** Rezultati istraživanja ukazuju na to da prekomerna ekspresija Ki-67

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predstavlja nezavisni prediktor LVI u UUT-UC. Ekspresija E-kaderina ne doprinosi značajno proceni verovatnoće prisustva LVI. To naglašava značaj određivanja Ki-67 proliferativnog indeksa u ovom tumoru u rutinskoj patološkoj praksi. Bolesnike sa prekomernom ekspresijom Ki-67, posebno kod tumora sa solidnim rastom i nodularnim tipom invazije, trebalo bi pažljivije pratiti nakon hirurškog lečenja.

Ključne reči:
urološke neoplazme; neoplazme, limfne metastaze; ki-67 antigen; kaderini; imunohistohemija; testovi, prognostička vrednost.

Introduction

Upper urinary tract urothelial carcinoma (UUT-UC) constitutes only 5% of malignant neoplasms arising from transitional epithelium, but is more invasive and worse differentiated than bladder cancer [1]. Therefore, there is a strong need to acquire as precise as possible assessment of disease progression and tumor invasiveness in every individual case.

In UUT-UC, lymphovascular invasion (LVI) is associated with established features of biologically aggressive carcinoma, such as advanced stage, high tumor grade, metastases to lymph nodes, sessile tumor architecture, tumor necrosis, and concomitant carcinoma *in situ* [2,3]. LVI may be associated with occult metastasis, and thus identify patients who are at increased risk of cancer recurrence and mortality despite apparently effective radical nephroureterectomy [2]. UUT-UC patients with LVI detected in primary tumor require to be followed-up more closely [4] and may be selected for postoperative adjuvant chemotherapy [3].

Abnormal cell proliferation, which results from deregulation of the cell cycle, is fundamental in tumorigenesis. Previous studies have demonstrated that cell proliferation, as detected by Ki-67 staining, is significantly associated with differentiation, tumor stage, tumor recurrence and prognosis in patients with UUT-UC [4,5]. However, the expression of metastatic phenotype requires activation of additional effector genes or suppression of local inhibitors over and above those required for uncontrolled growth alone [6]. LVI, as a critical step in the systemic dissemination of cancer cells [7], may be tightly linked to inactivation of molecules involved in intercellular adhesion. Decreased expression of E-cadherin, the invasion suppressor, even in a limited fraction of neoplastic cells, is sufficient to allow the onset of invasion [8].

In urothelial carcinoma, the loss of membranous expression of E-cadherin has been unanimously attributed to an aggressive neoplastic phenotype. Besides the correlation between decreasing E-cadherin staining and the depth of invasion and higher grade in urothelial carcinoma [9–11], recent studies has indicated that immunohistochemical determination of E-cadherin expression may be a useful diagnostic aid and prognostic factor for UUT-UC [6,9,12].

Accurate estimates of the clinical stage and prognosis are essential for patient counseling and informed decision making. This is of great importance in UUT-UC treatment, since this cancer is invasive at diagnosis in over 65% of cases [13]. In respect of indisputable major significance of LVI, the aim of our research was to investigate the predictive impact of Ki-67 and E-cadherin expression on LVI in UUT-UC.

Methods

We examined formalin-fixed, paraffin-embedded specimens from 106 patients who had undergone open type nephroureterectomy with removal of bladder cuff for UUT-UC between 1995 and year 2010. The mean age of patients was 64.2 ± 10.8 years; the youngest patient was 32 years old, the oldest 87 years. There were 70 (66.0%) male and 36 (34.0%) female patients. Of the investigated UUT-UC, 78 (73.6%) had pelvic localization and 28 (26.4%) were tumors of ureter. During nephroureterectomy, enlarged lymph nodes were resected; no standard lymphadenectomy was undertaken. All cases of UUT-UC were diagnosed at the Institute of Pathology, Faculty of Medicine, Niš, Serbia.

The histological sections were processed by standard techniques, and stained with hematoxylin and eosin (HE). HE-stained slides were used to assess histological grade, pathologic stage, growth pattern of the tumor (papillary/solid), pattern of invasion (nodular/infiltrative), lymphovascular invasion and the presence of necrosis and metaplastic changes within the tumor. The 2002 Tumor Nodus Metasthasis (TNM) classification system [14] was used for pathologic staging, and the 2004 World Health Organization classification was used for histological grading of UUT-UC [15].

LVI was defined as the unequivocal presence of cancer cells in endothelium-lined lymphatic and vascular channels without underlying muscular walls [3]. By positive invasion was considered the presence of at least one well characterized malignant cell surrounded by endothelial cells. In the case of intravascular tumoral thrombus, it was usually floating completely free in the vascular lumen, with fibrin or plasma precipitate or erythrocytes around it. It was composed of tightly cohesive cells with a smooth border and a shrunk cytoplasm, and the cells in the periphery had a shell-like aspect [2]. Routine light microscopic examination was considered sufficient for LVI detection and no immunohistochemical staining was used to identify LVI particularly.

Immunohistochemical analysis

Tumors were analyzed using the mouse monoclonal antibody against E-cadherin (Takara Biomedical, Kyoto, Japan) at dilution of 1:1500, anti-Ki-67 antibody (Dako, Glostrup, Denmark) at 1:100 dilution, and a standard avidin–biotin immunoperoxidase complex detection system according to the manufacturer's protocol (Dako LSAB2R system-HRP). In brief, 4 μm tumor tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed in 0.1 M citrate buffer (pH 6.0) in a microwave oven. Endogenous peroxidase activity was quenched with 0.3% hydrogen

peroxide in methanol. After applying primary antibody, the slides were incubated for 60 minutes at room temperature. This step was followed by extensive washes with phosphate-buffered saline. Subsequently, sections were incubated with the secondary biotinylated antibody and with the streptavi-din/avidin–biotin–peroxidase complex solution. Staining was developed using a liquid 3,3'-diaminobenzidine (DAB) subs-trate kit. Sections were counterstained with Mayer's hematoxylin. Negative controls were carried out by omitting the primary antibodies. The technique quality was assessed and areas with greater positivity were selected, avoiding pe-ripheral area measurement, necrosis or artifact.

E-cadherin expression was scored according to the estab-lished criteria [16, 17] that classify tumors as normal if staining was similar to that of normal urothelium (> 90% of the cells are dyed). Aberrant tumor expression was defined as negative (complete absence of immunoreactivity), focally positive (< 10% of the cells were stained), and heterogeneous (10–90% of the cells were stained). In each case, it was determined whether the membrane or the cytoplasm was stained.

Ki-67 labeling index was calculated as the number of po-sitive nuclei ×100 *per* the total number of nuclei in ten random high power fields (×400) in each tumor. This index was estab-lished by counting at least 2,000 cells in fields distant from necrotic areas. The results were classified into following gro-ups: low Ki-67 expression (< 20% of cell nuclei stained posi-tive for Ki-67) and Ki-67 overexpression (> 20%) [18].

Statistical analysis

All data analyses were processed using the Statistical Package for Social Sciences, version 15.0 statistical software (SPSS, Chicago, IL). A *p* value of 0.05 or less was conside-red indicative of a statistically significant difference. Conti-nuous variables like age were represented as mean ± SD, and differences in the age of the patients among different groups were compared using Student's *t*-test. Categorical variables were analyzed by χ^2 and Fisher's exact test with Yates cor-rection. Binary logistic analysis was performed in SPSS.

Results

Normal surrounding transitional cell epithelium displayed exclusively membranous expression of E-cadherin, with staining of the cell-cell borders. Fifty eight tumors (54.7%) maintained normal staining pattern, while 48 (45.3%) showed altered E-cadherin immunoexpression (Fi-gure 1A). Within the aberrant group, the expression of E-cadherin was positive heterogeneous in 40 (37.7%), positive focally in 3 (2.8%) and negative in 5 (4.7%) of tumors. All tumors positive for E-cadherin displayed membranous positivity, while in 14 (13.2%) of the tumors with altered expression, significant cytoplasmic staining was also obser-ved. High Ki-67 labeling index was found in 40 (37.7%) of the tumors (Figure 1B).

Lymphovascular invasion was detected in 37 (34.9%) UUT-UC (Figures 1c and d). The association of LVI with pathological features, Ki-67 labeling index, and E-cadherin expression of the examined tumors are shown in Table 1.

LVI demonstrated strong correlation with tumor charac-teristics associated with high grade ($\chi^2 = 19.485$; $p < 0.001$), advanced stage ($\chi^2 = 24.580$; $p < 0.001$) and architecture of the tumor, with higher LVI occurrence in sessile tumors with solid growth ($\chi^2 = 19.485$; $p < 0.001$), than in papillary neoplasms. Moreover, LVI was associated with nodular type of invasion ($\chi^2 = 30.883$; $p < 0.001$) and the presence of necrosis
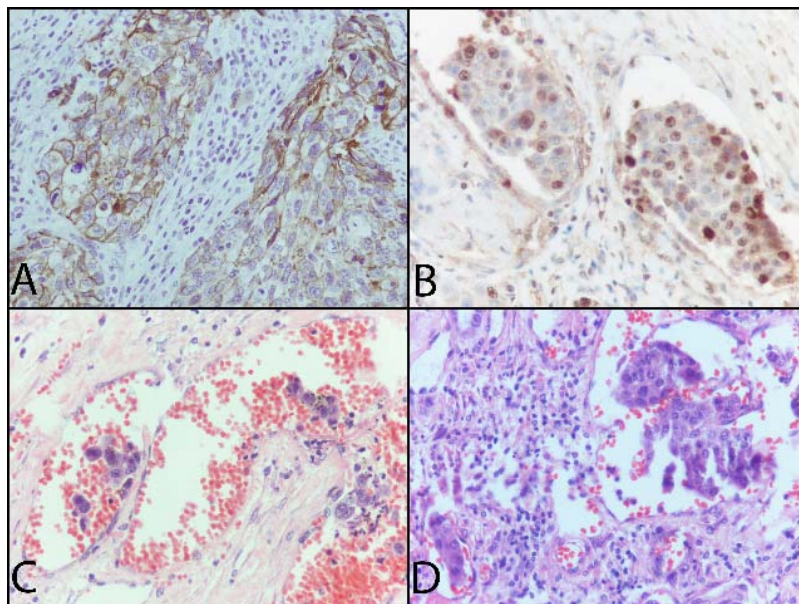
Fig. 1 – Representative photomicrographs of upper urinary tract urothelial carcinoma (UUT-UC), original magnification ×200: (A) Aberrant heterogeneous staining pattern of E-cadherin in UUT-UC; (B) Ki-67 immunostained nuclei of cancer cells in lymphovascular invasion; (C) and (D) Vascular invasion in UUT-UC infiltrating renal parenchyma, haematoxylin-eosin stain.

Table 1

The association of lymphovascular invasion with pathological characteristics, Ki-67 labeling index and E-cadherin expression and of UUT-UC

UUT-UC characteristics	LVI, n (%)		χ^2-test
	no	yes	p
Localization			5.835
pyelon	56 (52.8)	22 (20.8)	< 0.05
urether	13 (12.3)	15 (14.2)	
Multifocality			
no	47 (44.3)	26 (24.5)	0.052
yes	22 (20.8)	11 (10.4)	0.819
Grade			
low	40 (37.7)	5 (4.7)	19.485
high	29 (27.4)	32 (30.2)	< 0.001
Stage			
low	32 (30.2)	0 (0.0)	24.580
high	37 (34.9)	37 (34.9)	< 0.001
Growth pattern			
papillary	40 (37.7)	5 (4.7)	19.485
solid	29 (27.4)	32 (30.2)	< 0.001
Invasion pattern			
nodular	14 (13.2)	28 (26.4)	30.883
infiltrative	55 (51.9)	9 (8.5)	< 0.001
Necrosis			
no	47 (44.3)	15 (14.2)	7.543
yes	22 (20.8)	22 (20.8)	< 0.01
Ki-67 index			
< 20	53 (50.0)	13 (12.3)	17.805
> 20	16 (15.1)	24 (22.6)	< 0.001
E-cadherin staining			
normal	48 (45.3)	10 (9.4)	17.589
aberrant	21 (19.8)	27 (25.5)	< 0.001
E-cadherin type of expression			
negative	2 (1.9)	3 (2.8)	
focal	3 (2.8)	0 (0.0)	22.045
heterogeneous	16 (15.1)	24 (22.6)	< 0.001
homogeneous	48 (45.3)	10 (9.4)	
E-cadherin M/C			
negative	2 (1.9)	3 (2.8)	
membrane	59 (55.7)	26 (24.5)	3.687
membrane + cytoplasm	8 (7.5)	8 (7.5)	0.158

UUT-UC – upper urinary tract urothelial carcinoma; LVI – lymphovascular invasion.

($\chi^2 = 7.543$; $p < 0.01$), and was more frequently observed in neoplasms with ureteral than pelvic localization ($\chi^2 = 5.835$; $p < 0.05$). In addition, the presence of LVI was significantly associated with high Ki-67 labeling index ($\chi^2 = 17.805$; $p < 0.001$) and the loss of homogeneous membranous E-cadherin staining ($\chi^2 = 17.589$; $p < 0.001$), as well as with type of E-cadherin expression ($\chi^2 = 22.045$; $p < 0.001$).

Tumor characteristics and the level of immunohistochemical expression of the investigated markers, for which the association with LVI was observed, were tested in logistic regression analysis models. Stage, grade, growth and invasion pattern ($\chi^2 = 35.113$; $p < 0.001$), as well as the expression of Ki-67 and E-cadherin ($\chi^2 = 17.765$; $p < 0.001$) significantly predicted the presence of LVI. However, in the first model only solid growth ($p < 0.05$) and nodular type of invasion ($p < 0.05$) were good predictors of LVI (Table 2). In the second model, only Ki-67 overexpression was found to improve the prediction of

LVI ($p < 0.05$), while E-cadherin staining alteration did not demonstrate such quality (Table 3).

Discussion

UUT-UC is relatively rare disease, yet with a significant impact to mortality due to urothelial neoplasms. Poor prognoses have been reported for patients with tumors invading beyond the muscularis (pT3) and adjacent organs/perinephric fat (pT4), with 5-year survival rates of 54% and 19%, respectively [19]. Such outcomes indicate the importance of selecting patients at higher risk of disease-specific death, as well as adequate treatment strategies [20].

Invasion of tumor cells into blood vessels is an essential and important step in initiating metastatic cascade. LVI in primary tumor indicates that neoplastic cells have already invaded surrounding tissues [7]. Although further genetic altera-

Table 2

Binary logistic regression analysis of upper urinary tract urothelial carcinomas: tumor characteristics as model predictors

Tumor characteristics	B	S.E.	Sig.	Odds ratio	95.0% C.I.for odds ratio	
					lower	upper
Grade	-0.274	1.048	0.794	0.760	0.097	5.932
Stage	20.476	8.451E3	0.998	7.809E8	0.000	.
Growth pattern (solid)	1.692	0.864	0.050	5.432	1.000	29.514
Invasion pattern (nodular)	1.622	0.779	0.037	5.061	1.100	23.295
Constant	-42.502	1.690E4	0.998	0.000		

Table 3

Binary logistic regression analysis of upper urinary tract urothelial carcinomas: Ki-67 and E-cadherin immunohistochemical staining as model predictors

Parameters	B	S.E.	Sig.	Odds ratio	95.0% C.I.for odds ratio	
					lower	upper
Ki-67 index	1.459	0.607	0.016	4.303	1.309	14.151
E-cadherin expression (aberrant)	1.379	0.932	0.139	3.970	0.639	24.671
E-cadherin type of expression (heterogeneous)	0.092	0.428	0.830	1.096	0.474	2.536
Constant	-3.531	1.052	0.001	0.029		

tions are necessary for these disseminated cells to acquire the phenotype which will result in overt clinical metastases, LVI is unambiguous sign of disease progression and increased cancer mortality [2]. In recent study that comprised large international series of patients treated with radical nephroureterectomy for UUT-UC, LVI has been observed in 24% (349/1453) of the investigated patients [2]. In an investigation that *a priori* excluded lymph node positive patients from the study population LVI was found in only 13% (31/238) of patients [3]. In the present research LVI was detected in 34.9% of UUT-UC. The discrepancy may be due to study limitation caused by a relatively small number of investigated patients. This is a major reason for contemporary research of low-incidence diseases like UUT-UC to require multicentric collaboration.

There is a positive correlation between LVI and well established features of biologically aggressive UUT-UC [2]. Proportion of LVI increased with advancing tumor stage, high tumor grade, the presence of necrosis, sessile tumor architecture and the presence of squamous differentiation [2, 3]. The results of this study are in accordance with previous findings: LVI was associated with advanced stage, high tumor grade, solid growth and nodular invasion pattern. Moreover, the location of the primary tumor (renal pelvis/ureter) [21] and multifocality [22] were also proven to be independent variables affecting cancer-specific survival in UUT-UC. In the present study LVI was more frequently observed in ureteral tumors, but significant correlation between LVI and multifocality was not found.

LVI has been reported to be closely associated with metastases and a poor prognosis in urological malignancies [3, 23]. Recent study identified LVI as an independent predictor of clinical outcomes in non-metastatic patients who underwent radical nephroureterectomy for UUT-UC [2, 24]. In patients with localized UUT-TCC, LVI status may be a predictive marker

for recurrence-free and cancer-specific survivals [3]. Considering the importance of LVI assessment in UUT-UC diagnosis, we aimed to investigate the correlations between LVI and immunohistochemical expression of two well-known and frequently routinely applied immunohistochemical biomarkers: Ki-67 and E-cadherin.

Ki-67 is expressed during all phases of the cell cycle except G_0, rendering cellular expression of Ki-67 as a measure of tumor proliferation [25]. In UUT-UC, a significant association was observed between the overexpression of Ki-67 and the pathologic stage and tumor grade [4, 26], which was confirmed in our study. In a study by Fromont et al. [5] Ki-67 was the only one of the numerous investigated markers significantly associated with tumor stage in UUT-UC. In addition, besides the pathologic stage, Ki-67 overexpression was found to be an independent predictor of cancer specific survival in UUT-UC [4]. Our findings demonstrated significant association of Ki-67 labeling index and the presence of LVI in primary tumor. In logistic regression analysis Ki-67 expression, besides tumor growth and invasion pattern, was found to be the variable that improves the prediction of LVI. Our results strongly indicate mitotic index as a significant independent predictor of LVI. This emphasizes the importance of Ki-67 staining of UUT-UC sections in routine pathological practice, regardless if LVI has been observed in HE slides. We also found that Ki-67 index was significantly and independently associated with E-cadherin expression and tumor high grade, which concurs with previous results [4, 5, 26].

Mutation and inactivation of E-cadherin enables metastasis through induction of an epithelial-to-mesenchymal transition, invasiveness, and anoikis resistance [8]. In human tumors, loss or reduction of E-cadherin expression can be caused by somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the CDH1 promoter [8, 27]. Loss of E-cadherin

expression leads to a dissociation of cells from cohesive tissues and correlates with dedifferentiation and generation of invasive phenotype [9]. In the present study, preserved immunoreactivity of E-cadherin was recognized in 54.7% of the samples, which is similar to the results of previous investigations [4, 28]. Moreover, we observed that the majority of tumors with aberrant expression had a heterogeneous staining pattern with positive and negative areas within the tumor, in accordance with other studies [16, 29].

A reduced expression of E-cadherin has been linked not only with high grade and advanced stage, but also with disease progression and poor survival in UUT-UC [6, 12, 16]. Regardless of upper tract treatment modality, recurrence in the bladder consistently occurs in 20–50% of patients, necessitating the use of routine cystoscopic surveillance [30, 31]. Decreased expression level of E-cadherin was found to be the only independent predictor for intravesical recurrence [30]. However, despite the prognostic significance attributed to E-cadherin alteration, a recent study has not confirmed the association of E-cadherin with the parameters of biological aggressiveness and LVI [4]. Our findings implied that altered expression of E-cadherin is more frequent in UUT-UC with LVI. However, in logistic regression analysis aberrant E-cadherin staining was not recognized as independent predictor of LVI.

Conclusion

This study investigated the impact of Ki-67 and E-cadherin expression on lymphovascular invasion in primary UUT-UC. The choice to correlate these two markers to LVI was based on their well-established role in cancer growth, invasiveness and dissemination and, in addition, their availability in routine practice of immunohistochemical laboratories. Only Ki-67 expression was found to be a significant independent predictor of LVI in UUT-UC, while E-cadherin staining added no valuable information to LVI probability assessment. The evaluation of Ki-67 could identify a subset of patients with urothelial carcinoma of the upper urinary tract that might require closer follow-up after surgery.

Acknowledgements

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Graft stability after endothelial keratoplasty

Stabilnost kalema rožnjače posle endotelne keratoplastike

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Abstract

Bacground/Aim. Techniques for replacing the corneal endothelium have been improved. The host-graft interface is the key to graft adhesion and visual recovery. The aim of this study was to establish graft stability after Descemet stripping with endothelial keratoplasty (DSEK), compare it to the graft stability after endothelial keratoplasty with the intact posterior corneal layers (nDSEK) in the rabbit cornea, and to investigate the nature of wound healing. **Methods.** Adult white rabbits (n = 20) were divided in two experimental groups: ten rabbits underwent monocular DSEK, and ten rabbits underwent endothelial keratoplasty without Descemet stripping (nDSEK). On the second postoperative day a horizontal dislocation of the graft was tried using the Lindstrom roller in each animal. Corneas were processed for the light microscopy study. **Results.** Rolling the Lindstrom instrument over the corneal surface did not cause horizontal dislocation in any of the operated eyes. In the DSEK group light microscopy revealed the lack of inflammation and fibrosis at the clearly distinctive donor-recipient interface (DRI). Retrocorneal membrane was found in two eyes. In nDSEK group, the host Descemet`s membrane (DM) was intact without endothelial cells, with good graft apposition, without inflammation, fibrosis, or retrocorneal membrane. **Conclusion.** This study suggests that there is no difference in graft stability in DSEK compared to nDSEK in rabbit corneas. Wounds healed at DRI by hypocellular scarring only in both experimental groups.

Key words:
cornea; rabbits; ophthalmologic surgical procedures; transplantation; graft survival; histological techniques.

Apstrakt

Uvod/Cilj. Tehnike zamene kornealnog endotela se unapređuju. Ključ za adheziju grafta i oporavak vida je interfejs domaćina prema kalemu. Cilj rada bio je da se utvrdi stabilnost kalema rožnjače kod endotelne keratoplastike sa svlačenjem (DSEK) i uporedi sa stabilnošću kalema kod endotelne keratoplastike sa intaktnom zadnjom površinom (nDSEK) na zečijoj rožnjači, kao i da se ispita način zarastanja operativne rane. **Metode.** Eksperiment je izveden na kunićima soja činčile (n = 20), podeljenim u dve eksperimentalne grupe. U prvoj grupi (deset očiju) urađen je DSEK, a u drugoj (deset očiju) endotelna keratoplastika bez ljuštenja endotela. Drugog postoperativnog dana kod svih kunića izvršen je pokušaj horizontalne dislokacije kalema pomoću Lindstromovog rolera. Rožnjače su pripremljene za svetlosnu mikroskopiju. **Rezultati.** Pomeranjem Lindstromovog rolera preko površine rožnjače nije bilo moguće dislocirati nijedan kalem. U DSEK grupi, na svetlosnoj mikroskopiji nije bilo zapaljenja, niti fibroze na spoju strome primaoca i donatora (DRI), ali je DRI bio histološki uočljiv. Takođe, uočena je i retrokornealna membrana u dva oka. U grupi podvrgnutoj endotelnoj keratoplastici bez svlačenja Descemetove membrane (DM), DM primaoca je bila intaktna, bez endotelnih ćelija, sa dobrom apozicijom kalema, bez zapaljenja ili fibroze, a retrokornealna membrana nije uočena. **Zaključak.** Ova studija pokazuje da nema razlike u stabilnosti kalema kod DSEK, u poređenju sa stabilnošću kalema kod nDSEK i na zečijim rožnjačama. Zarastanje rane kod oba postupka odvija se stvaranjem hipocelularnog ožiljka u obe eksperimentalne grupe.

Ključne reči:
rožnjača; zečevi; hirurgija, oftalmološka, procedure; transplantacija; graft, preživljavanje; histološke tehnike.

Introduction

In the quest for better understanding of human corneal wound healing in the blooming era of refractive and lamellar keratoplasty procedures, animal model offered a valuable insight [1–10]. Although the rabbit cornea has some differences compared to the human, such as the lack of Bowman layer, abundant regenerative capacity of endothelial cells, and overall thinner cornea, the similarity of stromal corneal lamellar architecture, and keratocyte distribution, as well as the response to different agents, served as a good model for decades [4, 11–15].

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For more than 100 years, the only surgical technique to replace the diseased endothelium has been full-thickness penetrating keratoplasty (PK). Although useful vision could be achieved after PK, this procedure suffers from a wide range of complications. Graft failure is not uncommon and it accounts for up to 40% of all corneal transplants [16, 17]. Moreover, regrafts have higher failure rates than the first time PK, so an alternative would be desirable [18].

Since 1998, the technique for posterior lamellar keratoplasty (PLK) has evolved, maintaining the concept of replacing the corneal endothelium with an unsutured posterior lamellar graft through a small limbal incision in eyes that have not been previously grafted. The most commonly used modification of PLK is Descemet stripping with endothelial keratoplasty (DSEK). DSEK involves stripping of Descemet's membrane (DM) from the host cornea and placement of a donor graft, comprising a thin layer of the posterior stroma, DM and endothelium, onto the posterior surface of the host stroma. PLK may be better option than regrafting previously failed PKs, particularly when rapid visual recovery is important and an acceptable refractive outcome was achieved with the first time graft. It seems that in some cases stripping of DM is not necessary, but published data are limited and long term results are not known.

The host-graft interface is the key to graft adhesion and visual recovery. We compared PLK graft stability with and without DM stripping in a rabbit cornea and investigated the nature of wound healing by light microscopy.

Methods

Study design

A total of 20 white rabbits (weight 3–4 kg) with normal corneas were divided in two experimental groups: in the first group animals underwent monocular DSEK, and in the second one monocular PLK without DM removal. On the second postoperative day, a Lindstrom roller was used to try to dislocate graft horizontally in each animal, and after 7, 30 and 90 days corneas were harvested and evaluated using light microscopy. The rabbits were anaesthetised with an intramuscular injection of xylazine (5 mg/kg), and ketamine hydrochloride (35 mg/kg), and topically with tetracaine hydrochloride. The animals were heparinized (2 mL, 5,000 IU intravenously) immediately before surgery to prevent clotting of the aqueous humour. Postoperative treatment included topical 0.1% dexamethasone-neomycin four times *per* day, and 1% chloramphenicol ointment once. At the selected time points animals were killed by overdosing sodium pentobarbital (100 mg/kg), and the corneas were processed for histology (hematoxiline and eosin staining). Experiments were approved by "Zvezdara" University Medical Center Ethical Board, and all animals were housed and treated to Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research.

Surgical procedure

Donor

The donor cornea was prepared first, followed by the surgery of the recipient. Using the whole globe a 4 mm peripheral corneal incision was made with the perpendicular edge of a blade at approximately 2/3 stromal depth. From the bottom of the incision, a manual stromal dissection was made. Once the dissection was completed, the corneoscleral button was cut, transferred to a punching block, endothelial side up and cut with a 5 mm punch trephine. The button was placed on a spatula, endothelial side up, and covered with viscoelastic substance.

Recipients

The DSEK group: The recipient corneal epithelium was lightly marked with 5 mm trephine, to outline the area for Descemet stripping and placement of donor tissue. A 1.5 mm corneal incision was made 4 mm away from limbal area, and the anterior chamber was filled with air. A bent, 21 G needle was used to score DM in a circular pattern under the area of the epithelial reference mark. Reversed Sinskey hook was used to strip off DM and endothelium within the scored area and to remove it from the anterior chamber. The anterior chamber was filled with air. The initial corneal incision was lengthened to 5 mm, and the previously prepared donor graft was inserted into the anterior chamber laying on a spatula with stromal side up, and endothelial side protected with viscoelastic. Air was injected into the anterior chamber to press it up against the recipient cornea. The peripheral corneal incision was closed with 10–0 monofilament nylon suture. Air was left in place for two hours, and then partially replaced with balanced salt solution (BSS).

The nDSEK group: The procedure was the same as described above, except for leaving the recipient DM intact.

Results

Rolling the Lindstrom instrument over the corneal surface on the second postoperative day did not cause horizontal dislocation in any of the operated eyes.

The DSEK group

Seven days after DSEK

Tissue obtained on the day 7 after DSEK and stained with haematoxylin and eosine (HE) revealed that donor-recipient interface (DRI) could be determined, but was not conspicuous. Stromal collagen lamellae both of the recipient and donor were parallel, but with greater interalmellar space and with less keratocytes on the donor side. DM end the endothelium of the graft appeared unchanged.

Thirty days after DSEK

Thirty days after DSEK, DRI were still inconspicuous. In one specimen there was a deposit of amorphous material at DRI (Figure 1). Stromal collagen lamellae remained parallel, but with greater intetrlamellar space and less keratocytes in the donor graft. DM end endothelium appeared normal.

Ninety days after DSEK

HE staining revealed DRI still without scarring, with

parallel lamellae and evenly distributed keartocytes. In two specimens retrocorneal membrane was visible (Figures 2–5).

The nDSEK group

On days 7, 30 and 90 after surgery, histology revealed similar findings in all specimens. Recipient endothelium was absent, and DM retained normal appearance. Stromal collagen lamellae were parallel with the uniform distribution of keratocytes, both on recipient and donor side. Donor DM and endothelial cells were normal. Histology revealed good graft apposition (Figure 6).

Fig. 1 – Light microscopy after Descemet stripping with endothelial keratoplosty (DSEK) from the periferal part of the cornea (HE, ×20). Arrows indicate the interface between host and a donor stroma.

Fig. 2 – Light microscopy 2 months after Descemet stripping with endothelial keratoplasty (DSEK) at a higher magnification (HE, ×40): donor-recipient interface with hypocellular scaring (arrows).

Fig. 3 – Light microscopy one month after Descemet stripping with endothelial keratoplasty (DSEK) showing amorphous material at donor-recipient interface (HE, ×40).
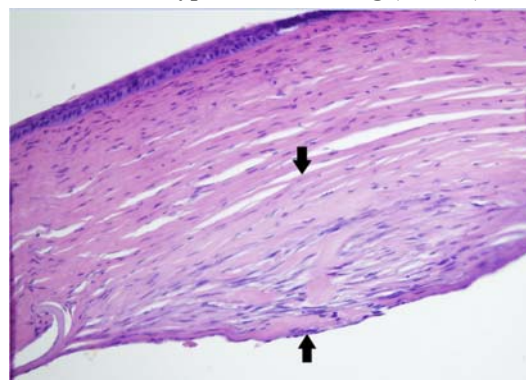
Fig. 4 – Light microscopy 3 months after Descemet stripping with endothelial keratoplasty (DSEK) showing globular retrocorneal membrane between arrows (HE, ×20).

Fig. 5 – Light microscopy 3 months after Descemet stripping with endothelial keratoplasty (DSEK) showing thin delicate retrocorneal membrane (arrow) (HE, x20).
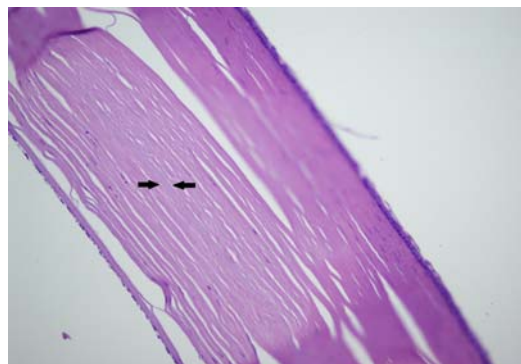
Fig. 6 – Light microscopy 3 months after posterior lamellar keratoplasty without Descemet's membrane (DM) removal, showing the absence of host endothelial cells, firm attachment of host DM to both host and donor stroma, and normal HE staining (arrows) (HE, ×20).

Discussion

The anatomy of the rabbit eye makes endothelial keratoplasty much more challenging compared to human. First, the peripheral convexity of the iris in the rabbit eye, makes an incision near the limbus impossible, as both the iris and the lens would be damaged. Therefore the incision had to be placed 4 mm away from the limbus in the clear cornea, where the anterior chamber is deeper, and allows more space for surgical manipulation. Second, the rabbit cornea is overall thinner than human (300 μm compared to 550 μm) which made the endothelial transplant extremely thin and fragile, with a tendency to twist inwards. As manipulation should be minimal, it was of utmost importance to be careful about the orientation of the stromal and endothelial side, in order to avoid the wrong orientation during insertion into the anterior chamber. Third, the aqueous humour in rabbits has strong tendency to clot during intraocular surgery, and seriously compromise its outcome. In order to prevent this the animals in both groups were heparinized immediately before the surgery. Besides, abundant regenerative capacity of corneal endothelial cells in rabbits limits the evaluation of viability of the endothelium in the late postoperative period [19].

Our study demonstrates that there is no difference in the graft stability with and without DM removal. The study also suggests that in both experimental groups wounds heal by hypocellular scar only. The retrocorneal membrane formation was observed only in the DSEK group.

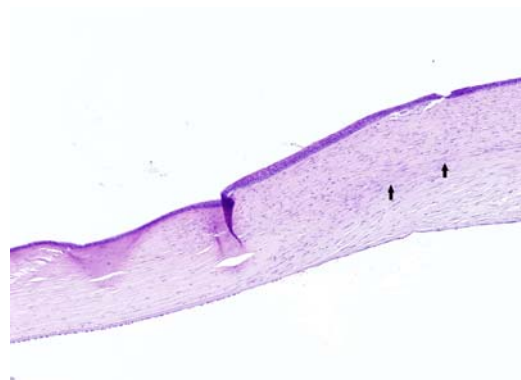Our results were obtained after endothelial keratoplasty in healthy eyes, without intra- or postoperative complications, which could be compared only to failed human grafts.

Light microscopy in our DSEK group, showed a firm attachment between the posterior donor and anterior host stromal tissue, lack of inflammation and fibrosis at DRI, and a barely perceptible DRI. Amorphous material found at DRI in one specimen could be related to collagen fibers rupture during DM stripping, which is more difficult to perform in a healthy rabbit cornea than in eyes with underlying endothelial pathologic abnormalities [20].

Caldwell et al. [21] analysed 10 failed grafts, with the DRI being mostly indistinctive, although in some specimens focal hypercellularity was visible in the areas of separation between the graft and the anterior stroma. The author assigned this to a proliferative response to the loss of apposition to DM or stroma. Contrary to this, in eyes enucleated 7 days after the surgery, we found a good apposition and hypocellularity near the DRI. It has been shown earlier, that lamellar cut across the cornea produced by microkeratome, as performed during laser-assisted *in situ* keratomileusis (LASIK), induces keratocyte apoptosis, not only peripherally at the site of epithelial injury, as would be expected, but also along both sides of the lamellar interface. The proposed mechanism is the diffusion of cytokines from the injured peripheral epithelium along the lamellar interface [10].

Several studies showed the presence of fibrocellular tissue at the DRI [22, 23]. Shulman et al. [22] showed that half of the failed grafts with interface fibrosis was associated with chronic stromal inflammation. The authors presumed that increa-sed intraocular manipulation might have contributed to the inflammatory response, which further led to stromal fibroblast activation by inflammatory mediators. In our study we did not notice either inflammatory cells, or fibrocellular scar. Contrary to the surgical procedure used in published studies where graft was folded, grasped (and eventually partially crushed) with forceps, and pulled through a very tight incision, we used the previously described technique where the graft was unfolded, flat, positioned on the spatula, endothelial side down, with the viscoelastic protection, and introduced into the anterior chamber-which did not cause any compression to the graft. As previously mentioned, manipulation of a very thin graft is extremely difficult, so we decided to use the technique where the graft could be minimally damaged.

We did not find any epithelial cells at the DRI, which is consistent with the early Melles` et al. [6] report. However, there are several reports on epithelial ingrowth and the presence of epithelial membrane at DRI [22–24]. In report by Bansal et al. [24] epithelial ingrowth was continuous with the stromal puncture incision for interface fluid removal, while in a Shulmans et al. [22] report there was an eccentric lenticule trephination, which resulted in the retention of a full-thickness donor cornea and its epithelium at one edge of the graft. We did not perform corneal incision for interface fluid removal. Opposite to microkeratome dissected tissue which could result in eccentric trephination, we prepared the graft by manual dissection, so that none of the epithelial cells could have access to the DRI.

In the DSEK group, DM was adherent in all but two cases, which we believe that this detachment is attributable to shearing stress from tissue cutting. Endothelium was preserved in all specimens.

The most frequent reason for endothelial keratoplasty graft failure is endothelial atrophy often preceded by its damaging during graft preparation and manipulation during implantation into the anterior chamber [25, 26]. Using the air bubble at the end of the surgery, to keep the donor disc in its position, potentially could harm the endothelium in the early postoperative period [11, 27]. The chronic endothelial cell loss may include slow migration of the central endothelium to the periphery, apoptosis and a slow, subclinical, immune-mediated destruction of the donor endothelium by the recipient. The use of the donor tissue that has been precut with a microkeratome or femtosecond laser by the eye bank and then shipped to the surgeon are becoming the popular way of performing endothelial keratoplasty throughout the world, although the long term effect on the donor endothelial cell counts is unknown [28].

In our experimental model, endothelium could not be evaluated, because of the immense regenerative capacity of the rabbit endothelium. Furthermore, we used grafts prepared from the fresh, whole globes with highly viable endothelial cells.

In the two of our DSEK cases, retrocorneal membrane was present after a 90-day follow-up.

In ten failed grafts Caldwell et al. [21] found 4 retrocorneal membranes, two of which were thin, and two other were prominent and localized at the graft edges. Shulman et al. [22] found retrocorneal membrane in one third of the 22 failed

grafts, while Sbarbaro et al. [29] described two membranes in three failed grafts. Similar to retrocorneal fibrous membrane in failed PK grafts, retrocorneal fibrous membrane in failed DSEKs can result from fibrous ingrowth or from fibrous metaplasia of endothelial cells. It is well-documented that disruption and separation of DM can provide the trigger for emanating stromal tissue from the wound edge [30]. While not necessarily contributing to graft failure, if it is limited to the edge of the graft, the retrocorneal membranes could lead to graft decompensation, if it is large and diffuse, as in the case of a large wound, or poor host and the graft DM alignment as in PKP or DSEK [31]. Clinically, the cornea appears cloudy and oedematous. Since the regenerative capacity of rabbit endothelial cells is large, we could expect more aggressive response to the poor alignment of graft. In one case we noticed a mature, globular retrocorneal membrane, at the edge of the graft which did not compromise the optical clarity. In the second specimen, the membrane was thin, and delicate so did not cause significant corneal edema.

One of the major concerns regarding endothelial keratoplasty is graft dislocation, which is very often followed by graft decompensation. It is now well-known that mastering the technique leads to lower percentage of dislocation. At the dawn of this technique the reported rate of dislocation was even 50% [25, 26]. Today, it is less than 1–2% [28]. It was presumed that thinner grafts were associated with a higher rate of early donor failure [26, 32]. Preliminary results of the transplantation of a bare DM without stromal carrier, show that the graft thickness does not play the major role in dislocation [33]. Melles [33] states that donor dislocation rarely happens when fresh tissue is used, which may suggest that eye banks preservation process affects the endothelial pump function. He also presumes that the most important surgical factor which prevents graft attachment is the use of hyaluronic acid (dextran in tissue storage media also has similar viscous feature).

The mechanism of graft adhesion is still unknown. The proposed mechanism by which a graft remains attached to the host stroma is wound compression by imbibition swelling of the exposed, posterior edges of both the donor and the recipient, stickiness of the stromal tissue at DRI, the suction force of the donor endothelium and fibrin deposition throughout the wound area in the early healing phase [34]. It has already been shown that fibronectin appears at the site of corneal stromal wound in rabbits shortly after wounding, and in case of lamellar keratoplasty, fibronectin has been detected at the interface of the graft and the recipient cornea [1, 9]. Stromal remodelling is supposed to be the mechanism of wound healing in later phases.

Rolling the Lindstrom instrument over the surface of the cornea on the second postoperative day, did not cause any graft dislocation. It seems that the proposed mechanisms for early graft adhesion were efficient enough to hold the graft in apposition in early postoperative period. If fibrotic wound repair was the only mechanism to hold the graft in place, it would be possible to dislocate the graft for a much longer postoperative period.

The hypocellular primitive scar lacks collagen fibrils as previously shown on post LASIK corneas [7]. The experiment

with rabbit corneas showed that in weeks after lamellar surgery, regions of healing stromal matrix were structurally disorganised, and contain collagen-free areas populated by abnormally large sulphated proteoglycan filaments, which might aid tissue restructuring because of their water-binding capacity [8].

There was no graft dislocation in the DSEK group. The possible explanation could be that we used fresh, unfolded tissue. Our grafts were manually prepared which could increase the stickiness of the lamellar stromal surfaces.

Light microscopy in the nDSEK group showed firm host DM attachment to both host and donor stroma and the disappearance of the host endothelial cells. There was no inflammation or retrocorneal membrane formation. In the previous reports on lamellar keratoplasty using full thickness donor material in rabbit eyes, it was shown that donor endothelial cells start to disappear 30 minutes after the surgery, and that disappearance is completed by 24 hours. There were very few inflammatory cells in the neighbouring recipient cornea. The DM remained intact. Our finding goes in line with the previous reports. There were no host endothelial cells, and no inflammatory response. DM remained intact, and showed normal staining for HE [35].

Although first descriptions of failed and detached grafts implicated that the retention of DM at DRI may weaken graft adhesion and cause primary graft failure [23], in eight histologic sections of failed grafts Caldwell et al. [21] show that the presence of DM does not hinder graft adhesion. Furthermore, it appeared that there was an increased adhesion in the areas of residual DM. In two of the cases residual host DM was more adherent to DSEK graft than to the anterior host cornea. There was no alteration in cellularity or inflammation in the areas of residual DM. From that Caldwell et al. [21] suggested that removal of DM might not be necessary.

Sbarbaro et al. [29] described histology of 3 failed endothelial keratoplasty grafts. In two grafts there was a delicate fibrous retrocorneal membrane, and the authors suggested that it could be a possible reason for endothelial keratoplasty failure. They also found a firm adhesion of residual host DM to the donor stroma and concluded that removing optically clear DM may not be necessary.

Price and Price [36] described initial series of endothelial keritoplasty without DM removal. They treated 5 eyes with failed PKs for endothelial decompensation after previously having clear corneal transplant. In all cases the graft adhered to and cleared the edema from the previous penetrating graft. Within the next 3 months of endothelial keratoplasty visual acuity (VA) had improved as well [36].

We recently showed histological and ultrastructural finding of endothelial keratoplasty without DM removal in a failed PK graft a year after the surgery. Light microscopy showed the absence of host endothelium, and good graft apposition. Electron microscopy revealed quiet keratocytes without prominent endoplasmatic reticulum. Host DM had the normal homogenous structure, without scar formation on either of its sides [37].

A recently performed study on a similar experimental model showed clear corneas and good graft apposition after both

DS/EK and non-Descemet stripping automated endothelial keratoplasty (nDSAEK). Analysing the fate of endothelial cells two weeks after the surgery, the authors expressed concerns regarding long-term graft adherence in nDSAEK cases [38]. In our study the follow up was much longer (12 weeks), and there was a good graft adherence throughout the observation period.

Clinical evaluation of nDSEK in selected cases showed very favourable outcome. Park and Chuck [39] reported a case of non-Descemet stripping Descemet membrane endothelial keratoplasty (nDMEK) in one patient with pseudophakic bullous keratopathy. During a 6-month follow-up a graft remained attached and corneal edema subsided, which resulted in a remarkable visual ocyty improvement [39]. Masaki et al. [40] reported a good clinical outcome of 19 nDSAEK for non-Fuchs-type bullous keratopathy. All corneas remained clear after one year of follow-up [40]. Similar results had Chaurasia et al. [41] in their series of 23 patients with the mean follow up of 7.4 months. Based on their favorable results regarding corneal clarity, visual acuity and no interface haze, the authors suggested non-DSAEK as safe option in cases of corneal decompensation when the Descemet membrane is healthy.

Leaving the intact DM from the failed penetrating grafts seems to offer some advantages. Scraping DM from the graft could be difficult and incomplete which may provoke stromal overgrowth and the formation of a retrocorneal membrane [20]. Furthermore, DM is a storage depot for bound basic fibroblast growth factor, and a mechanical injury to this membrane could convert it into a reservoir for the sustained release of mitogenic activity that may overcome rapid degradation of this factor [42].

Conclusion

In conclusion, our study showed that both removing or leaving Descemet's memberane does not destabilize the endothelial keratoplasty graft in the early postoperative period. Removing the Descemet membrane could initialize formation of retrocorneal membrane which could lead to the late graft decompensation, while leaving the Descemet membrane intact reduces such risk. Furthermore, leaving the optically clear Descemet membrane could increase the chance for a long-term graft survival.

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Pseudomonas aeruginosa serotypes and resistance to antibiotics from wound swabs

Serotipovi i rezistencija na antibiotike *Pseudomonas aeruginosa* iz briseva rana

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Abstract

Introduction/Aim. *Pseudomonas aeruginosa (P. aeruginosa)* is the most common cause of wound infections, following the disruption of the skin or mucous membranes integrity. The aim of this study was to analyze of the presence *P. aeruginosa* in wound swabs, antibiotics susceptibility testing, determination of the minimum inhibitory concentrations (MICs) of antibiotics, testing of the metallo-β-lactamases (MBLs) production, isolates serotyping and analysis of the most common serotypes resistance. **Methods.** A total of 90 outpatients and 55 intpatients wound swabs were cultivated. Wound swabs were taken from the patients with wound infections symptoms. Antibiotics susceptibility testing was performed to: meropenem, imipenem, piperacillin-tazobactam, ceftazidime, cefepime, amikacin, gentamicin, netilmicin, ofloxacin, ciprofloxacin and colistin (HiMedia). Polyvalent and monovalent antisera for agglutination (Biorad) were used in *P. aeruginosa* agglutination. **Results.** *P. aeruginosa* was isolated from 36.55% wound swabs (36.66% of the inpatients wounds and 36.36% of the outpatients). The analyzed isolates showed the highest degree of sensitivity to colistin (100%) and meropenem (93.44%) and the lowest to cefepime (19.54%). The majority of the inpatients isolates had 12 μg/mL (28.57%) MIC for piperacillin-tazobactam and 16 μg/mL (28.57%) for the outpatients. The most common MICs for ciprofloxacin were 0.19 μg/mL (31.81%) for the nosocomial isolates, and 0.25 μg/mL (28.57%) for the outpatients’ ones. The most common MICs for amikacin of the nosocomial isolates were 6 μg/ml (40.9%), and for the outpatients ones 4 μg/mL (33.33%). Five (9.43%) isolates produced MBLs. The most common serotypes were P11 (22.64%), P6 (15.09%) and P1 (11.32%). **Conclusion.** Neither the increased presence of *P. aeruginosa* was noticed in wounds swabs, nor the antibiotic resistance in the nosocomial isolates compared to those from outpatients. The analyzed isolates had the higest sensitivity to colistin and meropenem, and the lowest to cefepime.

Key words:
pseudomonas aeruginosa; wounds infection; serotyping; anti-bacterial agents; drug resistance.

Apstrakt

Uvod/Cilj. *Pseudomonas aeruginosa* (*P. aeruginosa*) spada među najčešće uzročnike infekcija rana nakon narušavanja integriteta kože ili sluzokože. Cilj ispitivanja bila je analiza prisustva P. aeruginosa u brisevima rana, zatim ispitivanje osetljivosti na antibiotike, određivanje minimalne inhibitorne koncentracije (MIC) antibiotika, ispitivanje produkcije metalo-β-laktamaza (MBL), serotipizacija izolata i analiza rezistencije najčešćih serotipova. **Metode.** Kultivisana su 90 ambulantno i 55 bolnički uzorkovanih briseva rana. Brisevi su uzimani kod bolesnika sa simptomima infekcije rana. Ispitivanje osetljivosti *P. aeruginosa* vršeno je na: meropenem, imipenem, piperacilin-tazobaktam, ceftazidim, cefepim, amikacin, gentamicin, netilmicin, ofloksacin, ciprofloksacin i kolistin (Himedia). U aglutinaciji *P. aeruginosa* korišćeni su polivalentni i monovalentni serumi (Biorad). **Rezultati.** *P. aeruginosa* izolovan je iz 36,55% briseva rana (36,66% rana bolničkog porekla i 36,36% ambulantnog). Izolati su pokazali najveći stepen osetljivosti na kolistin (100%) i meropenem (93,44%), a najmanji na cefepim (19,54%). Najveći broj izolata bolničkog porekla imao je MIC za piperacilin-tazobaktam 12 μg/mL (28,57%), a ambulantnog 16 μg/mL (28,57%). Najčešći MIC za ciprofloksacin kod bolničkih izolata bio je 0,19 μg/mL (31,81%), a ambulantnih 0,25 μg/mL (28,57%). Najčešći MIC za amikacin kod izolata bolničkog porekla bio je 6 μg/mL (40,9%), a ambulantnog 4 μg/mL (33,33%). Pet (9,43%) izolata proizvodilo je MBL. Najprisutniji bili su serotipovi P11 (22,64%), P6 (15,09%) i P1 (11,32%). **Zaključak.** Nije uočena češća kultivacija *P. aeruginosa* u brisevima rana, niti rezistencija na antibiotike kod izolata bolničkog porekla u odnosu na ambulantne. Analizirani izolati pokazali su najveći stepen osetljivosti na kolistin i meropenem, a najmanji na cefepim.

Ključne reči:
pseudomonas aeruginosa; rana, infekcija; serotipizacija; antibiotici; lekovi, rezistencija.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most common bacteria colonizing the hospital environment [1–5]. High genetic variability, flexible physiology, adaptability, metabolic potential, production of broad capsules [6], biofilm forming [7–9], control of external membrane permeability [10] and resistance to antibiotics and disinfectants allow the bacillus to be widely dispersed [11].

P. aeruginosa is a common cause of inflammation, if there is a disruption of the body's defense forces for any reason: malignant disease, chemotherapy, neutropenia, diabetes mellitus, cardiovascular diseases, alcoholism, smoking and obesity. It mainly causes infections of urinary and respiratory tract and wounds, especially burns [12–14]. It mostly causes inflammation of the skin and subcutaneous tissue if the integrity of the skin and mucous membranes is damaged. *P. aeruginosa* causes wound infections after surgical procedures, various types of injuries, burns and dermatitis [15, 16]. Primary wound colonization occurs after its contact with the external environment, and often during the surgical wound treatments in hospital environments. Infections are more common in diabetes mellitus and peripheral circulatory disorders, which cause the formation of chronic ulcers on calves [16].

There are multiple mechanisms of *P. aeruginosa* antibiotic resistance [17]. β-lactamases production is among the most important mechanisms of resistance. *P. aeruginosa* produces over 100 β-lactamases [18]. Metallo β-lactamases (MBLs) are clinically the most important β-lactamases. Among the Gram negative bacteria, *P. aeruginosa* most often produces MBLs. MBLs belong to the Amblers class B, subclass B1, group 3 by Bush and Sykes [19]. The main difference between MBLs and serine β-lactamases is that MBLs have a metal cofactor, unlike serine, they are sensitive to ethylenediaminetetraacetic acid (EDTA), but not to inhibitors of serine β-lactamases. MBLs have a broad spectrum of activities against many antibiotics, including all β-lactam antibiotics and carbapenems. The most important MBLs produced by *P. aeruginosa* are: IMP, VIM, GIM, SPM and AIM-1 [17].

Serological examination of *P. aeruginosa* serotypes is important for epidemiological analyses. Facts about serotypes facilitate the analysis of the prevalence of certain serotypes and locating sources of infection in hospitals. Sensitivity to antibiotics is easier to follow if there are data about the serotypes present in the specific area. Different serotypes are predominant in certain regions and have different clinical and epidemiological significance, primarily due to different antibiotic resistance [3].

The aim of the study was to analyze the presence of *P. aeruginosa* in wound swabs of inpatients and outpatients, antibiotic susceptibility testing, determination of minimal inhibitory concentrations (MICs) for piperacillin-tazobactam, amikacin and ciprofloxacin, production of MBLs, determination of the presence of certain serotypes and the most common serotypes resistance analysis.

Method

Isolation and identification of P. aeruginosa

During 2012, 145 wounds swabs were cultivated in the Healthcare Centre "Aleksinac" Microbiology Department, in Aleksinac, Serbia. A total of 90 outpatients swabs and 55 intpatients swabs were taken. Swabs were taken from inpatients in the Surgery, Gynecology and Obstetrics and Internal Medicine Departments. The patient's data were collected from medical records kept in the Healthcare Centre "Aleksinac" computer system. Outpatient subjects were patients who came ambulatory to the Microbiological Laboratory. Wound swabs were taken from the patients with signs of wound infection: redness, the presence of pus, pain or fever.

Wound swabs were put on blood agar plates and MacConkey plates, nutrient broth and thioglycollate broth (Hi-Media). The inoculated plates were incubated aerobically for 24 hours. Nutrient broth and thioglycollate broth were recultivated on blood agar and MacConkey plates. Identification of *P. aeruginosa* was done on the basis of their microscopic, cultural and physiological-biochemical characteristics [20, 21]. Isolated strains of *P. aeruginosa* were recultured on the trypticase soy agar (Torlak).

Antibiotic susceptibility testing

P. aeruginosa sensitivity testing to antibiotics was performed using the disc diffusion method according to CLSI standards [21]. Bacillus suspension of 0.5 McFarland density was poured onto the Müller-Hinton agar and dried. Then the commercial discs were placed on the agar surfaces: piperacillin-tazobactam (100/10 μg), imipenem (10 μg), meropenem (10 μg), colistin (10 μg), ceftazidime (30 μg), cefepime (30 μg), amikacin (30 μg), gentamicin (10 μg), netilmicin (30 μg), ofloxacin (5 μg) and ciprofloxacin (5 μg) (HiMedia). The resistance to antibiotics was read after 24 hours, based on the zone of inhibition around the disk. Susceptibility was marked as sensitive, intermediate and resistant. MICs for piperacillin-tazobactam, amikacin and ciprofloxacin were determined according to antibiotics tape manufacturer's instructions (Liofilmchem).

MBL production ability was tested by imipenem and imipenem-EDTA discs. Test was marked as positive if the difference in growth inhibition zone around the discs was bigger than 6 mm [21].

Agglutination

Polyvalent and monovalent antisera (Biorad) were used in agglutination. Agglutination kit contains 4 polyvalent and 16 monovalent antisera. Polyvalent antisera are PMA, PMF, PMC and PME. The PMA group includes the following serotypes: P1, P3, P4 and P6, the PME P2, P5, P15 and P16. The PMC group includes P9, P10, P13 and P14, while PMF includes P11, P12, P7 and P8. Agglutination was described as positive if it caused a positive slide agglutination reaction. Some isolates were poly-agglutinative, while some agglutinated only by polyvalent, but not monovalent antisera. Some isolates were not agglutinated with any antisera. Such isolates were described as non-typical. Isolates of *P. aeruginosa* were agglutinated from trypticase soy agar [22].

Statistical data processing

The files were created in the SPSS 12.0 package, where data analysis was done. For the results analysis we used χ²-

test, C contingency test and coefficient of parametric and nonparametric small and large samples.

Results

The study group consisted of 145 patients, 79 (54.48%) male and 66 (45.52%) female, of which there were 55 inpatients, 30 (66.66%) male and 25 (45.45%) female. Swabs of inpatients were collected mainly from the Surgery Department, 53 (96.36%), one swab (1.81%) was collected in the Gynecology and Obstetrics Department and one in the Department of Internal Medicine. There were 90 outpatients, 49 (54.44%) male and 41 (45.56%) female.

P. aeruginosa was cultured from 53 (36.55%) wound swabs. It was present in 33 (36.66%) cultured outpatient isolates, and in 20 (36.36%) intpatient isolates ($p = 0.99$), of which 29 (54.72%) were males and 24 (45.28%) females ($t = 0.97$; $p = 0.01$). The inpatients included 12 (60%) males and 8 (40%) females ($\chi^2 = 0.67$, $p = 0.67$; C = 0,089) and the out-

patients 17 females (51.51%) and 16 (48.49%) males ($\chi^2 = 1.46$, $p = 0.226$, C = 0.152).

The average age of patients from whose wound swabs *P. aeruginosa* was isolated was 67.32 ± 24.22 years, median (Me) 67 years, of males 66.65 ± 24.22 years (Me 70 years) and females 67.95 ± 24.35 years (Me 69 years). The largest number of patients was in the sixth to eighth decade of age (84.89%), ($\chi^2 = 91$; $p < 0.001$; C = 0.99) (Table 1).

The respondents were most frequently (78.62%) diagnosed with ulcus cruris: 74.54% of inpatients and 81.11% of outpatients (Table 2). A total of 72.44% respondents with ulcus cruris also had diabetes mellitus. The patients with isolated *P. aeruginosa* were frequently (77.35%) diagnosed with ulcus cruris (74.54% inpatients and 26.66% outpatients) and burns (16.98%). *P. aeruginosa* was isolated from 74.6% patients with ulcus cruris and diabetes mellitus.

Antibiotic resistance of *P. aeruginosa* inpatients and outpatients isolates is shown in Table 3.

The analyzed isolates of the whole group, as well as of the

Table 1

Demographic characteristics of the patients from whose wound swabs *P. aeruginosa* was cultivated

Sex	Patients' age (years), n (%)						
	31–40	41–50	51–60	61–70	71–80	81–90	Total
Male	1 (1.88)	1 (1.88)	3 (5.66)	3 (5.66)	18 (5.66)	3 (5.66)	29 (54.7)
Female	- -	2 (3.77)	5 (9.43)	10 (18.86)	6 (11.32)	1 (1.88)	24 (45.3)
Total	1 (1.88)	3 (5.66)	8 (15.09)	13 (24.52)	24 (45.28)	4 (7.54)	53 (100)

Table 2

Clinical diagnosis of the patients from whose wound swabs *P.aeruginosa* was cultivated

Diagnosis	Inpatients, n (%)		Outpatients, n (%)	
	Total	Positive	Total	Positive
Burns	3 (5.45)	2 (10)	7 (7.77)	7 (21.21)
Ulcus cruris	41 (74.54)	17 (85)	73 (81.11)	24 (72.72)
Postoperative wound	4 (7.27)	1 (5)	-	-
Posttraumatic wound	4 (7.27)	0 -	8 (8.88)	2 (6.26)
Other	3 (5.45)	0 -	2 (2.22)	-
Total	55 (100)	20 (100)	90 (100)	33 (100)

Table 3

The inpatients and outpatients *P. aeruginosa* isolates' resistance to antibiotics

Antibiotics	Inpatients, n (%)			Outpatients, n (%)		
	S	I	R	S	I	R
TAZ	28 (84.9)	1 (3.03)	4 (12.1)	14 (70)	-	6 (30)
I	30 (90.09)	1 (3.03)	2 (6.06)	16 (80)	1 (5)	3 (15)
M	31 (93.93)	-	2 (6.06)	19 (95)	-	1 (5)
CAZ	25 (75.75)	-	8 (24.24)	14 (70)	-	6 (30)
CP	3 (9.09)	1 (3.03)	29 (87.87)	6 (30)	2 (10)	12 (60)
G	19 (57.57)	1 (3.03)	13 (39.39)	9 (40)	1 (5)	10 (50)
A	22 (66.66)	2 (6.06)	9 (27.279	12 (60)	1 (5)	7 (35)
NM	16 (48.48)	3 (9.09)	14 (42.42)	13 (65)	-	7 (35)
OF	20 (60.6)	-	13 (39.39)	7 (35)	-	13 (65)
CC	22 (66.66)	-	11(33.33)	6 (30)	-	4 (70)
C	33 (100)	-	-	20 (100)	-	-

S – sensitive; I – intermediate sensitive; R – resistant; N – number; TAZ – pipercillin-tazobactam; I – imipenem; M – meropenem; CAZ – cefatzidime; CP – cefepime; G – gentamicin; A – amikacin; NM – netilmicin; OF – ofloxacin; CC – ciprofloxacin; C – colistin.

inpatient and outpatient origin respectively, showed the highest degree of sensitivity to colistin (100%), meropenem (93.44%), imipenem (86.7%) and piperacillin-tazobactam (79.24%). There is a slightly lower degree of sensitivity to ciprofloxacin, ofloxsacin, netilmicin and gentamicin. The largest manifested resistance was to cefepime. Nine (16.98%) (5 inpatient and 4 outpatients) isolates were sensitive or intermediately sensitive to all antibiotics, while 3 isolates (5.66%) were resistant to all antibiotics, except to colistin.

The values of MICs for piperacillin-tazobactam are shown in Table 4.

Table 5 shows the MIC values for ciprofloxacin, while MIC values of amikacin are shown in Table 6.

Two inpatients and three outpatients' isolates produced MBLs (9.43%) (t 32.28, p 0.05). However, the percentage of outpatients wounds isolates (10%) was higher than the inpatients wounds (9.09%). One nosocomial isolate was atypical and was sensitive to piperacillin-tazobactam and ceftazidime. Another isolate agglutinated only with PME polyvalent antisera and was sensitive to piperacillin-tazobactam, ceftazidi-

me, ciprofloxacin, amikacin, gentamicin, and colistin. Two outpatients isolates were P11, and one was atypical. All the three isolates were resistant to all the tested antibiotics, except to colistin.

Serologically identified *P. aeruginosa* isolates belonged to all serogroups: PMA, PME, PMC and PMF ($\chi^2 = 17.09$; $p < 0.001$; C0.92). The largest number of isolates belonged to PMA (33.94%) and PMF (24.52%) serogroups. The largest number of nosocomial isolates belonged to the PMA group (20.75%), while outpatients ones to the PMF (15.09%) group.

The following serotypes of *P. aeruginosa* were identified: P1, P3, P4, P6, P10 and P11. The most frequent serotypes were P11 (22.64%), P6 (15.09%) and P1 (11.32%) (Table 7), ($\chi^2 = 39.65$; $p < 0.001$; C = 0.98). Fifteen (28.3%) isolates were atypical. Isolates that showed a positive agglutination reaction only with a polyvalent serum reacted mostly with the PME group (16%).

Resistence to antibiotics of the most common serotypes P11, P1, P6 and of atypical isolates (non-typable – NT) is shown on Figure 1.

Table 4
Distribution of piperacillin-tazobactam minimum inhibitory concentration (MIC) values of inpatients and outpatients *P. aeruginosa* isolates

MIC (μmol/mL)	Inpatients isolates	Outpatients isolates
	n (%)	
64	-	1 (7.14)
32	1 (3.57)	1 (7.14)
24	2 (7.14)	1 (7.14)
16	2 (7.14)	4 (28.57)
12	8 (28.57)	2 (14.28)
10	-	1 (7.14)
8	3 (10.71)	1 (7.14)
4	7 (25)	1 (7.14)
3	3 (10.71)	1 (7.14)
2	2 (7.14)	1 (7.14)

Table 5
Distribution of ciprofloxacin minimum inhibitory concentration (MIC) values of inpatients and outpatients *P. aeruginosa* isolates

MIC (μmol/mL)	Inpatients isolates	Outpatients isolates
	n (%)	
0.75	-	-
0.50	-	1 (12.5)
0.38	2 (9.09)	-
0.25	5 (22.72)	3 (28.57)
0.125	6 (27.27)	1 (12.5)
0.19	7 (31.81)	1 (12.5)
0.094	1 (4.54)	-
0.032	-	-
0.018	-	-
0.016	1 (4.54)	-

Table 6
Distribution of amikacin minimum inhibitory concentration (MIC) values of inpatient and outpatient *P. aeruginosa* isolates

MIC (μmol/mL)	Inpatients isolates	Outpatients isolates
	n (%)	
16	2 (9.09)	1 (8.33)
12	1 (4.54)	- -
8	5 (22.72)	3 (25)
6	9 (40.9)	1 (8.339
4	4 (18.189	4 (33.33)
3	1 (4.54)	3 (25)

Table 7
P. aeruginosa serotypes present in the wound swabs

Serotype	Inpatients isolates	Outpatients isolates	Total
		n (%)	
P11	6 (11.32)	6 (11.32)	12 (22.64)
P6	4 (7.54)	4 (7.54)	8 (15.09)
P1	3 (5.66)	3 (5.66)	6 (11.32)
P10	2 (3.77)	-	2 (3.77)
P4	-	2 (3.77)	2 (5.66)
P3	1 (1.88)	-	1 (1.88)
NT	11 (20.75)	4 (7.54)	15 (28.2)
PMA	1 (1.88)	-	1 (1.88)
PMF	1 (1.88)	-	1(1.88)
PME	4 (7.54)	1 (1.88)	5 (9.43)
Total	33 (62.26)	20 (37.73)	53 (100)

N – number; NT – non-typable (atypical) isolates.
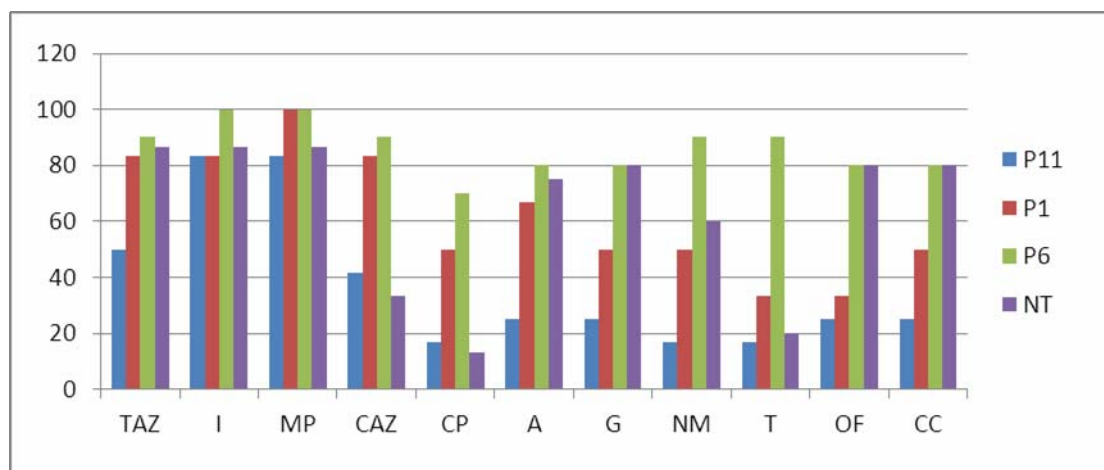

Fig. 1 – Resistance to antibiotics of the most common serotypes of *P. aeruginosa*
TAZ – pipercillin-tazobactam; I – imipenem; M – meropenem; CAZ – ceftazidime; CP – cefepime;
G – gentamicin; A – amikacin; NM – netilmicin; OF – ofloxacin; CC – ciprofloxacin; C – colistin;
NT – non-typable (atypical) isolates.

Discussion

P. aeruginosa is one of the most common pathogens responsible for wound infections all over the world [22–25]. According to the American Center for Monitoring Nosocomial Infections, multidrug-resistant *Pseudomonas* was given a threat level of serious threat in the Centers for Disease Control (CDC) Antibiotic Resistance (AR) Threat Report [26].

In Serbia, according to data of the Third National Study of the Nosocomial Infections Prevalence of October 2011, 13.3% of nosocomial infections are caused by *P. aeruginosa* [27]. In our study, *P. aeruginosa* with almost similar frequency was present in the inpatients and outpatients wound swabs. There was no significant difference in the isolation of bacilli in men and women. *P. aeruginosa* is the most frequently isolated in elderly patients, men in the eighth and women in the seventh decade; from wound swabs of patients with ulcus cruris and diabetes mellitus. This confirms data in the literature that it is most often present in wound swabs of patients with immune system disorder or skin defects. *P. aeruginosa* is the most common cause of burn infections. Among our respondents, bacillus was present in all outpatients wound swabs and in 66% outpatients ones with burns [28–31].

In studies in the USA 2,039 hospitals reported one or more health-care associated infection (HAIs) during 2009–2010, out of which 1,749 (86%) were general acute care hospitals, and 1,143 (56%) had fewer than 200 beds. These data were compared to data reported from HAIs occurring during 2007–2008. Central line–associated bloodstream infections, catheter-associated urinary tract infections, ventilator-associated pneumonia, and surgical site infections were included. There were 69,475 HAIs and 81,139 pathogens reported. Eight pathogen groups accounted for about 80% of reported pathogens: *Staphylococcus aureus* (16%), *Enterococcus* spp. (14%), *Escherichia coli* (12%), coagulase-negative staphylococci (11%), *Candida* spp. (9%), *Klebsiella pneumoniae* (and Klebsiella oxytoca; 8%), *Pseudomonas aeruginosa* (8%), and *Enterobacter* spp. (5%). The percentage of resistance was similar to that reported in the period of 2007 and 2008 yaers. Carbapenem-resistant *P. aeruginosa* was 2% [32].

In Iran, *P. aeruginosa* causes 73.9% burn infections and is their main cause [33]. By the 34. Gjødsbøl et al. [34] study in Sweden in 2006, *P. aeruginosa* causes 52.2% of *ulcus cruris* infections associated with varices, with tendency to increase.

Most of our isolates were susceptible to colistin (100%), meropenem (93.93%), imipenem (90.09%) and piperacillin-tazobactam (84.9%). Colistin has been recently re-

gistered in Serbia, and very rarly used therapeutically, which explains absolute sensitivity to this antibiotic. According the data from Iran, isolates from burns are resistant to colistin [35]. Our isolates had the highest resistance to cefepime (77.35%), despite it being the fourth-generation of cephalosporins and not often used in our hospital. Contrary to expectation, the resistance to piperacillin-tasobactam and imipenem was higher in outpatients than in inpatients isolates. This, however, does not apply to meropenem. Our test results show that carbapenems are important antibiotics that can be used in therapy of infections caused by *P. aeruginosa.*

Our isolates MIC values for piperacillin-tazobactam are similar for inpatients and outpatients isolates. Distribution of MIC values is wide, but most isolates MIC values were from 4 μg/mL to 16 μg/mL. MIC values for amikacin differ for inpatients and outpatients isolates. The largest number of hospital isolates (40.9%) had MICs 6 μg/mL, 81.8% of the isolates had the value of 4 μg/mL to 8 μg/mL. Most inpatients isolates had MIC values of 4 μg/mL, 8 μg/mL and 3 μg/mL. Our results confirm the hypothesis that amikacin is very efficient against *P. aeruginosa*, even against multi-resistant isolates. The MIC value we obtained was lower than in data recently published in Croatia. In a study that included 662 isolates, 90% of them had MIC 32 μg/mL [17]. In the Higgins et al. [36] study, which included imipenem resistant isolates, there were 70% isolates sensitive to amikacin, with $MIC_{90} > 64$ μg/mL. The largest number of inpatients isolates had MIC values 0.19 μg/mL, 0.125 μg/mL and 0.50 μg/mL [36]. The outpatients isolates had much higher MIC values of which none was significantly higher. Data from Croatia indicate that the MIC_{90} value of 32 μg/mL was the most often, but the analyzed isolates were imipenem resistant [17]. The resistance to ciprofloxacin of isolates from Switzerland was lower than ours [37].

In the Healthcare Centre "Aleksinac" the first *P. aeruginosa* isolates producing MBL were detected in 2011. The recent data indicate that the frequency of the MBL production was similar in the outpatients and inpatients isolates. In a Lepšanović et al. [38] study an isolate producing VIM-1 MBL was detected. MBL production was higher in the study at the Institute for Health Protection of Mother and Child "Dr.Vukan Čupić" Serbia in Belgrade (36.5%) [39]. Testing in Croatia in 2009 identified 3.6% *P. aeruginosa* strains producing MBL [40]. VIM-1 and VIM-2 MBLs are present in the most European countries [41–43]. A total of 1.3% of strains in Japan [17] and 30% in Canada [44] produce MBL.

The following serotypes *P.aeruginosa* were serologically identified in our studies: P1, P3, P4, P6, P10 and P11. The most frequent serotypes were P11 (22.64%), P6 (15,09%) and P1 (11.32%), while others were present in the smaller percentages. Totally 28.3% of strains were atypical (NT). Serotypes P11 (22.64%), P6 (11.32%) and P1 (11.32%) were most common in inpatients swabs. In outpatients samples P11 (15.09%), P6 (7.54%) and P1 (5.66%) serotypes were the most frequent ones. Similar results were obtained in the Tomanovic's et al. [45] study. P1 (21%), P6 (18%) and P12 (16%) were the most common serotypes. Testing in Slovenia included 208 clinical *P. aeruginosa* isolates on which serotyping and susceptibility testing was performed. The most often serotypes were P11 (36%) and P6 (14.4%), 25.6% of the isolates belonged to other serotypes, and 20.2% were poly-agglutinative [46]. The results from Croatia in 2009, after analysis of isolates mainly from respiratory tract samples, indicate that most common serotypes were P12 (58.6%) and P11 (17.1%), while other serotypes were less frequent, while 10.65% of the isolates were atypical [17].

The most common serotype P11 was the most resistant to almost all tested antibiotics, except to colistin. Atypical isolates were next according to their sensibility to antibiotics, and then P1 and P6. The highest resistance to antibiotics allows P11 serotype to be widely spread.

Conclusion

The data we obtained in the study on the *P. aeruginosa* presence in inpatients and outpatients wounds, antibiotic resistance, minimum inhibitory concentration value and metallo-β-lactamases production are different from what we expected. *P. aeruginosa* is present with similar frequency in inpatients and outpatients isolates, their resistance to antibiotics is similar, as well as minimum inhibitory concentration values and metallo-β-lactamases production. Contrary to expectations, isolates from outpatient's swabs produced more MBL than inpatients isolates. The spatial correlation of the surgical department and the surgical clinic explains it. The lack of health personnel and inadequate organization contributes to the spread of resistant strains.

Acknowledgements

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Surgical dislocation of the hip in patients with femoroacetabular impingement: Surgical techniques and our experience

Hirurška dislokacija kuka kod bolesnika sa femoroacetabularnim impingementom: hirurške tehnike i naše iskustvo

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Abstract

Background/Aim. Arthrosis of the hip is the most common cause of a hip joint disorders. The aim of this study was to present our experience in the application of a safe surgical dislocation of the hip in patients with minor morphological changes in the hip joint, which, through the mechanism of femoroacetabular impingement, cause damage to the acetabular labrum and adjacent cartilage as an early sign of the hip arthrosis. **Methods.** We have operated 51 patients with different morphological bone changes in the hip area and resultant soft tissue damage of the acetabular labrum and its adjacent cartilage. Surgical technique that we applied in this group of patients, was adapted to our needs and capabilities and it was minimaly modified compared to the original procedure. **Results.** The surgical technique presented in this paper, proved to be a good method of treatment of bone and soft tissue pathomorphological changes of the hip in patients with femoroacetabular impingement. We had no cases with avascular necrosis of the femoral head, and two patients had nonunion of the greater trochanter, 9 patients developed paraarticular ossification, without subjective symptoms, while 3 patients suffered from postoperative pain in the groin during more energetic physical activities. **Conclusion.** Utilization of our partly modified surgical technique of controlled and safe dislocation of the hip can solve all the bone and soft tissue problems in patients with femoracetibular impingement to stop already developed osteoarthritis of the hip or to prevent mild form of it.

Key words:
femoracetabular impingement; hip joint; orthopedic procedures; methods; treatment outcome.

Apstrakt

Uvod/Cilj. Artroza je najčešći uzrok poremećaja zgloba kuka. Cilj rada bio je da se prikažu naša iskustva u primeni hirurške dislokacije kuka kod bolesnika sa malim morfološkim promenama u predelu kuka koje mehanizmom femoroacetabularnog impingementa, izazivaju oštećenje labruma acetabuluma i njemu susedne hrskavice koji su znaci rane artroze kuka. **Metode.** Operisali smo 51 bolesnika sa različitim morfološkim koštanim promenama u predelu kuka i posledičnim mekotkivnim oštećenjima labruma acetabuluma i njemu susedne hrskavice. Hirušku tehniku koju smo primenili kod operisanih bolesnika, prilagodili smo našim potrebama i mogućnostima i minimalno modifikovali u odnosu na originalnu proceduru. **Rezultati.** Hirurška tehnika, prikazana u ovom radu, pokazala se kao dobra metoda lečenja patomorfoloških koštanih i mekotkivnih promena kuka kod bolesnika sa femoroacetabularnim impingementom. Nismo imali bolesnike sa avaskularnom nekrozom femoralne glave, a kod dva bolesnika bilo je prisutno produženo srastanje osteotomije velikog trohantera. Kod 9 bolesnika razvila se paraartikularna osifikacija, bez subjektivnih tegoba, dok je kod tri bolesnika trajao postoperativni bol u preponi pri jačem naporu. **Zaključak.** Primena naše delimično modifikovane tehnike hirurške dislokacije kuka može rešiti sve probleme kostiju i mekih tkiva kod bolesnika sa femoroacetabularnim impingementom, sa ciljem da se zaustavi već razvijen osteoartritis kuka ili da se spreče njegove blage forme.

Ključne reči:
femoroacetabularni sudar; kuk, zglob; ortopedske procedure; metodi; lečenje, ishod.

Introduction

Femoroacetabular impingement (FAI) is the pathophysiological mechanism of the hip, which is a consequence of minor morphological changes in the acetabulum and/or proximal femur which reduce the physiological distance between them and cause repeated impacts of the femoral head-neck junction on the anterior and superior acetabulum edge

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during hip movements. Secondary pathological changes occur due to daily repeated microtrauma of the labrum (tear, cystic degenerative changes and ossification), labrum adjacent articular cartilage and subhondral bone [1–11]. Extensive labrum damages cause groin pain and limit the function of the hip. Two basic forms of FAI were indentified, based on the localization of bone changes: cam and pincer type of FAI, while, mixed type of FAI is, actually, a combination of the previous two. Cam type of FAI, with aspherical femoral head configuration, is the result of localized thickening or cam at the femoral head-neck junction, often described in the literature as a "pistol grip" [5, 12] or "tilt" deformity of the femoral head [13], recognised in slip of the femoral head epiphysis [14–16], Legg-Calve-Perthes disease [17], femoral head avascular necrosis [18] and poorly healed fractures of the femoral neck [19]. Pincer type of FAI is seen in the overcoverage of the femoral head by the acetabulum, as a global overcoverage (protrusio acetabuli, coxa profunda) [2, 4], the local overcoverage in retroversio acetabuli [20] and in redirectional osteotomies of the pelvis or in the trauma of the acetabuli [20, 21]. The impact of the femoral neck on the edge of the acetabulum, during movements, generates a system of levers arm, and produces counter impact of the femoral head on the posterior wall of the acetabuli, causing acetabular cartilage damage, known as "counter cup" lesions [4, 20]. Mixed type of FAI, the most common one in clinical practice, is a combination of the previous two, with morphological changes on the acetabulum and the femoral head-neck junction at the same time.

Specified pathoanatomical changes of the femur and acetabulum are treated only surgically, with the aim of the prevention of an early development of the hip osteoarthritis. There are two basic surgical approaches in FAI treatment, widely accepted in recent time hip arthroscopy, is applied to small-scale bone and soft tissue changes in the hip and, the other one, is open method, applied to extensive changes in the hip. The open method of surgery, described by Ganz et al. [22] and Lavigne et al. [23], implies surgical dislocation of the hip.

The aim of this paper was to present and popularize the basics of the surgical techniques in patients with FAI in our material as well as the early results of surgical treatment.

Methods

From November 1999 until January 2011, we performed 54 open surgical dislocation of the hip in 51 patients with FAI. There were 36 women and 15 men with an average age of 36.1 ± 9.1 years (range 19 – 54 years) at the time of surgery. In three patients, surgery was performed bilaterally, with a period between the operations of 12 to 28 months.

The indications for the surgery were: several months of groin pain, positive impingement test and the presence of clear radiographic signs of bone hip changes.

There were 11 patients (8 men and 3 women) with cam type of FAI, 29 patients (3 men and 26 women) with pincer type of FAI, and 14 patients (4 men and 10 women) with mixed form of FAI. The avarage operative time was 50 min (45 to 80 min) with a blood loss of 250 mL (150 to 400 mL). The preservation of the femoral head blood flow was the basic postulate of open

surgical procedures, to prevent femoral head avascular necrosis. Medial circumflex femoral artery (ACFM) nourishes much of the femoral head in full, as a terminal branch profunda femoris artery [24, 25]. ACFM follows the lower edge of the obturator externus muscle and going below the hip external rotators and joint capsule over the posterior and superior femoral neck, it branches in 4 to 6 retinacular arteries that have been posted subperiostaly and 4 to 5 mm from the epiphyseal line, enters the femoral head through the nutritional holes.

Surgical technique

The patient is situated on the healthy side with leg holder between the legs, in our environment made of sponge, dimensions $50 \times 100 \times 15$cm, which elevates the operated leg from the operational table, relaxing the gluteal muscles and make easier access and soft tissue preparation on the hip. Skin and fascia lata incision is lateral. Posterior edge of the gluteus medius muscle, vastus lateralis muscle are identified. In the line of the greater trochanter attachment of these muscles, the line of osteotomy on the greater trochanter is marked by electrocautery, which was set to about 5 mm behind the posterior edge, thereby avoiding the trochanteric osteotomy compromise ACFM. The thickness of the osteotomized part of the greater trochanter is up to 1.5 cm, so that the atachments of the gluteus medius and *vastus lateralia* muscle remain on it. A greater trochanter is osteotomized with a saw and then lifted anteriorly. Careful preparation of the muscle fibers along the posteruir edge of the *musculus gluteus medius* is done, tendon of the *musculus piriformis* and its attachment to the trochanteric fossa is visulised and retracts posteriorly. Below this tendon the body of *gluteus minimus* muscle is visualised and then carefully lift by sharp dissection from the superior and anterior part of the joint capsule (Figure 1).
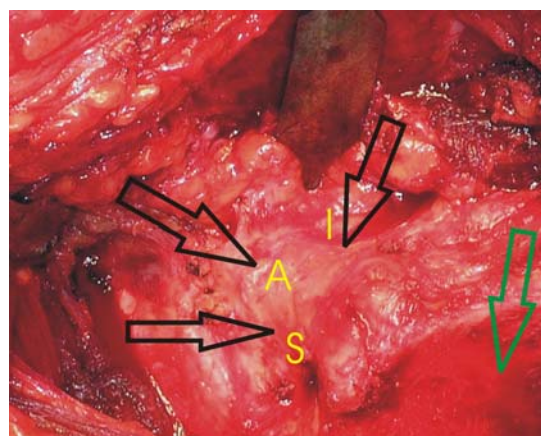
Fig. 1 – Completed dissection of the upper (S), anterior (A) and lower (I) part of the hip joint capsule (black arrow); position of the trochanteric osteotomy (green arrow).

Thus, the joint capsule is dissected, from the edge of the piriformis tendon muscle forward and downward to the lesser trochanter. After the joint capsule dissection had been completed, joint atrhrotomy is made, in the form of its near acetabular attachment, in order to prevent unnecessary damage

of the acetabular labrum in the line of capsules incison. A horizontal arm of the "Z" capsule incision, extends along the base of the neck of the femur following capsule attachment to the base of a greater trochanter, to the upper edge of the lesser trochanter. Another horizontal arm of the "Z" incision of the hip joint capsules follows its attachment to the upper edge of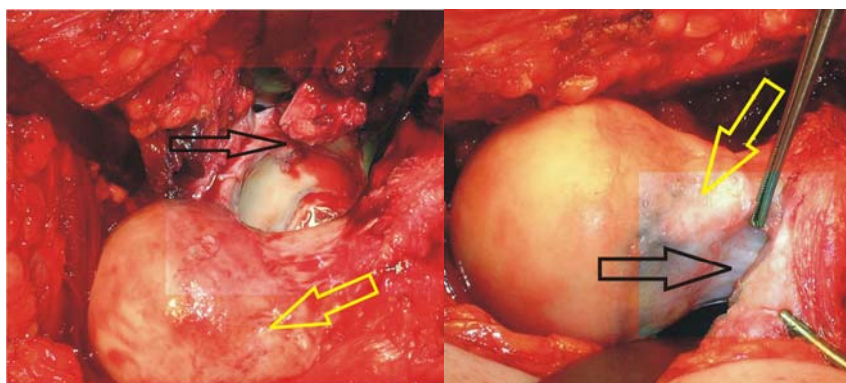 the acetabulum, and extends posteriorly to the edge of the *musculus pirifiromis* tendon, and if necessary, the approach to the hip joint, can be extended lifting the tendon of *musculus piriformis* (Figure 2). Homann retractrors are set on the anterior edge of the acetabulum above the acetabular *labrum* and the other one in the supraacetabular region, then moving the thigh in flexion,

lifting them from the base and from the subchondral acetabular bone, which is konwn as a "degloving phenomenon". Surgical correction of primary and secondary pathological changes of the hip is determined by preoperative radiographic findings and intraoperative changes of the hip. After 2005 in all cases, where the labrum damage was not too extensive, we did reinsertion of the labrum using 2–4 bone anchors, with the prior osteotomy and refreshment of the anterior and superior edge of the acetabulum. Cam osteohondral changes on the anteror and/or superior part of the femoral head and neck junction were osteotomized, following the line of the undamaged articular cartilage of femoral head,

Fig. 2 – Left: Vertical arm of "Z" capsulotomy (black arrow). Right: Horizontal acetabular arm (black arrow) and horizontal trochanteric arm of "Z" capsulotomy (yellow arrow); the femoral head and neck are shown.

adduction and internal rotation under the visual control, the area of conflict-impact of the femoral head and neck with the edge of the acetabulum is identified.

After this trial of the FAI mechanism, the controlled anterior dislocation of the hip is approached, flecting, externally rotating and adducting the femur, then, crossing the thigh of the operated leg over the healthy one and puting the lower leg of the operated side in a sterile bag made from compresses. After the hip dislocation completition, it is possible to completely visualise 360° circumference of the acetabulum and femoral head, manipulating with the leg in additional adduction or abduction of the thigh (Figure 3).

strictly taking care not to compromise the zone of the retinakular blood vessels entry in the femoral head (Figure 4). After completion of the planned surgical treatment, we performed orthopedic repositioning of the femoral head, reconstructed joint capsule and screwed a part of the osteotomized greater trochanter with two cortical screws.

Results

In all of the operated patients *labrum* lesions were located in the anterior or anterosuperior region of the acetabulum. In 11 hips anterior and/or superior acetabular edge were

Fig. 3 – Left: Access to the 360 ° circumference of the hip, severe acetabular *labrum* lesion (black arrow), cam-deformity at the junction of the femoral head and neck (white arrow). Right: Cam deformity of the femoral neck (yellow arrow), the position of retinacular blood vessels in relation to cam-deformity (black arrow).

Using the dental hook, *labrum* lesion and the part of the articular cartilage, adjacent to the *labrum* lesion is identified,
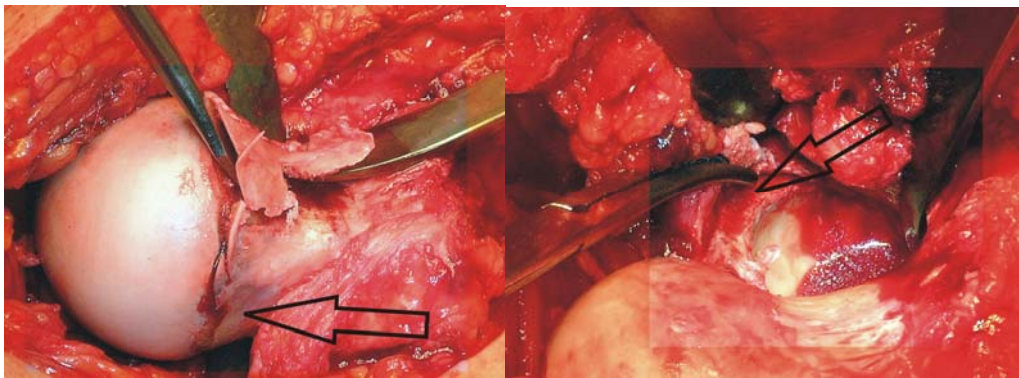
osteotomized and labrum was refixed to the bone with 2–4 anchor sutures and in 43 hips the damaged part of the *lab-*

Fig. 4 – Left: Osteochondroplasty of cam-deformity at the femoral head-neck junction, position of the retinacular blood vessels of the femoral head (black arrow). Right: partially resected badly damaged acetabular *labrum* and osteotomy of the anterior edge of the acetabulum (black arrow).

rum was resected with planned osteotomy of the edge of the retroverted acetabulum.

In 20 patients with cam and mixed type of FAI, lifted acetabular cartilages from subchondral bone were found, so-called "degloving phenomena", in the *labrum* lesion area (15–20 mm width, and 5–15 mm depth towards the fossa acetabuli) without any appropriate therapeutic possibility to solve this problem. In pincer type FAI patients, chondral lesions occurred in two places: one, in the impact area, as the actabulum *labrum* lesions, and the damaged zone of cartilage roughness, softening and hyperemia, and the other one, the counter-cup acetabular cartilage lesions, in the postero-inferior acetabular region, with, also, softening, hyperemia and fisurization of the acetabular articular cartilage.

Femoral head-neck junction osteohondral cam deformity, were found in anterior and mostly, superior part, in all patients with cam type of FAI and, only, in the anterior part of the femoral head-neck junction in the patients with mixed type of FAI. Macroscopically, cam deformity was clearly demarcated by a semicircular line, protruded above the femoral head circumference and compromized sphericity of the femoral head and neck circumference, covered with rough, hyperaemic cartilage tissue. Cam deformity was osteotomized up to the level of the femoral neck taking care not to osteotomise more than 30% of the thickness of the neck [26]. In patients with pincer type of FAI, it was very difficult to determine, how much of the acetabular edge osteotomy was enough to prevent postoperative re-impact of the femoral neck on the edge of the acetabulum on one side, and how to avoid anterior insufitient coverage of the femoral head due to excessive resection of the acetabular edge on the other side. Osteotomy of the femoral head-neck junction was done in all the patients with pincer type of FAI.

Our clinical results (Table 1) confirm the validity of the method applied in the treatment of symptomatic hips with different types of FAI. All the patients showed improvement in internal rotation of the thigh in the hip joint, hip flexion was not significantly changed, and the groin pain ceased, except for three women operated with pinzer type FAI with protrusion of the acetabulum, where a mildly positive impingement tests were found, and the reason for this as assumed, was the lack of radical resection of the anterior edge of the acetabulum.

Postoperative complications: neuropraxia of the main nerves of the leg (*nervus ischiadicus*, *nervus femoralis*), postoperative deep vein thrombosis of the operated leg, patients with avascular necrosis of the femoral head as the result of violation of its vascularization were not found. In 9 of the patients asymptomatic postoperative paraartikular osifications were developed as incidental radiographic findings at radiographic control examinations. Trochanteric osteotomies were healed on the average of 6–8 weeks, but in two patients, due to premature full weight bearing on the operated leg, slow healing of osteotomy of the greater trochanter was observed. In one patient it was enough to restrict weight bearing on the operated leg for up to 2 months, osteotomy to heal, and in one patient, 9 months after the prime operation, refixation of the great trochanter of the femur was done, which healed 3 months after the second operation.

Table 1

Early clinical preoperative and postoperative results in operated patients with different forms of femoroacetabular impigement

Parameter	Preoperative	Postoperative
Positive impingement test (n)	54	0
Groin pain (n)	54	3
Hip internal rotation (°)	0–15	20–35
Hip flexion (°)	70–90	80–90

n – number of patients.

Discussion

The interest in the problem of femoroacetabular impingement in the world is increasingly progressing as measured by the number of published papers on this subject. So, 10 papers were published in 2004, 44 papers in 2007 and, in recent years, the number of publications, per year, has exceeded 100 [27]. In our country, unfortunately, only one paper on this subject was published [28], and the number of treated patients, compared to the literature data, is significantly lower than the world's epidemiological data. This paper presents our initiative in approaching the femoroacetabular impingement and our desire to pay much more attention to this matter, in the future.

Athroscopic FAI surgery is the most commonly used method in the treatment of mild types of FAI, which is often combined with the minimally invasive open method of femoral head and neck cam defomrity resection [29], which could not be performed due to technical reasons at our institution.

This is confirmed by our early results presented in a series of patients, due to the fact that patients did not have complications such as avascular necrosis of the femoral head.

Surgical debridement of the hip is indicated in young adults with or without initial arthrotic changes to the hip, for which, the problem of hip pain can be solved and the development of arthrosis stopped or prevented. Osteochondroplastic of femoral neck bears the risk of femoral neck fracture if more than 30% of its thicknes is being osteotomized [26, 30]. In all the presen-

ted patients, femoral head and neck osteochonroplastic were done without iatrogenic postoperative femoral neck fracture.

Conclusion

Based on the previously described facts and material and our partly modified surgical technique of controlled and safe dislocation of the hip in treatment of patients with all types of femoroacetabular impingement, we believe this method to be an excellent therapeutic method. Utilization of this method can solve all bone and soft tissue problems in patients with femoroacetabular impingement aimed to stop already developed osteoarthritis of the hip, or to prevent mild forms of it.

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Effects of Rusanda Spa balneotherapy combined with calcipotriol on plaque psoriasis

Efekti balneoterapije u Banji Rusanda kombinovane sa kalcipotriolom na plak psorijazu

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Abstract

Background/Aim. Treatment of psoriasis is very complex and there are no still universal, nor unique treatment modalities. Apart from conventional treatment, which includes topical calcipotriol (vitamin D3 analogue), balneotherapy is drawing increased attention worldwide. Being part of climatotherapy, balneotherapy is defined as the use of natural environmental factors in the treatment of health conditions, whereas in the treatment of psoriasis it means the use of mineral baths and peloids. The aim of this study was to examine the therapeutic efficacy of mineral waters and peloids of the Rusanda Spa on plaque psoriasis in patients also treated with calcipotriol. **Methods.** The study included 60 patients divided into two groups. The first group included patients treated with mineral waters, peloids and calcipotriol in the Rusanda Spa, while the second one included those treated only with calcipotriol. The study took 21 days, and each patient was followed up for at least one month after ending the treatment. The treatment efficacy was measured by psoriasis area severity index (PASI) scores on the days 0, 7, 14 and 21 during the treatment and 30 after the end of the therapy. **Results.** After a 3-week treatment in the Rusanda Spa, the first group showed a decrease in PASI score by 59.45%, whereas in the group of outpatients treated by calcipotriol it was 39.34%. On the day 30 following the treatment, the first group presented with the PASI score reduction of 58.44%, and the second group of 34.78%. The therapeutic efficacy of mineral waters and peloids combined with calcipotriol showed to be significantly higher in regard to monotherapy with calcipotriol ($p < 0.05$). In regard to clinical symptoms, the best results were obtained in the reduction of desquamation ($p < 0.001$). **Conclusion.** The results of our study show that in the treatment of plaque-type psoriasis, topical calcipotriol combined with Spa Rusanda balneotherapy is more effective than topical calcipotriol alone. Randomized controlled trials are needed to confirm the effects of balneotherapy as monotherapy in treatment of this type of psoriasis.

Key words:
psoriasis; mud therapy; baths; serbia; vitamin d; administration, topical; treatment outcome.

Apstrakt

Uvod/Cilj. Lečenje psorijaze veoma je složeno i još se traga za univerzalno ili jedinstveno efikasnim terapijskim metodama. Osim konvencionalne terapije, koja kod lokalnog lečenja uključuje i primenu kalcipotriola, analoga vitaminu D3, u svetu se pažnja sve više posvećuje i balneoterapiji. Pod pojmom balneoterapije kao sastavnom delu klimatoterapije, podrazumeva se delovanje prirodnih činilaca spoljašnje sredine na ljudski organizam, a u terapiji psorijaze konkretno se misli na upotrebu mineralne vode i peloida. Cilj rada bio je da se ispita terapijski učinak mineralne vode i peloida Banje Rusande na plakozne promene psorijaze kod obolelih koji su istovremeno lečeni kalcipotriolom. **Metode.** Ispitano je 60 osoba koji su bile razvrstane u dve grupe. Prva grupa je obuhvatila one koji su lečeni mineralnom vodom, peloidom i kalcipotriolom u Banji Rusandi, a u drugoj grupi su bile osobe lečene kalcipotriolom. Ispitivanje je trajalo 21 dan, a ispitanici su praćeni najmanje mesec dana po obustavi terapije. Za procenu terapijske efikasnosti korišćena je standardna metoda određivanja *psoriasis area severity index* (PASI) skora 0, 7, 14, 21. dana terapije i 30. dana po završetku terapije. **Rezultati.** U prvoj grupi lečenih u Banji Rusandi, nakon tronedeljne terapije došlo je do sniženja vrednosti PASI skora za 59,45%, dok je u grupi lečenih kalcipotriolom ovo sniženje iznosilo 39,34%. Tridesetog dana po obustavi terapije procenat poboljšanja PASI skora iznosio je 58,44% u grupi lečenih u Banji Rusandi i 34,78% u grupi lečenih kalcipotriolom. Terapijski učinak mineralne vode i peloida u kombinaciji sa kalcipot-

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riolom pokazao se značajno efikasnijim od monoterapijske primene kalcipotriola ($p < 0,05$). Od kliničkih simptoma bolesti najbolji terapijski rezultati postignuti su u smanjivanju deskvamacije ($p < 0,001$). **Zaključak.** Rezultati naše studije pokazali su da je kombinovana primena topijskog kalcipotriola i balneoterapije u Banji Rusandi u lečenju psorijaze tipa plaka efikasnija nego topijska primena kalci-

potriola kao monoterapije. Potrebne su randomizirane kontrolisane studije koje bi potvrdile efekte balneoterapije kao monoterapije u lečenju ovog tipa psorijaze.

Ključne reči:
psorijaza; lečenje blatom; kupke; srbija; vitamin d; lokalna primena; lečenje, ishod.

Introduction

Treatment of psoriasis is still very complex and brings together a large number of experts in various fields to find effective therapeutic methods that provide the required level of safety for patients [1, 2]. The treatment may be topical, systemic or combined. Topical treatment has several advantages over systematic methods: it is easier to apply, provides fast action which is directed only to the skin, as well as a lower degree of risks from adverse reactions. Topical treatment includes emollients, keratolytics, corticosteroids, dithranol, various tar preparations, retinoids (tazarotene), immunomodulators (tacrolimus and pimecrolimus), phototherapy ultraviolet A – long wave and ultraviolet B – short wave rays (UVA, UVB light), climate therapy and D3 analogues (calcipotriol). Effects of calcipotriol (vitamin D analogue) in the treatment of psoriasis have been published over the past 25 years, pointing to a long term scientific interest in this drug [3–5].

Climatic treatment includes balneotherapy and sun exposure [6]. Balneotherapy factors, such as mineral water and/or peloids exert favorable therapeutic effects on plaque psoriasis [7–11]. A combination of multiple local and/or systemic medications aims to increase the efficiency of psoriasis treatment (PUVA – psoralen +UVA).

The aim of this study was to determine whether therapeutic use of mineral waters and peloids of Rusanda Spa combined with topical calcipotriol has greater effects on clinical symptoms and the course of vulgar psoriasis (plaque form) compared to monotherapy with calcipotriol.

Methods

The study included 60 consecutively examined patients with the diagnosis of plaque psoriasis examined and treated at the Clinic of Dermatovenereology Diseases of the Clinical Center of Vojvodina in Novi Sad. The subjects were randomized into two groups of 30 patients each.

The study group included the patients treated with mineral water, peloids and calcipotriol in Rusanda Spa. The control group included the outpatients treated only with calcipotriol.

Inclusion and exclusion criteria

Inclusion criteria were: plaque psoriasis affecting the extremities and/or trunk and age over 16 years. Exclusion criteria were as follows: all other forms of psoriasis, age under 16 years, cytostatic therapy in the previous 4 weeks, systemic retinoid therapy in the past 12 months, local corti-

costeroid therapy, topical therapy with retinoids or vitamin D3 analogues in the last three months and liver or kidney diseases. In the group of patients undergoing treatment with mineral water and peloids, exclusion criteria also included contraindications to spa treatment, infectious diseases, cancer, frequent and heavy bleeding, cachexia, pregnancy, severe cardiovascular disorders, as well as uncooperative behavior and reported adverse effects.

The study took 21 days, and each patient was followed-up for at least 1 month after ending the therapy.

The study group was treated in Rusanda Spa and patients were subjected to the following treatment schedule: 20-minute mineral baths twice a day, 20-minute 3 cm thick layers of hot peloid packs with polyethylene pressure (38–42°C) once a day, and calcipotriol ointment twice a day. The control group of patients received only calcipotriol ointment twice a day.

Treatment efficacy assessment

In order to assess the therapeutic efficacy, a standard psoriasis area and severity index (PASI) quantitative rating score was used for measuring localization, extent and severity of psoriatic lesions on days 0, 7, 14 and 21 during the treatment and 30th after the end of therapy. A reduction in PASI score ≤ 20% showed a low response rate, 21–40% a satisfactory response rate, 41–60% a good response rate, 61–80% a very good response rate, and 81–100% showed excellent responsiveness [12–14].

Statistical analysis

Data analysis included descriptive and inferential statistics. The following numerical homogeneous characteristics were calculated and presented: the size of sample, arithmetic mean, median (Med), mode (Mod), range and standard deviation (SD), while categories were presented as absolute and relative numbers. Comparison of mean values was performed using Student's t-test. The differences between the obtained and expected frequencies were calculated using Pearson's $\chi 2$ test of agreement. A statistical significance was set at $p < 0.05$.

Results

The water of Rusanda Spa is alkaline (pH 9.3–9.4) oligomineral, hypothermal mineral waters rich in sodium bicarbonate. Characteristics of Rusanda Spa mineral water are shown in Table 1.

Table 1

The composition of mineral waters and peloids in Rusanda Spa

Mineral water	mg/L	Peloid	mg/kg
Sodium	1235.0	Silicon dioxide	408.0
Potassium	276.4	Titanium dioxide	3.2
Lithium	0.02	Aluminium oxide	110.0
Ammonium	9.0	Manganate	0.8
Calcium	40.0	Iron oxide	26.0
Magnesium	60.0	Iron (III) oxide	23.4
Strontium	0.1	Calcium oxide	48.0
Manganese	0.05	Magnesium oxide	54.0
Iron	0.6	Sodium oxide	23.4
Aluminum	0.2	Potassium oxide	36,0
Hydrocarbons	2200.0	Phosphorus pentoxide	4.5
Chloride	980.0	Sulfate	9.0
Bromides	7.0	S-sulfides	2.5
Iodides	0.8	Chlorine	22.0
Fluoride	1.2	Carbon dioxide	78.0
Phosphate	0.1	Water	41.0
Sulfate	192.0	Nitrate	3.5
Metasilicic acid	14.3	Ammonium	0.8
Metaboric acid	46.8		

Demographic characteristics of patients

The study group treated in Rusanda Spa with mineral baths, peloids, and calcipotriol, included 18 (60%) female patients, which was not statistically significantly higher incidence than men ($\chi2 = 1.2$; $p > 0.05$). The patients' age ranged from 24 to 75 years, the mean age 55.46 (SD = ±12.13) years. In 12 (40%) examinees lesions were located only on the extremities, in 13 (43.3%) on the trunk and extremities, and the remaining 5 (16.7%) presented with lesions only on the trunk.

In the group of patients treated with calcipotriol, there was a predominance of male patients (16 of 30 patients), but it was not statistically significant higher number than females. The age of examinees in this group ranged from 20 to 67 years. The average age was 41.73 (SD = ±13.33) years. In 13 (43.3%) patients lesions were located only on the extremities, while the remaining 17 (56.7%) patients presented with lesions on the trunk and extremities.

There was a statistically significant difference between the two groups in relation to age ($t = 4.1726$; $p = 0.0001$), and no significant difference in sex distribution ($\chi2 = 1.071$; $p > 0.05$). Also, no significant differences were found in the distribution of lesions within the analyzed groups ($\chi2 = 5.573$; $p > 0.05$).

Distribution of psoriasis area severity index scores during the treatment

In the study group treated in Rusanda Spa with mineral baths, peloids, and calcipotriol, the PASI scores on day 0 (baseline PASI score) ranged from 2.40 to 17.40. The average PASI score was 9.89. Seven days after the beginning of the therapy, PASI scores ranged from 1.60 to 13.80. The average score was 8.13. On the day 14 of the treatment, PASI scores ranged from 1.60 to 11.60, while the average score was 5.91. After 21 days from the initiation of the therapy, PASI scores ranged from 0.80 to 10.60, while the average score

was 4.01. Thirty days after the treatment ended, PASI scores ranged from 0.80 to 10.60, with the average score of 4.11.

In the group of patients treated with calcipotriol, baseline PASI scores ranged from 2.40 to 16.40, with the average score of 8.54. On the day 7 of the therapy, PASI scores ranged from 2.40 to 15.60, with the average score of 7.84. After 14 days of the treatment, PASI scores ranged from 2.40 to 12.40, and the average score was 6.48. After 21 days of the treatment, PASI scores ranged from 1.60 to 10.40. The average score was 5.18. Thirty days after the end of the treatment, PASI scores ranged from 2.00 to 12.60, with the average score of 5.57. PASI scores are shown in Table 2.

In the study group treated with mineral baths, peloids and calcipotriol, the mean score for erythema at baseline was 3.25, and 2.11 at the end of therapy; this means that after treatment the mean scores for erythema decreased by 35.1%. Based on these results, therapeutic efficacy for erythema may be regarded as satisfactory. The mean scores for infiltration ranged from 3.49 at baseline to 1.39 at the end of the treatment, with a percentage improvement of 60.4, which is a very good therapeutic response. PASI scores for desquamation at the end of therapy decreased from 3.15 to 0.49, with a percentage improvement of 84.5, that is an excellent therapeutic response.

The PASI score for erythema in the group treated only with calcipotriol was 2.87 at baseline, and 2.13 immediately after cessation of the therapy. These results indicate that the average scores for erythema decreased by 25.8% and the therapeutic efficacy may be regarded as satisfactory.

In regard to infiltration, after treatment, the control group presented with an average PASI score decrease by 42.6% and it is considered as good therapeutic efficiency. At the end of the therapy, in patients treated with calcipotriol the PASI score reduction for desquamation was 49.9%, which was also considered as good therapeutic response. Table 3 shows PASI scores for erythema, desquamation and infiltration.

Comparative analysis of the mean PASI scores between the two groups showed no statistically significant difference du-

Table 2
Psoriasis area severity index (PASI) scores in the patients treated with mineral baths, peloids and calcipotriol in Rusanda Spa (group 1) and in the patients treated with calcipotriol (group 2)

Days	PASI score (Group 1)	PASI score (Group 2)	p^*
Day 0	9.89	8.54	
med	9.80	8.40	
mod	9.60	8.40	
SD	3.97	3.66	
Day 7	8.13	7,84	
med	8.00	7.40	
mod	8.00	7.20	
SD	3.56	3.54	
Day 14	5.91	6,48	
med	6.30	6.40	
mod	3.20	2.40	
SD	2.71	2.80	
Day 21	4.01	5.18	0.042
med	4.00	4.80	
mod	3.20	4.80	
SD	2.09	2.28	
Day 30 after treatment	4.11	5.57	0.016
med	4.00	5.15	
mod	3.20	2.40	
SD	2.06	2.47	

*p – statistically significant (< 0.05).

Table 3
Psoriasis area severity index (PASI) scores for erythema, infiltration and desquamation before and after the treatment in the patients treated with mineral baths, peloids and calcipotriol in Rusanda Spa (group 1) and in the control group treated with calcipotriol (group 2)

Plaque psoriasis changes	PASI score (Group 1)	PASI score (Group 2)	p
Erythema before treatment	3.25	2.87	
Erythema after 21 days of therapy	2.11	2.13	
Infiltration before treatment	3.49	2.78	0.049
Infilration after 21 days of therapy	1.39	1.60	
Desquamation before treatment	3.15	2.89	
Desquamation after 21 days of therapy	0.49	1.45	< 0.001

*p – statistically significant (< 0.05).

ring treatment at days 0, 7 and 14. Using Student's *t*-test, a statistically significant difference was determined after 21 days of treatment ($t = -2.080$; $p = 0.042$) and 30 days after ending the therapy ($t = -2.487$, $p = 0.016$) in favor of the group treated with the combination therapy (balneotherapy and calcipotriol).

There was no statistically significant difference between the groups in terms of reduction of erythema and infiltration at the end of the treatment, but there was a statistically significant difference in the reduction of desquamation in favor of the group treated with a combination therapy (Student's *t*-test).

Adverse effects

Slight irritation at the site of calcipotriol application was reported during the first week of the therapy by 3 (10%) patients from the study group and by 2 (6.6%) patients from the control group, but discontinuation of the planned treatment was not necessary. In regard to the observed adverese effects, there were no significant differences between the two groups ($\chi2 = 0.218$, $p > 0.05$).

Discussion

After a 3-week treatment, the study group treated in Rusanda Spa showed a decrease of PASI scores by 59.45%, while in the control group treated only with calcipotriol a decrease was 39.34%. The therapeutic effects of peloids and mineral baths in combination with calcipotriol proved to be significantly more effective than monotherapy with calcipotriol.

It is well-known that mineral waters and peloids exhibit mechanical, thermal and chemical effects when applied to the skin. They reduce the thickness of the stratum *corneum* and stratum *lucidum* of the epidermis, increase the number of lymphocytes, histiocytes, and eosinophils, increase the permeability of the skin, reduce the activity of inflammatory processes, improve microcirculation and enhance the immune processes [15, 16]. It was found that mineral waters inhibit mast cell activation *in vitro*, that may explain their beneficial anti-inflammatory effects. It is assumed that inhibition of mast cells is achieved by the effects of mineral waters on nerve endings and the substance P in the skin [15, 16].

The immunomodulating effects of sulfur-rich water are expressed through the inhibition of proliferation of T-lymphocytes. Bicarbonate and silicium waters reduce degranulation of basophilic granulocytes and suppress the effect on cytokine production. This effect is attributed to selenium, zinc and copper. Although immunomodulating effects of mineral waters have only been established *in vitro*, they can be compared with the pharmacological effects of local immunomodulators, e.g. with imiquimod [9].

The knowledge about positive effects of mineral waters and peloids on skin has encouraged investigations on their efficacy in the treatment of many skin diseases such as atopic dermatitis, seborrheic dermatitis, chronic eczema and related conditions.

Psoriasis is also one of the diseases that have been successfully treated with balneotherapy, and the object of numerous studies, particularly when dealing with a combination of balneotherapy with other therapeutic modalities, e.g. phototherapy [9]. A small number of studies have examined the effects of monotherapy with mineral waters and peloids, and the only compatible study of this kind has been conducted in Prolom Spa with satisfactory therapeutic results. The study included 35 patients and after 3 weeks of therapeutic use of mineral waters and peloids a 38.75% PASI score reduction was established [17]. Compared with the results of our study, these results indicate a lower therapeutic efficacy of Prolom Spa treatment, which may be explained by the fact that in our study balneotherapy was combined with topical application of calcipotriol. It is possible that the therapeutic efficacy was affected by the composition of mineral water, as well as its alkalinity.

It is well-known that alkaline water makes the skin soft and smooth. In combination with heat, it stimulates the transport of substances through the skin in both directions, which possibly increases the transportation of calcipotriol and efficiency of combined therapy over monotherapy as was in our patients. The best results were achieved in reducing desquamation, followed by reduction of infiltration and erythema. After a 3-week therapy, desquamation was reduced by 84.5% and no significant differences were found in a 30-day follow-up period. This effect was statistically significant compared to calcipotriol monotherapy. In the study of Paravina et al. [17] the reduction of desquamation after three weeks of balneotherapy was only 42.71%. Although the average reduction in induration in the study group treated with balneotherapy and calcipotriol was 60.4%, and it was higher than in the study of Paravina and et al. [17], it was not significantly higher than in the group treated exclusively with calcipotriol. Induration reduction in the group treated only with calcipotriol (42.5%) was similar to the results in the above mentioned study (43.59%), indicating a similar level of efficiency of calcipotriol monotherapy and balneal monotherapy in regard to infiltration.

As a clinical sign of the disease, erythema showed the lowest response rate in both groups of patients: neither type of therapy has proved significantly more effective than the other; similar results were obtained in the study conducted in Prolom Spa [17].

The effectiveness of balneotherapy in the treatment of psoriasis has been reported in many studies conducted in the world. If we take into account that our results regarding effectiveness were assessed as good, it can be said that they are in accordance with the results of other authors. In Argentina, 55 patients with psoriasis were treated with mineral baths, peloids and/or algae in Copahue Thermal Complex. Twice a day mineral baths lasted for 10 days on the average and the patients showed improvements in terms of the reduction of erythema and desquamation, which was confirmed by histopathological analysis [10]. Beneficial effects of balneotherapy have been reported in Bulgaria, where they use hypothermal water causing skin vasodilatation [18]. In Jagodina Spa, 54 patients with vulgar psoriasis underwent a combination of balneotherapy, topical dithranol and phototherapy (20 minute baths, application of 1.5% to 3% dithranol for 10 to 30 minutes, and exposure to UV rays with wavelengths of 300 – 340 nm from 1 to 20 minutes). Three weeks later, 73.3% of patients showed a significant improvement of skin lesions [17]. An investigation in Marikostinovo Spa included 100 patients treated with a combination of mineral water and sulfide peloids. Both procedures lasted 10 to 20 minutes a day for three weeks, and 3% of respondents showed a complete regression of skin lesions, in 5% there was a significant improvement, 83% showed moderate improvement, in 5% the therapy showed no effects, and in 4% of patients the condition got worse [18].

A large number of investigations worldwide studied effects of balneotherapy and heliotherapy on chronic stationary psoriasis. The best results were achieved at the Dead Sea in Israel, which is characterized by a high salinity (about 30%), high concentration of minerals in the air (magnesium, bromide and other minerals) and a high number of sunny days per year. The most comprehensive study at the Dead Sea was conducted in 1995 including 1.448 patients with psoriasis. After four weeks of bathing in sea water and sun exposure, 88% of subjects showed improvement. The degree of improvement varied from 80% to 100% reduction in PASI score [11]. Another prospective study included 100 patients with psoriasis treated at the Dead Sea for four weeks. In 75% of patients there was a complete regression of skin lesions. In further follow-up, after the end of the therapy, 68% of these patients were in complete remission during the next four months, 43% of patients were in remission after six months, and in 10% of patients complete remission lasted for eight months after treatment. By monitoring the length of remission after treatment, it was observed that after climatic and heliotherapy this period was shorter than after some other forms of therapy, such as with cyclosporine or PUVA. However, contrary to this, a comparative study, although including a small number of patients, showed that the efficacy of heliotherapy during four weeks was higher than with UVB phototherapy [19]. Attempted treatment by bathing in the Dead Sea and UVB phototherapy (wavelengths of 311 nm) also gave good results. After 35 treatments (three to five times per week), the PASI score decreased from 17.7 to 5.2. The most common side effect of this therapy was rash, found in 87% of cases, and it was caused by ultraviolet radiation. Af-

ter treatment, 55% of patients had recurrence after six months, and 68% of patients after one year [20].

A combination of three favorable factors for psoriasis, sun, sea water and air, is applied in patients with psoriasis at the Black Sea in Bulgaria. In a group of 177 patients, the therapy lasted 20 days and covered sun exposure of 5–6 hours *per* day, and bathing in sea water from 5– 15 minutes a day. Complete regression of skin lesions was found in 68.9% of patients, 17.1% presented with a significant improvement, and a moderate improvement was found in 9.5% of patients. Only 4.5% of patients did not respond to the therapy (no processing done in terms of statistical significance) [21].

Salt water soaks have a significant impact on transforming growth factor beta1 (TGFβ1) which is decreased in psoriatic epidermis models. If the affected skin is exposed to 30% sodium chloride solution, TGF-β1 mRNA expression significantly increases, even without additional skin exposure to UVB rays ($p = 0.0024$), compared to untreated psoriatic epidermis models or epidermis treated with 3% sodium chloride solution. If combined with UVB rays, a significant increase in the expression of TGFβ1 mRNA, apart from using 30% sodium chloride, is evident when using Dead Sea salt water in comparison with 3% sodium chloride ($p = 0.00014$) [22]. A study conducted in spa resorts in Germany showed that a significant reduction in PASI score occurred in psoriatic patients exposed to UVB rays and various concentrations of sodium chloride, compared to patients only exposed to UVB radiation, after 6 weeks of treatment. The best results were found in patients treated with highly concentrated saline spa water baths, sodium chloride concentration between 25% and 27% ($p < 0.001$) [23, 24]. A German study compared the effectiveness of salt water *versus* tap water. Ten psoriasis patients with chronic plaques on the elbows were included in the study. One elbow was soaked in 24% NaCl solution and the other in tap water. Subsequently, broadband UVB irradiation was administered. Balneophototherapy was performed 4 times weekly with a total of 30 treatments. A highly significant ($p < 0.001$) decrease of the clinical baseline score was observed after 30 treatments; however, there was no significant ($p > 0.5$) difference in clearance of the psoriatic lesions between the sites soaked in salt water and tap water [25]. If we examine the efficacy of psoralens dissolved in a warm-water bath followed by exposure to UVA irradiation (bath PUVA) or salt water UVB phototherapy compared with tap-water UVB phototherapy or UVB irradiation alone in psoriasis, we come to the conclusion that bath PUVA and salt water UVB phototherapy are comparably effective treatments in psoriasis and superior to UVB and tap-water UVB phototherapy ($p < 0.001$) [26].

The question is to what extent pretreatment with salt water soaks alters inflammatory and/or carcinogenic effects of UVB phototherapy. Therefore, the impact of balneophototherapy on cyclooxygenase (COX-2) gene expression and apoptosis in normal and psoriatic skin were investigated. Compared with untreated controls, COX 2 gene expression (COX-2 mRNA) was significantly increased in UVB irradiated normal and psoriatic epidermis models. UVB-exposed and non-exposed 30% NaCl and 30% Dead Sea water-treated normal and psoriatic epidermis models showed significantly higher COX-2 mRNA, when compared with controls and 3% NaCl. In UVB-exposed 30% NaCl and 30% Dead Sea water-treated normal and psoriatic epidermis surviving mRNA was significantly decreased when compared with controls and 3% NaCl. Although balneophototherapy using high-concentrated salt water solutions is associated with increased epidermal COX-2, mRNA expression, apoptosis of keratinocytes is enhanced possibly due to the down-regulation of surviving mRNA expression [27].

The control group of our patients treated only with calcipotriol presented with the PASI score reduction of 8.2% after 7 days of treatment, 24.12% after 14 days of treatment, 39.94% after 21 days of treatment, and 34.78% after thirty days from ending the treatment.

It was found that the effectiveness of therapy is achieved by binding of calcipotriol to the vitamin D receptor (VDR) in keratinocytes, and then interacting with regions of DNA inhibiting cell proliferation. Expression of VDR in keratinocytes is directly proportional to the therapeutic response to calcipotriol. This was determined by immunohistochemical analysis in vivo. Calcipotriol therapy statistically significantly increases the expression of VDR, the impact of drugs on the proliferation and differentiation of keratinocytes is higher than the impact on inflammation in dermis [4]. In a study conducted by van Rossum et al. [28] a four-week therapy with calcipotriol reduced the proliferation of keratinocytes by 11.7% (no processing done in terms of statistical significance). Given these results, it may be assumed that the results of our research, in both groups, would be even better if the therapy lasted longer.

One of the first multicenter studies on the efficacy of calcipotriol in the treatment of plaque psoriasis was conducted in the US in the mid-nineties [29]. It included 277 patients aged 19 to 83 years. At the end of the first week of treatment, statistically significantly lower scores ($p < 0.001$) were obtained and they were present at the end of 8 weeks of therapy. None of the patients reported worsening of symptoms [29]. These results also indicate that the efficacy of calcipotriol depends on the duration of treatment. In Singapore, a research included 30 patients: after six weeks of treatment their PASI score was reduced by about 70% [30]. A multicenter study conducted in several European countries included 308 patients with psoriasis. They were treated with calcipotriol ointment twice a day. The average age of patients was 46.3 years, and the mean duration of the disease was 18 years. The average reduction in PASI score after the first week of therapy was 28.4%, and 48.8% after three weeks of therapy [31]. Good results in the treatment of psoriasis were also achieved combining calcipotriol and heliotherapy, regardless if it was natural or artificial light [32, 33].

In a European multicenter study conducted in 2004, including 972 patients, the most frequent side effect was calcipotriol induced pruritus reported by 10.9% of patients [34]. Two studies also showed side effects of topical calcipotriol in 11.4% and 19.8% of respondents, respectively. They manifested as irritation and itching of the skin [35, 36]. In our study, unwanted skin irritation and itching were observed in 10% of

patients treated with balneotherapy and calcipotriol, and in 6.6% of patients treated only with calcipotriol.

A limitation of our study is that we did not examine the effects of balneotherapy as a monotherapeutic modality in the treatment of plaque-type psoriasis. At the moment, there is a limited number of studies whose results suggest that balneotherapy, as a monotherapy, is superior to conservative therapy.

Conclusion

In conclusion, the results of our study show that in the treatment of plaque-type psoriasis, topical calcipotriol combined with Rusanda Spa balneotherapy is more effective than topical calcipotriol alone. Randomized controlled trials are needed to confirm the effects of balneotherapy as a monotherapy in treatment of this type of psoriasis.

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Determination of 5-caffeoylquinic acid (5-CQA) as one of the major classes of chlorogenic acid in commercial tea and coffee samples

Određivanje 5-kafeoilhinske kiseline (5-CQA) kao jedne od najznačajnijih klasa hlorogenske kiseline u komercijalnim uzorcima čaja i kafe

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Abstract

Background/Aim. Tea and coffee are one of the most widely consumed beverages in the world due to their beneficial health effects which are largely associated with their phenolic compounds composition, including chlorogenic acid. The main aim of this study was to determine 5-caffeoylquinic acid (5-CQA), as one of the major classes of chlorogenic acid, in various commercial tea and coffee samples present at the Serbian market. **Methods.** A high-performance liquid chromatography (HPLC) method for determination of 5-CQA in plant extracts was applied to determine the content of this active compound in commercial tea and coffee samples. Mobile phase was aqueous 1.5% acetic acid – methanol (80:20, v/v) with the flow rate of 0.8 mL/min. Run time was 15 min and column temperature 25°C. The detection was performed at 240 nm. **Results.** The HPLC method was modified and revalidated. The 5-CQA content varied depending on the type of tea (white, green, black tea and mate) and the processing technology. Green tea had the highest 5-CQA content (16 mg/100 mL) among the analyzed tea samples. The content of 5-CQA in coffee samples ranged 0–36.20 mg/g of coffee and 0–46.98 mg/100 mL of beverage, showing that the content varied depending on the type of coffee, coffee processing technology and the formulation. **Conclusion.** The modified and revalidated HPLC method showed a good accuracy, repeatability, selectivity and robustness. The highest amount of 5-CQA was determined in green tea in comparison to white, black and mate tea because the increased oxidation level decreases the amount of 5-CQA. The obtained results for commercial coffee samples indicated that the formulation was the most important factor determining the amount of 5-CQA. It can be concluded that plant material selection, processing conditions and formulation have great influence on the amount of chlorogenic acid (5-CQA) in the final tea and coffee products.

Apstrakt

Uvod/Cilj. Čaj i kafa su jedni od najčešće konzumiranih napitaka širom sveta zahvaljujući svom lekovitom dejstvu koje se u velikoj meri pripisuje fenolnim komponentama koje sadrže, uključujući i hlorogensku kiselinu. Glavni cilj istraživanja bio je određivanje 5-kafeoilhine kiseline (5-CQA), kao jedne od najznačajnijih klasa hlorogenske kiseline, u različitim komercijalnim uzorcima čaja i kafe prisutnih u slobodnoj prodaji na tržištu Republike Srbije. **Metode.** Metoda za određivanje 5-CQA u biljnim ekstraktima primenom visokoefikasne tečne hromatografije (HPLC) korišćena je za ispitivanje sadržaja ove aktivne komponente u komercijalnim uzorcima čaja i kafe. Mobilnu fazu predstavljao je vodeni rastvor 1.5% sirćetne kiseline – metanol (80 : 20, v/v) sa protokom od 0,8 mL/min. Vreme analize iznosilo je 15 minuta, a temperatura analitičke kolone 25°C. Detekcija je vršena na 240 nm. **Rezultati.** HPLC metoda je modifikovana i revalidovana. Sadržaj 5-CQA varirao je u zavisnosti od vrste čaja (beli, zeleni, crni i mate čaj) i tehnološkog postupka prerade. Najveći sadržaj 5-CQA ustanovljen je u uzorku zelenog čaja (16 mg/100 mL). Sadržaj 5-CQA u uzorcima kafe kretao se u opsegu 0–36,20 mg/g kafe i 0–46,98 mg/100 mL napitka ukazujući da je sadržaj zavisio od tipa kafe, tehnološkog postupka prerade i formulacije. **Zaključak.** Modifikovana i revalidovana HPLC metoda pokazala je dobru preciznost, ponovljivost, selektivnost i postojanost (robusnost). Najveći sadržaj 5-CQA u uzorcima čajeva je utvrđen u uzorku zelenog čaja u poređenju sa belim, crnim i mate čajem jer sa većim stepenom oksidacije opada sadržaj 5-CQA. Rezultati dobijeni za uzorke kafe ukazivali su da je na količinu 5-CQA najviše uticala formulacija. Može se zaključiti da selekcija biljnog materijala, kao i uslovi tehnološke obrade i formulacija pokazuju značajan efekat na sadržaj hlorogenske kiseline (5-CQA) u finalnim proizvodima čaja i kafe.

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Introduction

Tea and coffee are among the most widely consumed beverages in the world. It is estimated that about 3 million tons of tea is produced and consumed annually, among which 76% to 78% is black, 20% to 22% is green and less than 2% is oolong [1, 2]. Due to its attractive aroma and pleasant taste it has been used for thousands of years as a refreshing drink, while nowadays the scientific interest in this plant is increasing due to its potential beneficial health effects. Herbal teas are widely used for a great number of health problems, as an additional or sometimes only "medicine" given [3, 4]. Different types of tea are gained from the same *Camellia sinensis* (Theaceae) plant, with different production processes: white and green tea (the least processed, both unfermented), oolong tea (partially fermented) and black tea (fully fermented). Another plant originating from South America whose leaves are important source of purine alkaloids, but also chlorogenic acid, is *Ilex paraguariensis*, Aquifoliaceae (Paraguayan tea or mate) [5–7].

Coffee is a bitter drink made from roasted seeds of plants *Coffea* spp., Rubiaceae. This plant grows in over 70 countries, primarily in equatorial South America, Southeast and South Asia and Africa [8]. Brazil is the largest manufacturer and exporter of green coffee seeds making 35% of the world coffee production. There are several types of coffee species used for commertial production, but the most important are *Coffea arabica* and *Coffea canephora* [9]. After harvest, green coffee beans are processed in one of the three ways: dry ('natural' or 'unwashed' coffee), wet ('washed' or 'parchment' coffee) or semidry method [10].

Both tea leaves and coffee seeds have very diverse chemical composition. Various positive health effects of *Camellia sinensis* and *Coffea* spp. are attributed to the presence of purine alkaloids (caffeine, theobromine and theophylline), but also to their phenolic components. Antioxidant, hypoglycemic, antiviral and hepatoprotective activities are some of the potential beneficial properties associated with the chlorogenic acid and other phenolic compounds. Some studies demonstrated the connection between chlorogenic acid and reduced risk of cardiovascular diseases, diabetes mellitus type 2 and Alzheimer's disease [11–13].

Farah et al. [14] found that the consumption of green coffee significantly reduced blood pressure, improved vasoreactivity and reduced accumulation of adipose tissue and glucose metabolism due to high content of chlorogenic acid (5–12 g/100g. Similar effects are associated with the tea consumption [11].

The major classes of chlorogenic acids are caffeoylquinic acid (CQA), dicaffeoylquinic acid (diCQA) and less frequently, feruloyilquinic acid (FQA). Each class has at least three isomers. The most abundant and responsible for many beneficial health effects is considered to be 5-CQA [12]. It is the most responsible for pigmentation and the flavour of tea and coffee [15]. During tea fermentation, some of the phenolic compounds, including 5-CQA, are oxidized and tea undergoes changes in color, taste, scent and aroma [16]. Also, when it comes to coffee, it degrades by heat into several different compounds which determine the quality of coffee [17]. Beside the processing method, the type of coffee has great effect on the content of 5-CQA in commercial samples (the content is lower in type *Coffea arabica* than in *Coffea canephora*) [18].

There is a general lack of information on the content of 5-CQA, as one of the major antioxidants, but responsible also for many other health benefits, in commercial tea and coffee preparations of domestic and foreign manufacturers available at the Serbian market. Accordingly, the aim of this study was to apply high-performance liquid chromatography (HPLC) method for chlorogenic acid (5-CQA) determination in herbal infusions for quantifying 5-CQA in commercial tea and coffee samples present at the Serbian market. This research will also give the information on approximate chlorogenic acid (5-CQA) intake by consumers which is important due to its beneficial health effects.

Methods

Standard of 5-CQA (≥ 95%) was purchased from Sigma Aldrich (St. Louis, USA), methanol (95%, v/v) from Sigma (Deisenhofen, Germany) and glacial acetic acid from Zorka Pharma a.d. (Šabac, Serbia). Ultra pure water was used for the preparation of solutions (Milli-Q-quality). All solvents and reagents were of an analytical grade unless indicated otherwise.

Tea samples analyzed in this paper are presented in Table 1. Infusions were prepared by adding 200 mL of boiling water to

Table 1

Commercial tea samples used in the study

Sample number	Sample name	Producer	Serial number
1	Aromatized white tea with melon and orange	Fructus d.o.o., Serbia	002 10383 T2 A
2	White tea	Teekanne, Polland	L16CW02:39K CH:401498
3	Earl Grey – aromatized black tea	Teekanne, Polland	L19LW04:02K CH:402560
4	Indian tea – black tea	Vitamin, Horgoš, Serbia	-
5	Black tea	Kirka Pharma, Novi Beograd, Serbia	-
6	Black tea	Welton, Holland	-
7	Black Earl Grey tea	Winston Tea Company LTD, London, UK	CH:877594 L22 KW03:00 S
8	Green tea	Macval Tea d.o.o. Novi Sad, Serbia	-
9	Green tea	Welton, Holland	-
10	Mate tea	Neiner's Gesundheit & Wellness GmBH	9570; L.: 520902305

each tea bag allowing it to infuse for 10 minutes. After cooling, 2 mL of each extract was filtered through 0.45 μm membrane filter directly into the vial, while 20 μL of filtrate was injected into the high performance liquid chromatography (HPLC) system for analysis.

Coffee samples analyzed in our research are shown in Table 2. Infusions were prepared by the same way as described for the tea samples. Sample preparations used in our study are thoroughly presented and described in recent publications performing similar investigations [19, 20]. These extraction methods were chosen due to similarity with a manufacturer's recommendations for the product preparation, so 5-CQA intake could be evaluated more reliably.

HPLC conditions working standard solutions of 5-CQA (0.1001 – 1.001 mg/mL) were injected into the HPLC system and a linear standard curve was constructed by plotting concentrations *versus* peak areas (y = 251.81 ×–0.994). A high value of coefficient of correlation R = 0.999 showed an excellent correlation between concentrations and peak areas. Limit of detection (LD = 0.0127 mg/mL) and limit of quantification (LQ = 0.0373 mg/mL) were also calculated based on the standard deviation of the response and the slope of the calibration curve. Accuracy of this method was tested by comparing the measured and known values of concentrations for standard solutions of 5-CQA. According to the recovery value of 98.74% the method showed accep-

Table 2

Commercial coffee samples used in the study

Sample number	Sample name	Producer	Serial number
1	Jacobs 2 in 1	Kraft foods, Czech Republic	VM26 10052013
2	Jacobs original 3 in 1	Kraft foods, Czech Republic	VM26 22042013
3	Jacobs intense 3 in 1	Kraft foods, Czech Republic	VM26 05042013
4	Grand coffee 3 in1	Droga Kolinska, Slovenia	18.07.13 16:23 WII
5	Mokate coffee drink 3 in 1	Mokate Sp., Polland	21062013A12
6	Doncafe 3 in1	Strauss coffee, Romania	281011011
7	Grand aroma	Grand prom, Serbia	2811M08 18:22
8	Nescafe 3 in 1 classic	Nestle, Romania	128209731 3
9	Nescafe 3 in 1 strong	Nestle, Hungary	100588384 43336638
10	Nescafe 3 in 1 mild	Nestle, Hungary	128009731 9

The stock solution of 5-CQA was prepared by weighing 100.1 mg of the standard substance and dissolving in 10 mL of methanol (95%, v/v). Working solutions were prepared by diluting 0.1, 0.3, 0.6, 0.8 and 1 mL of the stock solution to 10 mL with methanol to obtain different concentrations of 5-CQA (0.1001–1.001 mg/mL). HPLC analysis was performed using modified and revalidated HPLC method for chlorogenic acid (5-CQA) determination in herbal infusions [21] using different detection wavelength (240 nm).

Qualitative and quantitative determination of 5-CQA in commercial tea samples was carried out using an Agilent HP 1100 HPLC-diode array detection (DAD) system equipped with autosampler (Agilent, Waldbronn, Germany). The analytical column was the Zorbax CB-C18 (4.6 × 150 mm, *id*, 5 μm particle size). Mobile phase was aqueous 1.5% acetic acid-methanol (85 : 15) with a flow rate of 0.8 mL/min. Run time was 15 min and column temperature 25°C. The detection was performed at 240 nm.

HPLC method used for the analysis in our study was developed and validated for chlorogenic acid (5-CQA) determination in mate tea extracts obtained by supercritical carbon dioxide extraction [21]. When applied on tea and coffee infusions, this method showed lower selectivity for coffee samples, so the analysis had to be performed with different mobile phase composition and on a different wavelength (240 nm). Therefore, the applied method needed to be revalidated due to changed chromatographic conditions. Procedures used for re-validation of HPLC method for determination of 5-CQA in commercial tea and coffee samples are described in USP 24 [22] and other literature [23–25]. Under determined

table accuracy. Repeatability of the method was tested by analyzing three different concentrations of 5-CQA standards in six repetitions. The relative standard deviations (RSD) ranged from 0.22% to 0.52% for retention time and from 0.03% to 0.38% for peak area, showing good repeatability. By comparing the chromatograms of 5-CQA standards and tea and coffee samples as well as the obtained signal spectrums, the selectivity of the method was evaluated. The chromatograms showed no other signals with the same retention time as the signal deriving from 5-CQA standard, which confirmed high selectivity of the method. Analyzing the effects of slightly changed parameters of the used HPLC conditions, such as different column temperature (± 1°C), flow rate (± 0.05 mL/min) and wavelength of detection (± 3 nm), the robustness of the method was confirmed.

The statistical analyses were done by MS Excel for Windows, v. 2007 software and also by ANOVA (Duncan's test, SPSS, version 17). Level $p < 0.05$ was considered statistically significant.

Results

Representative chromatogram of analyzed commertial tea sample (sample No. 6) is presented in Figure 1. The results of 5-CQA content in commercial tea samples obtained by the revalidated HPLC method are shown in Table 3.

The results of 5-CQA content in the examined coffee samples are presented in Table 4 and the representative chromatogram of the coffee sample No. 2 is shown in Figure 2.

Table 3

Content of 5-coffeoylquinic acid (5-CQA) in the examined tea samples

Sample number	Tea type	5-CQA content (mg/100mL)
1	White tea	0.659 ± 0.003^b
2		2.160 ± 0.022^d
3	Black tea	2.979 ± 0.009^d
4		0.939 ± 0.011^c
5		0.327 ± 0.023^a
6		0.562 ± 0.008^b
7		6.110 ± 0.012^e
8	Green tea	7.374 ± 0.013^e
9		16.020 ± 0.033^g
10	Mate	9.700 ± 0.010^f

*Data are expressed as average ± standard deviations of triplicate measurements. Statistically significant differences were noted by different superscript letters ($p < 0.05$). Same letters – no statistically significant differences.

Table 4

Content of 5-coffeoylquinic acid (5-CQA) in coffee samples

Sample number	Sample name	5-CQA content (mg/100 mL)	5-CQA content (mg/g of coffee)
1	Jacobs 2 in 1	-	-
2	Jacobs original 3 in 1	7.446 ± 0.010^a	7.299 ± 0.022^a
3	Jacobs intense 3 in 1	46.982 ± 0.033^e	36.196 ± 0.068^e
4	Grand coffee 3 in1	34.940 ± 0.028^d	28.757 ± 0.051^d
5	Mokate coffee drink 3 in 1	-	-
6	Doncafe 3 in1	10.012 ± 0.009^b	8.939 ± 0.012^a
7	Grand aroma	22.188 ± 0.012^c	8.875 ± 0.010^a
8	Nescafe 3 in 1 classic	9.309 ± 0.011^b	11.822 ± 0.009^b
9	Nescafe 3 in 1 strong	23.074 ± 0.018^c	14.906 ± 0.011^c
10	Nescafe 3 in 1 mild	11.245 ± 0.009^b	27.112 ± 0.031^d

*Data are expressed as average ± standard deviations of triplicate measurements. Statistically significant differences were noted by different superscript letters ($p < 0.05$). Same letters – no statistically significant differences.
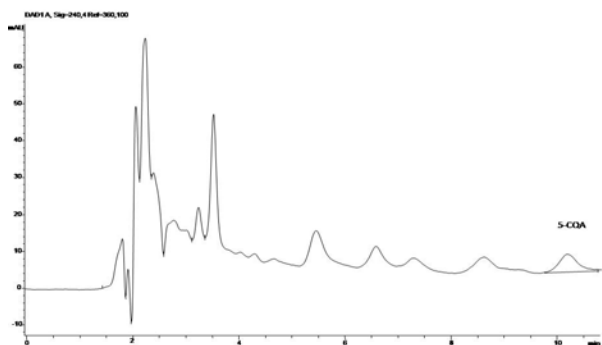

Fig. 1 – High performance liquid chromatography – array detection (HPLC-DAD) chromatogram of the analyzed commercial tea sample (sample number 6).
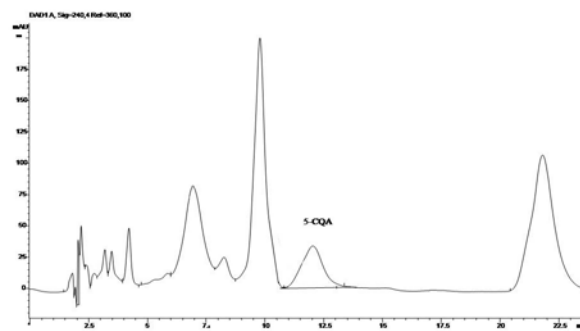

Fig. 2 – Representative high performance liquid chromatography – array detection (HPLC-DAD) chromatogram of the analyzed commercial coffee sample (sample number 2).

Discussion

The results of our study obtained by HPLC analysis of different tea samples showed that the highest amount of 5-CQA was determined in green tea, which was expectable due to its minimal processing during production, where oxidation of phenolic compounds, including chlorogenic acid (5-CQA), was prevented. Differences between the same types of tea, but originating from different producers, were also observed and showed the great effect of the quality of the selected plant material and final formulation. In our study the highest amount of 5-CQA was observed in Welton green tea, which was twice as high as in green tea sample from a domestic producer and many times exceeded the amount of 5-CQA in most of the analyzed samples.

Comparing the results of 5-CQA content in white tea infusions, it was observed that these values were higher than those found in a similar research conducted in Croatia (0.065

– 0.356 mg/100 mL) [26]. The content of 5-CQA in black tea ranged 0.33 – 6.11 mg/100 mL, which was in good agreement with the results obtained in a similar research in UK (6.2 mg/100 mL) [27]. The values for 5-CQA content in green tea were found to be between the results attained in Brazil (0.13 – 0.19 mg/100 mL) [12] and in UK (23.1 mg/100 mL) [27]. In mate tea sample the amount of 5-CQA corresponded to the results obtained in Brazil (5.97 – 12.69 mg/100 mL) [28]. This value was slightly higher than the one established in another research in Brazil (7.5 mg/100 mL) [12] and significantly lower than the result from a recently published study conducted also in Brazil (52.5 mg/100 mL) [29].

These differences can be attributed to different brewing conditions, quality of selected plant material as well as technological processes and formulation [9].

The content of 5-CQA in different coffee samples varied from 0 to 36.20 mg/g of pure coffee (specified on the commercial package). Ky et al. [18] showed that the highest content of 5-CQA was found in samples derived from *Coffea canephora* species, as well as in those processed by the semidry method [18]. The study performed in Brazil in 2008 showed that the 5-CQA content was slightly higher in samples that were treated with semidry method and ranged 31.63 – 48.45 mg/g of coffee than in the samples that were treated with wet method, where the content was from 29.67 to 42.00 mg/g of coffee [30]. Our results also confirmed that coffee species and processing technology of the grain had a notable influence on 5-CQA content.

The concentrations of 5-CQA in our samples ranged 0 – 46.89 mg/100 mL of beverage. Different range of concentrations could be the result of different composition of commercial samples, which is typical for each manufacturer and the product [31]. In a study conducted at the Department of Toxicology at University of California in 2006, 7 commercial coffee samples from the USA were analyzed. The content of 5-CQA ranged from 2.13 to 7.06 mg/g of commercial sample and the content highly depended on the sample composition [32]. Our results were consistent with this study, showing that the formulation of the sample (percentage of coffee) had a major influence on the content of 5-CQA, beside different coffee type and technological processing of the grain. This can be explained by comparing samples number 4 and 10 (Table 4). The mass of the sample number 4 was 18 g (weight of pure coffee was 2.43 g) and the mass of the sam-

ple number 10 was 16 g (weight of pure coffee is 0.80 g). Although the content of 5-CQA (mg/g of coffee) was similar, after consumption of sample number 4 higher quantities will enter the human body (commercial sample number 4 contained three times more coffee than sample number 10). Also, lower quality of coffee beans (containing less 5-CQA) can be compensated by making a formulation with greater representation of coffee in relation to other components as shown in Table 4 for samples 9 and 10. Coffee sample number 9 contained significantly less 5-CQA (mg/g of coffee) in comparison to sample number 10, but coffee amount in formulation (3.10 g) was almost four times higher than in sample number 10 (0.80 g). The results showed that the consumption of sample number 9 could lead to two times higher intake of chlorogenic acid (5-CQA).

Conclusion

During this study a HPLC method was modified, revalidated and successfully applied for 5-CQA determination in commercial tea and coffee samples. The method showed good accuracy, repeatability, selectivity and robustness. HPLC analysis of tea samples showed that the highest amount of 5-CQA was obtained in green tea in comparison to white, black and mate tea because the increased oxidation level decreases the amount of 5-CQA. The results of coffee samples analysis indicated that the content of 5-CQA depended on the type of coffee (*Coffea canephora* species contained more 5-CQA), the technological processing of coffee (higher amount was determined in coffee treated with semi-dry process) and the formulation. The obtained results of this study led to a conclusion that adequate plant material selection, processing, brewing conditions and product formulation have the major influence on the amount of chlorogenic acid (5-CQA) in the final tea and coffee products.

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GENERAL REVIEW

Allergic asthma and rhinitis comorbidity

Komorbiditet alergijske astme i rinitisa

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Introduction

Allergic respiratory diseases, asthma and rhinitis, are rightly called the 21st century epidemic and modern age diseases. Although modern medicine offers a variety of preventive and therapeutic strategies, the prevalence of these diseases has been dramatically increasing in both developed and undeveloped countries across the world, especially in children and young adults. The World Health Organization (WHO) estimates there are 300 million asthmatics in the world, and 500 million people with the symptoms of rhinitis. Very often, asthma and rhinitis coexist in one patient. Numerous contemporary studies, guided by the concept „one airway – one disease", have revealed the epidemiological, pathophysiological and clinical correlation of asthma and rhinitis. A greater scientific interest in these diseases is due to a constantly growing number of the affected in the world, accounting for describing the last three decades as „the age of respiratory allergy". The coexistence of the upper and lower airway disorders is not a new discovery, as it had been recognized over two thousand years ago. It is therefore amazing that it took centuries to establish the concept of concurrence of asthma and rhinitis, and realize the importance of their diagnosis and adequate treatment [1].

Definition of asthma and allergic rhinitis

The Global Initiative for Asthma (GINA) was promoted in 1995, proposing a new definition of asthma, which has been used and quoted in modern literature ever since. According to this definition „Asthma is a chronic inflammation of the airways, with a variety of cells playing an important role in it, including mastocytes, eosinophils, T-lymphocytes, and epithelial cells. In hypersensitive subjects, this inflammation induces recurrent episodes of wheezing, suffocation, chest tightness and cough, particularly during the night and/or early in the morning. These symptoms are related to a widely spread and changing, either spontaneously or by medicaments, at least partially reversible obstruction of the air flow through the airways. The inflammation also induces hyperreactivity of the airways to diverse stimuli, existing even when neither the disease symptoms, nor bronchial obstruction are manifested". The fundamental feature of asthma is a long-term, chronic inflammation which induces hyperreactivity and obstruction of the airways [2].

In 2001, the WHO published the guide „Allergic Rhinitis and its Impact on Asthma" (ARIA), representing a directive for prevention, diagnosis and treatment of rhinitis. Allergic rhinitis is defined as an inflammation of the nasal mucosa, mediated by the IgE mechanism, which is clinically manifested by the symptoms of sneezing, nasal secretion, blocked and itching nose, often accompanied with eye symptoms [3, 4].

Epidemiology of asthma and allergic rhinitis

In 2004, it was reported that about 300 million people of all ages suffered from asthma. This number has been increasing from year to year, becoming doubled or tripled in many countries. The disease severity is unevenly distributed, being mild in most cases, while severe asthma forms are registered in the minority of the patients (around 15%). It is assessed that another 100 million people will be affected by the disease until 2025 [5, 6].

Allergic rhinitis is the most common disorder of respiratory organs, owing its importance to its significantly in-

creasing frequency, effects on the life quality and working ability of the affected, and high diagnostic and treatment costs. This disease is therefore recognized as a global health issue. It has been estimated that about 20–35% of the total world population suffer from allergic rhinitis, taking the fifth position on the morbidity list. The ARIA directive reports that about 500 million people all over the world have allergic rhinitis, which is four to six times as many as asthma cases, particularly in children [4].

Many studies have reported a coexistence of these two allergic disorders of the respiratory tract–asthma and rhinitis, in the same patient. The prevalence of allergic respiratory diseases has been doubling every ten years since the 80s of the 20th century. The reported prevalence varies from study to study, depending on the applied diagnostic criteria, the environment and age of the tested population. The ratio of "three rhinitis cases: one asthma case" has been registered in all countries, in both children and adults. The 1st International Study of Asthma and Allergies in Childhood (ISAAC) study revealed the presence of asthma in 25–35% subjects with rhinitis, and rhinitis symptoms in 75–90% of asthmatics. Rhinitis was found in 62.61% of atopic, and in 45.4% of non-atopic asthmatics, confirming that the concomitance of rhinitis and asthma was not limited exclusively to allergy [7–9]. In their study, Zvezdin et al. [10] reported that in 733 patients with diagnosed allergic asthma, the frequency of concomitant rhinitis was 64%, while in those with nonallergic asthma, it was 28%.

Many studies report the highest incidence of asthma in children and young adults at 20–29 years of age (7–11%). The highest incidence of rhinitis was also registered in children, adolescents, and young adults. In our population, the mean age of the patients with asthma and rhinitis, examined in two studies, was 29.71 years, and the majority of the affected patients belonged to the age group of 18–30 years (52%) [10, 11].

Causes of allergic respiratory diseases

The real cause of the increasing number of allergic subjects is unknown. Allergic diseases are complex and develop through interaction of internal factors of an organism, and external environmental factors. There are a lot of factors contributing to the development of asthma and rhinitis, which most commonly act in synergy. Generally, they are classified into predisposing, causing and contributing ones, representing a combination of internal and external factors [2].

Atopy is the major predisposing factor. It is defined as a disposition of an organism to produce increased quantities of IgE antibodies in response to different substances from the external environment. The atopic constitution has been confirmed to occur more frequently in some families, so the children with this constitution have a greater susceptibility to develop an allergic disease, even 16–20 times as high as non-atopic subjects. If a parent has allergy to an inhalant allergen, the probability that a child will also develop it amounts to 30–50%, increasing to even 60–80% if both parents suffer from allergy. Over 90% of asthmatic children have atopy. Asthma is usually associated with other atopic diseases, al-

lergic rhinitis and atopic dermatitis. Atopic infants, hypersensitive to eggs, house dust and house dust mites are more at risk of developing asthma later in life [12, 13]. Of 733 subjects with diagnosed asthma in our country, 34.9% had a positive family history, more frequently on the mother's side [10].

Sex is considered a predisposing factor. Until the age of five, boys are more frequently affected than girls, explained by the fact they have narrower airways and an elevated tonus of the bronchial musculature, reacting more intensely to diverse stimuli, as well as a huger level of IgE antibodies. After puberty, the frequency is higher in females, which is explained by hormone effects associated with the reproductive and menopausal period, administration of oral contraceptives, greater exposure to internal allergens and indoor irritants, and a higher susceptibility to smoking effects 13. Zvezdin et al. [10] more frequently confirmed the comorbid disease of the airways in females (64.3%), in the ratio of 1.8:1 in favour of females.

Causing factors are inhalant allergens, which induce sensitisation of the airways, and stimulate production of specific T-lymphocyte clones and specific IgE antibodies. At a repeated contact with the allergen to which a subject has already been sensitised, the disease exacerbates, the inflammatory process is initiated, and characteristic symptoms of the disease emerge. Inhalant allergens are generally classified as internal, external, and professional. An exposure to inhalant allergens contributes to both an exacerbation of the symptoms, and the genesis of the disease itself. Hypersensitivity to at least one of the standard inhalant allergens has been registered in 79.5% of the patients diagnosed in the Institute for Pulmonary Diseases of Vojvodina, most of them hypersensitive to internal allergens (77.5%), predominantly house dust mites (76.1%) [14].

Contributing factors are those which increase the risk of the disease when they come in contact with a causing factor. Contributing factors are smoking, outdoor respiratory irritants (air pollution), indoor irritants, early allergic sensitisation, obesity, infections, nutritional factors, socio-economic status, life style, risk factors related to pregnancy, delivery, and early neonatal period of life, and many others. The role of smoking as a factor contributing to asthma exacerbation, recurrent hospitalisations, bad treatment response and disease control has been unquestionably confirmed for both the active and passive smoking, although some studies failed to establish a positive correlation. A higher risk of the disease is registered in children whose mothers smoked in pregnancy and early childhood. Smokers have higher IgE levels than nonsmokers. Smoking cessation has been established by the WHO as a sole reliable primary prevention measure to prevent the development of allergic diseases [15]. Among the subjects of the study carried out in our country, there were 73.9% nonsmokers, 12.2% *ex* smokers, and 13.9 % active smokers [11].

Classification of asthma

Many attempts have been made to classify asthma in regard to its etiology. The most common classification of asthma to extrinsic and intrinsic has been in use since the

first half of the 20th century. Atopy is defined as a genetic hypersensitivity and hyper production of IgE antibodies. The term is used to describe both the clinical condition and predisposition. Recently, more suitable terms "allergic" and "nonallergic" have replaced the former terminology, and they are included in the official nomenclature of the European Academy of Allergy and Clinical Immunology [16]. In time, it has become quite clear that asthma has heterogeneous manifestations, explained by the phenotype, defined as the interaction of the genotype and the environment. Some phenotypes have been described: aspirin-induced asthma, inflammatory-eosinophil and non-eosinophil (neutrophil) asthma, which respond to the applied corticosteroid treatment differently from exacerbations, and are confirmed by the induced sputum method and detection of inflammatory markers, which are predictive of the risk, and particularly of sensitising environmental substances [2]. According to its immunological mechanism and inducing agent, asthma is classified as allergic or nonallergic. Allergic asthma usually starts earlier in life and is characterised with an elevated specific IgE antibody level, positive "prick" test, positive family history, and other recurrent allergic diseases (atopic dermatitis, food allergy, allergic rhinitis). According to the Global Initiative for Asthma, 70–90% of the patients have allergic asthma [2]. The investigation carried out in the Institute for Pulmonary Diseases of Vojvodina has established that of 733 asthmatics, 79.5% had allergic, and 20.5% nonallergic asthma [10]. Nonallergic asthma usually develops later in life, after the forth life decade, more frequently in females, and is characterised with a negative skin test finding, no clinical or family history of allergy, normal serum IgE level, more commonly registered aspirin idiosyncrasyand nasal polyps, and usually a more dramatic clinical course. Intrinsic asthma is considered a form of auto-allergy (usually develops after a viral infection episode), i.e. the affected subjects are allergic to an unidentified allergen. Except a different etiological immune mechanism involved in two asthma types, they both have similar pathophysiological and pathoanatomical changes of the airways, and an identical therapeutic approach. The positive skin test finding to inhalant allergens does not necessarily mean the disease is allergic in type, or the disease or its exacerbation are caused by the allergen. The measurement of the total IgE antibody levels in the serum does not have a diagnostic value for atopy confirmation [2].

Specific entities of asthma include professional, seasonal (pollen), aspirin-, cough-, and effort-induced asthma [2, 17]. Professional asthma is defined as asthma induced by inhalant allergens present in the work environment. About 200 specific allergens have been identified so far, and this number has been constantly increasing. It has been estimated that professional asthma makes about 7% of the total adult asthma cases. The clinical, functional and pathological changes in professional asthma resemble those in nonprofessional asthma, and the therapeutic approach is also identical. An adequate and objective confirmation if the diagnosis is very important, and it is obtained by a specific challenge test, performed in high-specialised medical institutions, or by peak expiratory flow (PEF) monitoring measurement four times a day over two weeks, in the same time period at work

and rest [2, 18]. Pollen, seasonal asthma is characterised by the onset of the symptoms during the pollination season, most frequently associated with allergic conjunctivitis and rhinitis [17]. Aspirin-sensitive asthma is a specific clinical syndrome in which asthmatic attacks are induced by aspirin and other non-steroid anti-inflammatory drugs. The mechanism is not allergological and is defined as a pseudo-allergic reaction (idiosyncrasy). Aspirin asthma is registered in 5–10% of adult asthmatics. These drugs inhibit cyclooxygenase, so the metabolism of the arachidic acid is mediated by lipoxygenase, inducing an increased production of cysteinyl leukotrienes and the development of bronchospasm. Having taken the incriminated drug, a severe asthma attack usually develops in an hour. Once developed, the drug intolerance persists during the lifetime. It is confirmed by the oral or inhalatory challenge test, if forced expiratory volume during the first second (FEV1) of a forced expiratory maneuver decreases for over 20%, or for 15% with the signs of bronchospasm, sneezing or conjunctivitis. These patients tolerate paracetamol, sodium salicylate, chloroquine, choline salicylate [19]. Cough-Variant asthma is one of the most common causes of chronic cough, persisting over two months. Cough is a sole symptom, often irritating in character, lasting day and night, provoked by effort or infection. It may develop in both children and adults. The bronchial challenge finding is usually negative, diurnal variability of the symptoms and lung function are absent. It is established by the positive bronchial challenge test finding, the presence of eosinophils in the induced sputum, but it is most often confirmed clinically, when cough disappears after the treatment with inhalant corticosteroids [2, 17, 20]. Exercise-induced asthma typically develops in younger adults, 5–10 minutes after exercise (rarely during exercise), with the symptoms of dyspnea and cough, lasting for about 30 to 45 minutes, and it most frequently disappears spontaneously. This type of asthma is considered to be induced by accelerated and increased ventilation, breathing through the mouth, cold air inhalation. A reduced hydro layer of the airways induces its hyper viscosity, which is a stimulus for mastocyte degranulation and a release of mediators. Cooling down on exercise induces vasoconstriction of peribronchial vascular plexuses; upon discontinuation of exercise, there develops heating, oedema, hyperemia of the mucosa and lumen constriction. Oscillations of the temperature and osmotic pressure stimulate both the local and central nerve reflexes. The accurate diagnosis is very difficult to establish. Some patients, most frequently children, develop the symptoms only at exercise. To confirm the diagnosis, they are submitted to exercise tests, including an easily performed 8-minute running protocol [17, 20].

According to the GINA, asthma is classified into four types, depending on the severity of clinical symptoms, applying no inhalant corticosteroids: occasional (intermittent), mild permanent (persistent), moderate persistent, and severe persistent. The severity of asthma is assessed in relation to the frequency and duration of the symptoms, intensity of the air flow limitation in the airways (FEV1), and variability of the bronchial obstruction (PEF variable). This staged classification has a clinical relevance, as the treatment approach,

although individual ("everyone has one's own asthma"), predominantly depends on the disease severity stage [2, 17]. In 2006, a new classification of asthma, related to the achieved disease control, was proposed. Depending on the exhibited clinical symptoms, this classification differentiates three asthma types: controlled, partially controlled and uncontrolled [2].

Classification of allergic rhinitis

Etiologically, rhinitis is classified as allergic and nonallergic [acute and chronic infectious, idiopathic, professional, drug- or food-induced, hormonal, irritant, emotional, atrophic and nonallergic rhinitis with eosinophil syndrome (NARES)] [1]. Since the mid 90s of the 20th century, several programmes and guidelines for the diagnosis and management of rhinitis have come out, supported by international allergology associations and the WHO. The first consensus, published in 1994 in Copenhagen (Rhinitis Management Group), proposed to classify the allergic rhinitis as seasonal and rhinitis persisting through the year [21]. The initiative of the WHO "Allergic Rhinitis and its Impact on Asthma", launched in 2001, was more widely supported and accepted [3]. The mission of this initiative was to promote the latest achievements in this field throughout the world, aiming to change the approach to these concomitant diseases and contribute to their better management. The initiative proposed a new classification of allergic rhinitis as intermittent and persistent, which were not synonymous with former classification classes. In intermittent rhinitis, the symptoms are manifested for less than four days a week, and less than four weeks a year, in persistent rhinitis more than four days a week, and more than four weeks a year. The new classification is therefore more appropriate, as most patients have a combined inhalant allergy [3, 4].

The severity assessment of allergic rhinitis does not depend on the applied treatment. The objective factors for the severity assessment of allergic rhinitis are the type and severity of symptoms, visual analogue scale (VAS), nasal obstruction measurement [(peak inspiratory flow, acoustic rhinometry and i rhinomanometry)], inflammation assessment (exhaled nitric oxide (NO) measuring, cells and mediators present in the nasal lavage fluid and nasal biopsy sample), nasal challenge test with histamine, metacholine, allergens, hypertonic solution, capsapicin or cold dry air, smell test. To assess the severity of rhinitis, VAS is applied in every-day clinical practice. A patient assesses the severity of each symptom scoring on the 0–10 scale, simply adding up the selected scores to provide the severity assessment score ("symptom score") of allergic rhinitis. The scores of 0–3, 4–7, and 8–10 characterise mild, moderate, and severe allergic rhinitis, respectively [4].

Allergic respiratory diseases: the diagnosis

It has been generally assessed that allergic respiratory diseases are insufficiently diagnosed and inadequately treated all over the world. To diagnose both allergic rhinitis and asthma is a continuous process, which often requires numerous and recurrent examinations of patients. The diagnosis is established on the basis of a patient's history, physical and objective examination, and additional examinations. Asthma-suggesting symptoms include suffocation attacks, wheezing, tight chest and cough during the day or most often at night, preceded by provoking factors (exercise, cold, exposure to allergens or irritants, specific food or drug intake, positive or negative emotions, premenstrum, etc.). The symptoms are episodic in character, appearing after an exposure to allergens, and season-variable. Rhinitis-suggesting symptoms are sneezing, dripping nose, nasal teasing and itching, blocked nose. The information of the family, personal and professional history are also important [4].

The following diagnostic procedures are recommended to establish the diagnosis of asthma: clinical examination (symptoms and physical examination, complete history), lung function tests (spirometry, PEF, bronchodilatatory test, specific and nonspecific bronchial challenge), which establish the diagnosis of asthma, the severity of bronchial obstruction and their reversibility/irreversibility, allergological tests ("prick" skin test, specific IgE antibody measurements), which identify the risk factors for exacerbations, in some patients, radiological examinations [chest X-ray, sometimes computed tomography (CT) of the chest], measurements of inflammation markers (inflammatory cells and mediators in an induced sputum sample, bronchoalveolar lavage fluid and bronchial biopsy sample, measurement of mediators or their metabolites in the blood and urine, measurement of NO concentrations in the exhaled air) [2].

The diagnostic procedures for allergic rhinitis include: clinical examination (history taking and anterior and posterior rhinoscopy), allergy status tests ("prick" skin test, measurement of specific IgE antibody levels), nasal mucociliary clearance tests (saccharine test, measurement of the cilia frequency in the nasal mucosa, electronic microscopy) in some cases, rigid and flexible endoscopic examination of the nasal cavity, radiologic examinations, smell tests, nasal flow tests [peak nasal inspiratory flow (PNIF), anterior and posterior rhinomanometry], bacteriological diagnostics, cytological examination of scraped nasal mucosa and analyses of nasal secretions for inflammatory mediators (cytokines, chemokines…), measurements of mediators and enzymes in the peripheral blood (histamine, prostaglandine D2 (PGD2), leukotriene C4 (LTC4), LTD4, LTE4, tryptase, quinines, eosinophil cationic protein (basally or after allergen challenge), nasal challenge tests (risky in patients with asthma) [4].

Allergic respiratory disease: therapy procedures

Inflammatory lesions in allergic asthma and rhinitis are very complex, so it is not always sufficient to inhibit only one mechanism in the treatment of these diseases. Multiple therapeutic approaches are required to achieve a good control of both diseases. The treatment objectives in asthma, according to the GINA, are reduction or elimination of the symptoms, improvement of life quality, prevention of complications, improvement of the course and prognosis of the dis-

ease, long-term control of the disease using as few medicaments as possible in the lowest possible doses. The treatment includes education, prevention, pharmacological treatment, and immune treatment [2].

Preventive measures include the general preventive measures, avoiding the contact with allergens. The general measures mean evasion of all the factors which increase the nasal and bronchial hyperreactivity and the symptoms, such as: smoking, sudden external temperature changes, air conditioners, strong and irritating scents and light. The patients are advised how to avoid the contact with allergens and reduce their concentration (for some allergens or in cases of an established professional disease, these measures may be very efficient). It is also necessary and very important to educate the patients about their diseases' primary features, way of life, treatment mode, adequate application of inhalant substances, what to do in exacerbations, particularly of asthma. The patient-physician partnership is very important [2]. Medicamentous treatment includes a specific combination of drugs for each disease. A uniform approach is not possible due to different severity stages of the disease, predisposing factors, patients' age, length of the disease. An individual approach is applied for each patient. The therapy of asthma included several pharmacological groups of drugs (inhalant and oral corticosteroids, β2-agonists, anticholinergics, theophylline (xanthines), antileukotrienes and anti- IgE therapy (omalizumab)), as well as the treatment of rhinitis (oral and intranasal H-1 antihistaminics, intranasal and oral corticosteroids, antileukotrienes, intranasal chromones, oral and intranasal decongestives, anticholinergics). The choice of the pharmacological treatment depends on the severity and frequency of symptoms, patients' motivation and compliance, availability of the drugs and their possible undesirable side effects.

Modern pharmacotherapy of allergic respiratory diseases provides a better quality of life of the affected patients, and normal personal, social, professional and physiological functioning. As asthma is a chronic inflammatory disease, it is considered it should be continuously and permanently treated. The staged treatment approach means that the medicaments are applied depending on the achieved effects and response to the treatment applied so far. The kinds and doses of the applied drugs are increased with the increasing severity of the disease, and the next, higher treatment level is indicated when a satisfactory control of the disease is not achieved by the former one. The major treatment objective is the best disease control with fewest drugs [2, 4].

Asthma and rhinitis: correlation mechanisms

The interpretation of respiratory allergy has been significantly changed due to the recognized correlation between rhinitis and asthma, including their epidemiology, anatomy, physiology, immunopathogenesis, and particularly therapy.

The relationship between the upper and lower part of the respiratory system was recognized 2000 years ago. In the 2nd century, Galen recommended frequent elimination of the nasal content to free the lungs, and in the 19th century Bostock emphasized that the respiratory system is a united entity. After these historical observations had been made, there succeeded a long period of silence until three decades ago, when „the age of respiratory allergy" started, marked with a great interest of scientists and clinicians in this field, and a great number of published papers on allergic diseases of the respiratory system. Respiratory allergy is nowadays considered the disease of the entire respiratory tract, which is clinically manifested as asthma and/or rhinitis. Many terms have been proposed in the literature to describe this phenomenon: combined allergic rhinitis-asthma syndrome, allergic rhino-bronchitis/allergic asthma-rhinitis association, allergic rhinitis/asthma syndrome, allergic rhinitis and asthma comorbidity, allergic rhinitis and asthma – the same disease, chronic allergic inflammatory respiratory syndrome, united airway disease, generalised respiratory inflammation, total respiratory inflammation, and other terms. In 2011, the WHO promoted the ARIA initiative, and the World Allergology Organization formulated the Combined Allergic Rhinitis and Asthma Syndrome (CARAS) initiative. These two organizations and their initiatives enabled a uniform approach in the diagnosis, classification and treatment of these, until recently separated diseases [1].

The relationship between rhinitis and asthma has first been recognized empirically, in the clinical practice, and then confirmed epidemiologically, as well. In the 80s of the 20th century, it was for the first time reported that 28–60% of asthmatics had nasal symptoms (compared to 20% of the general population), and 19–38% of the patients with allergic rhinitis might have asthma (compared to 3–5% in the general population) [21]. In 1999, it was reported that about 80% patients with asthma had rhinitis, while asthma was registered in 38% of the patients with allergic rhinitis, and similar findings were also reported by the ISAAC study [3, 22]. It has been estimated that about 5% of the general world population concurrently have both allergic respiratory diseases. In our region, the frequency of comorbid rhinitis in asthmatics amounts to 63.9% [10].

The symptoms of allergic rhinitis usually precede those of asthma. The longitudinal study by Settipane et al. [23] followed the patients with a positive allergy test finding and rhinitis symptoms, reporting that in ten years, 50% of the examined patients had identical symptoms, while 20% (those with perennial rhinitis) developed asthma. The study carried out in our country reports the symptoms of allergic rhinitis preceded asthma in three fifths (60%) of the examined population over the period ranging from 1–27 years, while a concurrent (within the same year) onset of both diseases was observed in two fifths (40%) of the examined [10]. Similar results were also obtained in a former study including fewer patients. A concurrent onset of both diseases occurred in 37%, and rhinitis preceded in 63% of the cases, over the period ranging from 1–11 years. All patients had confirmed allergy to internal allergens and characteristics of persisting allergic rhinitis [11]. The history of pollen fever in childhood increases the risk of asthma three times, or even six times in females, and other factors which also increase this risk are atopy status, patients' age at the moment of the onset of atopy (the younger the patients are, the higher the risk), and severity of rhinitis symptoms [22, 24].

According to Linneberg et al. [25], the relationship between rhinitis and asthma is less evident in pollen hypersensitivity than in hypersensitivity to internal allergens, which is explained by greater dimensions of the pollen seed (10–20 μm) compared to house dust particles (≤ 3 μm). All the patients in our study with concomitant asthma and rhinitis also had allergy to house dust mites [11].

Numerous studies have confirmed the presence of nonspecific bronchial hyperreactivity in patients with allergic rhinitis but no symptoms of asthma, as compared to the patients without rhinitis. The oldest study dating back to 1965 showed that even 73% of the patients with rhinitis had in increased bronchial reactivity to metacholine or histamine. Diverse studies have reported an elevated bronchial reactivity, with no respiratory symptoms, in 24–60% of the patients with rhinitis, who also developed asthma more frequently. Bronchial hyperreactivity (BHR) is higher in subjects with perennial rhinitis and hypersensitive to internal allergens, and in those with a longer history of rhinitis symptoms. Many authors opine the presence of bronchial hyperreactivity in patients with rhinitis may be helpful in identifying those with an increased risk of asthma. Bronchial hyperreactivity may be characteristic of the stage between rhinitis and manifested asthma [24, 26, 27].

Probably, there is a junction between the lower and upper part of the respiratory tree, which may be due to a reduced protective function of the nose, neural interrelation, or inflammatory propagation from the upper into the lower respiratory tree area [28]. Montefort et al. [29] performed bronchial biopsy in atopic asthmatics, atopic non-asthmatics and healthy subjects, and registered histological lesions specific for the asthmatic inflammation in non-asthmatics, which were less intense than in asthmatics.

Although it is out of question that allergic rhinitis may affect the onset and clinical course of asthma, the mechanism of this relationship and mutual influence has not been completely enlightened yet. These mechanisms are generally classified as indirect and direct. Indirect mechanisms are a nasal obstruction and oral breathing, with the important preparation function of the nose impaired. Direct mechanisms include: aspiration of the postnasal secretion with mediators and/or cells into the lungs (post nasal «drip»), resorption of inflammatory cells and/or mediators into the systemic circulation, and the nasobronchial reflex. Oral breathing due to a nasal obstruction induces bronchospasm, while nasal breathing with the preserved nasal function reduces asthma symptoms on exercise. This mechanism is explained by inspiration of the air which has not been formerly hydrated, heated, and purified from allergens and pollutants, which is the major function of the nose [30–32].

The concept that inflammatory cells and mediators descend by gravitation into the lower respiratory tree sounds logical. Investigations on animal models (downward head positioning and intubation-prevented secretion flow) have revealed an inhibited bronchial hyperreactivity, thus proving that the upper respiratory tree inflammation affects nonspecific bronchial hyperreactivity [31]. Littel et al. [33] have pointed out the possibility of a systemic propagation path of spasmogenic substances, instilling higher metacholine doses into the asthmatics' nose, thus increasing the bronchial resistance. They blocked this effect by a vasoconstrictor, showing that the vascular transit of mediators induced a constriction of the lower airways. Another possible mechanism is the presence of inflammatory cells and mediators in the systemic circulation and respiratory system, which are produced by the bone marrow in response to the nasal stimulus. Reversely, Braunstahl at al. [32] have in their study confirmed an inflammatory reaction in the nasal mucosa 24 hours after a segmented bronchial challenge test.

The causes of a significantly increased frequency of allergic respiratory diseases have not been definitely established yet. Numerous factors have been reported as possible agents which affect the expression of these diseases, varying greatly, depending on the geographical region and population features and age. The factors considered important or possible inducing agents of allergic respiratory diseases include: family history (genetic factor), type of allergic sensitisation, active and mothers' smoking in pregnancy and postpartum, living conditions (dirty and humid apartments), climate and life style.

The genetic factor is considered crucial for both diseases. A person whose family member suffers from asthma has three to four times greater risk to develop this disease, too; if a family member has rhinitis, the risk of this disease is five to six times higher. Atopy is inherited autosomally and dominantly (via the gene located on the 11th chromosome, but only if maternally). Some studies have revealed the maternal asthma, accompanied with rhinitis and smoking, may additionally increase the risk of asthma [10].

The exposure to tobacco smoke in childhood and active smoking are significant risk factors. Lundback [8] have confirmed that both smokers and ex-smokers with a positive family history of atopy have seven times increased risk to develop rhinitis and/or asthma. In our study, 80% of the patients never smoked, 12% were ex-smokers, and others (8%) were current smokers [5, 10, 11].

The early (in the first years of life) contact with allergens is a significant risk factor, explained by the immunological vulnerability in this period, in which the internal allergens (house dust, house dust mites, animal hair, mold), are more important than the external (pollens) ones. Almost all in the studies world have reported similar results – most patients with allergic respiratory diseases are hypersensitive to house dust mites. The percentage of allergies to external (pollens) allergens varies from country to country, because it depends on geographical features, climate and vegetation. In European countries, hypersensitivity to grass pollens has been most frequently established, and then to tree and weed pollens (allergy to tree pollens is more common in northern, and to weed pollens in southern European countries). The results of the studies performed in our country show the patients with both diseases are mostly sensitive to house dust mites (76.1%), and less to pollens, and the symptoms of the diseases, particularly those of rhinitis, emerged earlier in life [10, 34].

It was long ago when clinicians had empirically observed that an effective treatment of rhinitis and improved nasal function resulted in improved symptoms and a better control of asthma. Since the mid 80s of the 20th century,

several trials have attempted to prove this effect, i.e. the anti inflammatory corticosteroid treatment of rhinitis improves the symptoms of asthma, lung function and bronchial hyper-reactivity. The first study which confirmed the positive effects of nasal beclomethasone dipropionate and chromone on rhinitis symptoms, and those of nasal corticosteroids on asthma symptoms in the subjects with confirmed allergy to external allergens was published in 1987. (Welsh et al.[35]). It was followed by the study of Aubier et al.[36] in 1992, having confirmed that nasal corticosteroids reduced bronchial hyper-reactivity, unlike orally inhaled ones. In the same year, Corren et al.[37] came to a similar conclusion in their study of seasonal rhinitis and asthma, finding a significantly increased hyperreactivity in the patients receiving a placebo, while in those treated by nasal corticosteroids it remained unchanged.

Numerous studies have been assessing the effect of rhinitis anti-inflammatory treatment on the course and control of asthma. Most studies have reported the positive results of anti-inflammatory corticosteroid treatment of allergic rhinitis on bronchial hyperreactivity and asthma symptoms, although some trials have not confirmed this positive effect[38]. The results of the trials carried out in our country have demonstrated that corticosteroid treatment of rhinitis significantly contributes to improved asthma and rhinitis symptoms, as well as lung function parameters, and is an integral part of the treatment of asthma[11,39]. Three large-scale prospective studies in the United States have reported fewer hospital and emergency admissions of asthmatics treated for comorbid rhinitis, compared to asthmatics receiving no therapy for rhinitis. The diagnosis and treatment of rhinitis is obligatory and should be included in the treatment of asthma. Untreated rhinitis is one of the causes of frequent asthma exacerbations, its difficult control and treatment, as well as a bad prognosis of the disease[40,41].

Conclusion

All the studies performed so far conclude rhinitis is a risk factor and predictor of asthma, which it usually precedes. Some authors consider rhinitis and asthma are different clinical manifestations of the same systemic disease. They may occur simultaneously, or the symptoms of rhinitis may follow those of asthma, although more rarely. The ARIA guidelines suggest the patients with asthma should be evaluated for the presence of rhinitis, and *vice versa*. The presence of one of these diseases increases the probability for the development of the other. About 50% of the patients with early developed, long-term rhinitis and allergy to internal inhalant allergens will develop asthma as well. It is therefore important to identify the subjects predisposed for asthma among those with rhinitis. All possible predictive factors known so far cannot confirm the risk with certainty. It is difficult to establish whether rhinitis is the first manifestation of respiratory allergy, or it has a direct role as an inducing agent of asthma. In addition, both asthma and allergic rhinitis are modern-age diseases, most common in well-developed countries, in younger-age, active population. The relationship between asthma and allergic rhinitis has not been explained only theoretically, but its practical relevance has been recognized, as well. The recommendations of the world guidelines (ARIA, GINA and others) for every day clinical practice have been discussed, recognizing the need of additional studies in the diagnostic and therapy management of asthma and comorbid allergic rhinitis. The ultimate imperative is better life quality of the affected patients and their families, and positive effects on the global factors, such as the health care system and financial expenses.

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Non-functional parathyroid cyst – diagnostic pitfall: A case-report

Nefunkcionalna cista paratiroidne žlezde – dijagnostička zamka

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Abstract

Introduction. Parathyroid cysts are relative rare and they may be misdiagnosed with thyroid nodules. Parathyroid cysts are characterized by elevated level of parathyroid hormone (PTH) in cystic fluid. **Case report.** We reported about middle-aged woman with palpable node in the left thyroid lobe. Ultrasound showed anechoic 40 × 25 mm lesion in the left thyroid lobe. Fine needle aspiration (FNA) obtained 13 mL colorless, watery cystic fluid. PTH value in cystic fluid was ten fold more in comparison with serum PTH. Serum PTH was slightly elevated, D vitamin was under the reference range, serum calcium and phosphorus were normal as well as thyroid hormones. Thyroglobulin antibodies (TgAb) and thyroid peroxidase antibodies (TPOAb) were not detected. Radionuclide parathyroid scintigraphy indicated at physiological metaiodbenzyl-guamidine (MIBG) distribution. After six months of vitamin D supplementation, serum calcium, phosphorus, vitamin D and PTH were normal. This finding was indicative that was a nonfunctional parathyroid cyst. **Conclusion.** This case report points out that thyroid cystic lesions with thin walls, and reverberation in ultrasound, must be observed as a potential parathyroid cyst. These cysts require caution during diagnostic aspiration because of danger of hypercalcemic crises due to FNA, which can be a life-threatening condition.

Key words:
parathyroid diseases; cysts; biopsy, fine needle; hypercalcemia.

Apstrakt

Uvod. Paratireoidne ciste relativno su retke i mogu da izgledaju kao koloidno degenerisani čvorovi u štitastoj žlezdi. Za paratireoidne ciste karakteristčan je povišen nivo paratireoidnog hormona (PTH) u cistčnoj tečnosti. **Prikaz bolesnika.** Prikazali smo sredovečnu ženu sa palpabilnim čvorom u levom režnju štitaste žlezde. Ultrazvučnim preglednom ustanovljena je anehogena, ovalna promena 40 × 25 mm. Aspiracijom je dobijeno 13 mL bezbojne, bistre tečnosti. PTH u cistčnoj tečnosti bio je desetostruko viši u odnosu na vrednost PTH u serumu koji je bio blago povišen, vitamin D snižen, kalcijum, fosfor i tireoidni hormoni u granicama referentnih vrednosti. Tireoglobulinska i tireoperoksidazna (TPO) antitela nisu nađena. Scintigrafija paratireoidnih žlezda pokazala je fiziološku distribuciju radiofarmaka. Posle šest meseci nadoknade D vitamina, nalaz PTH, D vitamina, kalcijuma, fosfora i TSH u serumu je bio u granicama referentnih vrednosti. Ovi nalazi ukazivali su da je opisana promena u levom lobusu odgovarala nefunkcionalnoj cisti paratireoidne žlezde. **Zaključak.** Ovaj prikaz pokazuje da cistčne lezije u projekciji štitaste žlezde, tankih zidova, sa reverberacijom na ultrazvučnom pregledu, mogu da budu paratireoidne ciste. Prilikom aspiracije paratireoidnih cisti moguća je hiperkalcemična kriza koja može da bude životno ugrožavajuće stanje.

Ključne reči:
paratireoidne žlezde, bolesti; ciste; biopsija tankom iglom; hiperkalciemija.

Introduction

Parathyroid cysts have been relatively rare described in clinical practice, and till now, less than 300 cases have been reported in the literature. They are often confused with thyroid nodules [1]. Parathyroid cysts could be diagnosed by ultrasound and verified by elevated parathyroid hormone (PTH) in aspirated cystic fluid [2]. First description of macroscopic characteristic of parathyroid cysts was described in 1880 [3]. About 40% to 80% parathyroid lesions have been diagnosed by finding parathyroid cells in aspirated cystic fluid, about 8% to 30% are misdiagnosed as a thyroid lesion, and 8% to 16% have no enough cell elements for cytological diagnose [4, 5]. Cystic fluid is watery, colorless and looks as

lymph fluid, called "water from rocks" [1, 6]. Usually, there is no relapse after fine needle aspiration (FNA) and evacuation of cystic fluid after one-year follow-up [7].

Case report

We examined a 40-year-old female with discomfort in the lower part of the anterior cervical region. In her left thyroid lobe there was elastic, palpable node, approximately 40 mm. An ultrasound examination revealed anechoic and oval-shaped lesion measuring 24 × 40 mm in the left thyroid lobe, with reverberation and without register of blood flow (Figure 1). In the right thyroid lobe there was a 5 mm hypoechoic, colloid node.

Fine-needle aspiration was performed in order to evacuate the cystic fluid and to make cytology analyses. Fluid obtained by aspiration was watery, colorless, and it suspected to parathyroid cyst. In 13 mL of cystic fluid, the level of PTH was 766.22 pg/mL (serum reference range 15–65 pg/mL). Serum PTH was slightly elevated: 88 pg/mL [chemiluminescent microparticle immunoassay (CMIA), ARCHITECT ci8200, Abbot]. Serum calcium was 2.24 mmol/L, phosphorus 0.91 mmol/L and vitamin D was under the reference range: 12.6 ng/mL. Thyroid tests were normal, thyroid peroxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb) were not detected (Table 1).

Table 1

Laboratory results

Parameter	Baseline (reference range)	After 6 months
PTH (serum), pg/mL	88 (15–65pg/mL)	53.6
PTH (cystic fluid), pg/mL	766.22	
Calcium, mmol/L	2,24 (2.1–2.55)	2.13
Phosphorus, mmol/L	0,91 (0,79–1,42)	0.94
25 OHD, ng/ mL	12.6 (30–100)	33.2
TSH, μU/mL	1.75 (0.35–4.94)	1.3
FT4, pmol/L	12.2 (9.0–19.1)	
TPOAb, IU/mL	2.4 (< 5.61)	1.4

PTH – parathyroid hormone; TSH – thyroid stimulation hormone; FT4 – free thyroxine; TPOAb – thyroid peroxydase antibodies.

After one week ultrasound showed a resicdue of 2 mL of cystic fluid (Figure 2).

Cystic content was completely evacuated with repeated FNA. After the second aspiration ultrasound showed two hypoechoic solid structure lesions, 4 and 5 mm in diameter, without cystic content (Figure 3).

Radionuclide parathyroid scintigraphy 666 Tc99m methoxyisobutylisonitrile (MIBI) in 20, 60, and 120 min after applications indicated at physiological MIBI distribution (Figure 4).

After a 6-month vitamin D supplementation, serum calcium, phosphorus and PTH were normal, as well as serum vitamin D (Table 1). This finding was indicative that was a nonfunctional parathyroid cyst. Initially, the elevated PTH was a consequence of the low level of serum vitamin D.
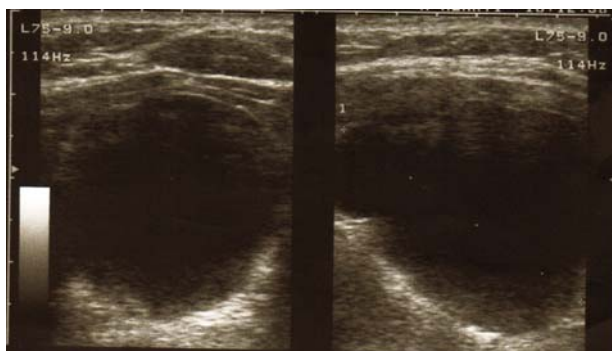

Fig. 1 – Ultrasound image of the left thyroid lobe – a cyst in the transversal section (left half of the panel) and longitudinal section (right half of the panel).

Fig. 2 – Ultrasound image in the longitudinal section – Directly to the bottom half of the left lobe is a remnant of the cyst.
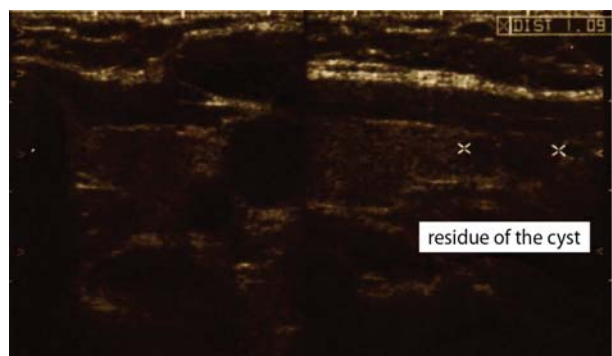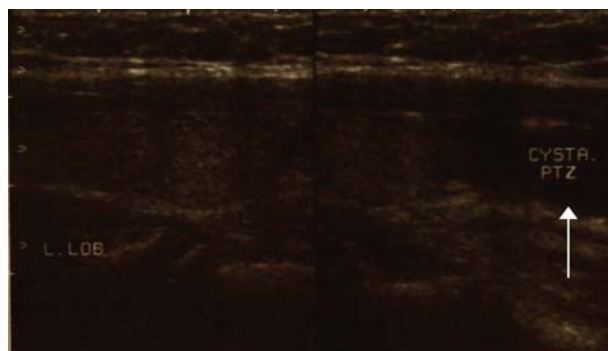

Fig. 3 – The area of the left lobe after repeated evacuation – a minimal residue of the cyst (transversal section on the left and longitudinal on the right panel).
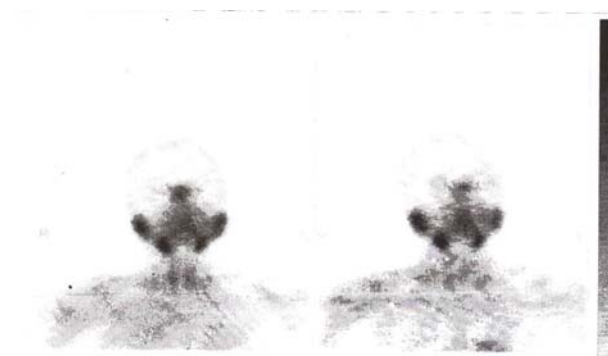

Fig. 4 – Radionuclide parathyroid scintigraphy [666 Tc99m methoxyisobutylisonitrile (MIBI)] indicated a physiological MIBI distribution.

Discussion

Parathyroid cysts are often misdiagnosed as a solitary thyroid adenoma or nodular goiter. They could be nonfunctional or functional. In both of them, the level of PTH in cystic fluid is high [6]. Nonfunctional cysts are often located in the inferior parathyroid glands, particularly in the left inferior gland, while localization of functional cysts is unpredictable [8, 9]. They could be small, from some millimeters to more than 50 mm [10]. A true ontogenetic parathyroid cyst, from the remnants of the third or fourth branchial clefts, has thin wall, lined by secretory epithelium and contains watery cystic fluid. A cyst formed by the coalescence of micro-cysts may contain hemorrhagic fluid. Cystic degeneration of parathyroid adenomas could be presented as pseudocysts. Their content is a brown fluid and they are rather functional [1, 6, 11]. Just a few cases of intrathyroidal parathyroid cyst reported till now. The latest was a case of primary hyperparathyroidism due to intrathyroidal parathyroid cyst which was confirmed by founding of elevated PTH in needle aspirate [12]. Other etiologies of parathyroid cysts have been proposed without any supporting evidence [13]. Case report from 1965 was described that administration of high doses of calcium acetate, vitamin D2, vitamin D3 or extract of parathyroid gland can caused appearance of parathyroid cysts in rats [14, 15]. The both, nonfunctional and functional cysts have a high PTH content in cystic fluid. Sometimes, for PTH dosage, regarding Hook effect, cystic fluid must be in a diluted form [6]. In our sample of cystic fluid, the PTH level was tenfold higher than the the reference serum range and dilution was not necessary. Functional cysts could be associated with high serum calcium, phosphorus and PTH, and they could appear spontaneously due to infarction or necrosis of parathyroid adenomas or could be worsening up to hypercalcemic crisis during or after FNA [9, 15]. Ultrasound with FNA is the first line of investigation approach. The routine morphologic and functional diagnostic tools in case of functional cystic adenoma, such as ultrasonography or (99m) Tc-sestamibi-scintigraphy, cannot distinguish between cystic parathyroid adenoma and carcinoma [16]. Elevated levels of PTH and C-terminal/mid-region of PTH itself are necessary for diagnose confirmation [1]. Fine needle aspiration could be the first choice procedure for the diagnosis and treatment for nonfunctioning parathyroid cysts, but for recurrent cases ablation with 99% ethanol under ultrasound guidance can be a subsequent treatment modality [17]. Surgical treatment is recommended for functional and recurrent nonfunctional parathyroid cysts [1].

Conclusion

This case report elucidates the diagnostic pitfalls in fine needle aspiration of thyroid lesions. Also, some of thyroid cystic lesions with thin walls, and reverberation in ultrasound, have to be observed as a potential parathyroid cyst. These cysts require caution during diagnostic aspiration because of danger of hypercalcemic crises due to fine needle aspiration, which could be a life-threatening condition.

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CASE REPORT

Rare solitary fibrous tumor of the stomach: A case report

Redak solitarni fibrozni tumor želuca

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Abstract

Introduction. Solitary fibrous tumors are rare soft tissue tumors of submesothelial origin and variable malignant potential. The most common localization is pleural, whereas only 0.6% are of extrapleural localization. Solitary fibrous tumor of the peritoneum, especially of gastric serosa is an extremely rare form of this tumor. **Case report.** We presented a 65-year-old female patient with solitary fibrous tumor of the stomach. Histopathological analysis of removed tissue showed the presence of tumor tissue built of spindle cells, elongated nuclei with moderately abundant cytoplasm. Cells were in a noncohesive arrangement, in smaller areas distributed in the form of palisade. There were amounts of hipocellular connective tissue, hyalinised, with small foci of dystrophic calcification. Mitoses were rare (less than 3/10 HPF). Blood vessels surrounded the connective tissue. Reviewed material did not contain elements of the parent organ. Immunohistochemically there were positivity on CD34 and vimentin, and negativity to S100, SMA, CD117, dezmin, and Ki-67 is < 2%. The change was diagnosed as a solitary fibrous tumor. **Conclusion.** Considering that benign solitary fibrous tumors of extrathoracic localizations are extremely rare neoplasms with unpredictable biological behavior and the possibility of recurrence, a long-term clinical and endoscopic follow-up on yearly basis of patients with this disease is recommended.

Key words:
fibroma; peritoneal neoplasms; stomach; diagnosis; immunoenzyme techniques; tumor markers, biological; antigens, cd 34; vimentin; immunohistochemistry.

Apstrakt

Uvod. Solitarni fibrozni tumori su retki mekotkivni tumori submezotelijalnog porekla, sa varijabilnim malignim potencijalom. Najčešće su pleuralne lokalizacije, dok je svega 0,6% smešteno ekstrapleuralno. Solitarni fibrozni tumor peritoneuma, posebno gastrične seroze, veoma je retka forma ove vrste tumora. **Prikaz bolesnika.** Prikazana je bolesnica stara 65 godina, sa solitarnim fibroznim tumorom želuca. Patohistološka analiza otklonjenog tkiva pokazala je postojanje tumorskog tkiva sagrađenog od vretenastih ćelija, izduženih jedara sa srednje obilnom do oskudnom citoplazmom. Tumorske ćelije bile su u nekohezivnom rasporedu, u manjim područjima raspoređene u vidu palisada. Između opisanih tumorskih ćelija bila je prisutna veća količina hipocelularnog vezivnog tkiva koje je mestimično bilo hijalinizovano, sa veoma malim fokusima distrofijskih kalcifikata. Mitoze su bile veoma retke (manje od 3/10 HPF). Bili su prisutni i krvni sudovi koje je okruživalo vezivo. U pregledanom materijalu nije bilo elemenata ishodišnog organa. Imunohistohemijski, dobijena je pozitivnost na CD34 i vimentin, negativnost na S100, SMA, CD117, dezmin, a Ki-67 je < 2%. Promena je dijagnostikovana kao solitarni fibrozni tumor. **Zaključak.** S obzirom na činjenicu da su benigni solitarni fibrozni tumori sa ekstratorakalnom lokalizacijom ekstremno retke neoplazme sa nepredvidivim biološkim ponašanjem i mogućnošću ponavljanja, preporučuje se dugoročno kliničko i endoskopsko praćenje na godišnjem nivou kod bolesnika sa tim oboljenjem.

Ključne reči:
fibrom; peritoneum, neoplazme; želudac; dijagnoza; imunoenzimske tehnike; tumorski markeri, biološki; antigeni, cd 34; vimentin; imunohistohemija.

Introduction

Solitary fibrous tumors are a rare group of spindle cell neoplasm which includes fibrous-fiber cell solitary fibrous tumors and associated lesions such as giant cell lipomatous hemangiopericytoma and angiofibroma [1]. Initially described solitary fibrous tumors were of pleural origin localized in the chest cavity [2]. Today are described extrathoracic localiza-

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tions of this tumor, such as: retroperitoneal, abdominal cavity, the head and neck [3–5]. This type of tumor most frequently occurs in middle-aged people of both sexes [6]. Extrathoracic solitary fibrous tumors, unlike thoracic, are generally followed by symptoms in the form of pain or pressure, depending on the size and location of the tumor mass [4, 7]. Solitary fibrous tumors, of any localization are usually of benign origin and surgical resection is curative therapy for almost the majority of these tumors [8]. Solitary fibrous tumors can recur, and metastasize after surgical resection, because in some cases the biological behavior of the tumor does not correlate with histopathology, and therefore the long-term clinical follow-up is mandatory for all patients [6]. These tumors independent on their localizations, appears to have a common characteristic of immune reactivity to the CD34.

So far, in the available literature there are only a three cases of solitary fibrous tumor with extrathoracic localization, in the area of gastric serosa [9–11].

Case report

After preoperative examination a 65-year-old female patient was operated at the Clinic for Abdominal, Endocrine and Transplantation Surgery, in the Clinical Center of Vojvodina, Novi Sad, Serbia. Specimen removed was a spherical formation with referral clinical diagnosis- "Gastrointestinal stromal tumor" and sent to histopathological examination. By macroscopic examination received material was described in the form of spherical fragment size $2.5 \times 2.3 \times 1$ cm, smooth, pale yellowish surface, on serial sections swirling material, white grayish color. The material was processed in its entirety.

After routine histological processing of materials: fixed in 10% formalin, dehydrated, embedded in paraffin, cut to a thickness of 4 micrometers, hematoxylin and eosin staining method, histological analysis were performed. Tumor tissue built of spindle cells with elongated nuclei and with moderate abundant to scant cytoplasm was observed by histological analysis of the samples. Tumor cells were scheduled in a non-cohesive arrangement in a small areas and in the form of the palisade. Between described tumor cells there was a large amount of hipocellular connective tissue, hyalinised, with very small foci of dystrophic calcification. Mitoses were very rare (less than 3/10 HPF). There were also blood vessels surrounding the connective tissue. Reviewed material did not contain elements of the parent organ, in one of the clips the tumor was surrounded by a muscle layer which might originate from the wall of the parent organ (Figure 1).

After standard staining methods, special immunohistochemical staining methods to S100, SMA, CD117, Desmin, CD34, Vimentin, DOG1, PDGFRA and Ki-67 were done (Table 1). The following immunoprofile was obtained: S100-, SMA-, CD117 – (Figure 2), desmin-, DOG1- (Figure 3), PDGFRA- (Figure 4), CD34 + (Figure 5), vimentin + (Figure 6), Ki-67+ < 2% (Figure 7).

Described histological structure of the tumor tissue was diagnosed as a benign mesenchymal tumor, with respect to the histological description and the obtained immunohistochemical profile it was in favor of solitary fibrous tumor.

Table 1

Applied antibodies for immunohistochemistry

| Antibody	(Clone)	Source	Dilution	Result
CD34	(QBEnd 10)	DAKO, Carpinteria,CA,USA	1:40	+
Vimentin	(V9)	Fremont, CA94538-6406, USA	1:50	+
Desmin	(D33)	Fremont, CA94538-6406, USA	1:50	-
DOG1	(rabbit monoclonal)	Termo Fisher Scientific Inc.Waltham, USA	1:25	-
PDGFRA	(rabbit polyclonal)	Termo Fisher Scientific Inc.Waltham, USA	1:100	-
S-100	(rabbit polyclonal)	Fremont, CA94538-6406, USA	1:300	-
CD-117	(rabbit polyclonal)	Fremont, CA94538-6406, USA	1:100	-
SMA	(alfa-sm-1)	Novocastra,Newcastle, UK	1:50	-
Ki-67	(rabbit polyclonal)	Fremont, CA94538-6406, USA	ready-to-use	+ < 2%
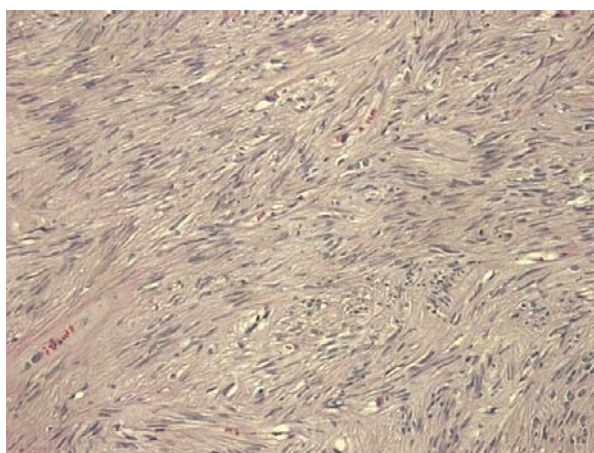
Fig. 1 – Tumor is surrounded by muscle layer that may originate from the wall of the parent organ (HE, ×100).
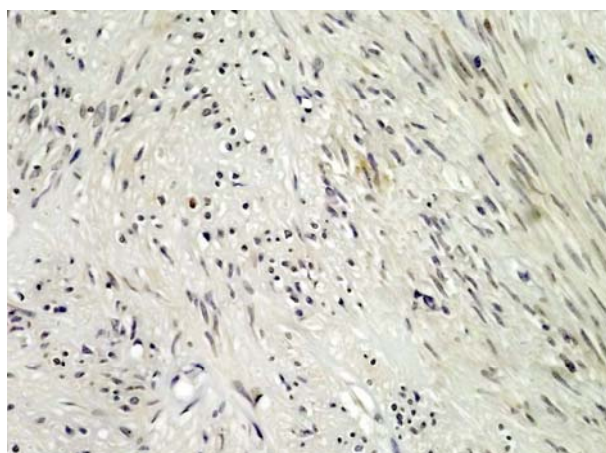

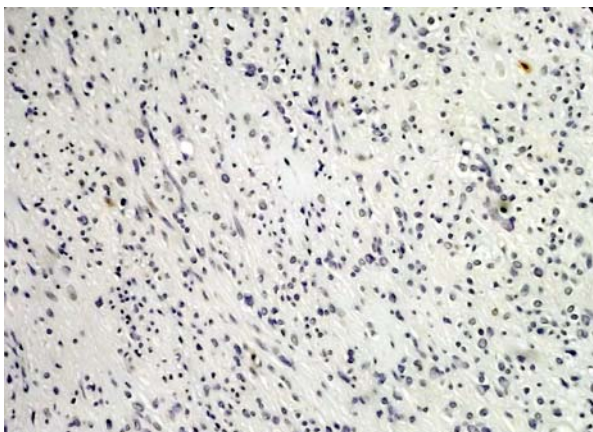**Fig. 2 – Tumor tissue (immunohistochemical stain for CD 117, ×400).**

Fig. 3 – Tumor tissue (immunohistochemical stain for DOG1, ×400).

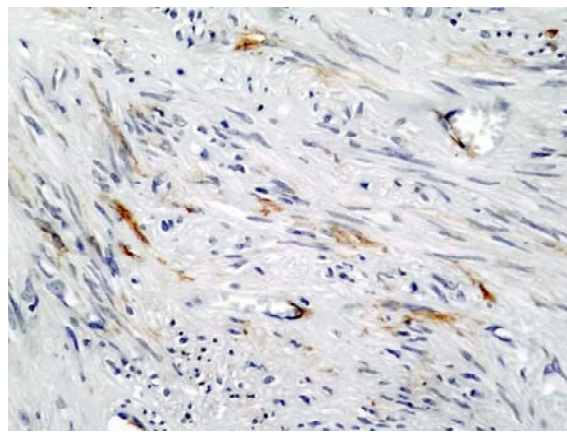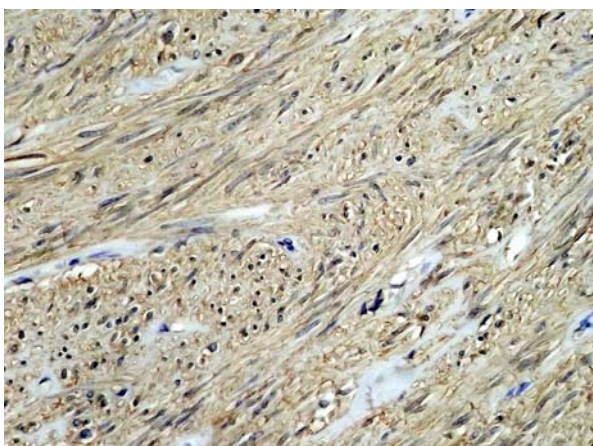**Fig. 4 – PDGFRA immunoreactivity of resected tumor (×400).**

Fig. 5 – Immunohistochemical feature of the tumor: CD34 positivity in tumor cells – nuclear positivity 20% (×400).
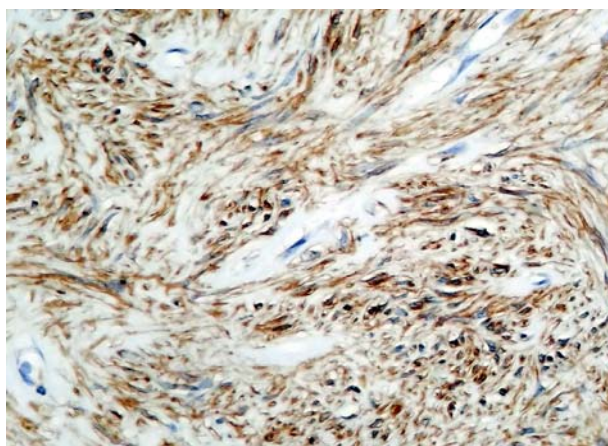

Fig. 6 – Tumor cells showing immunohistochemical positivity for vimentin: cytoplasmic positivity 60% (×400).

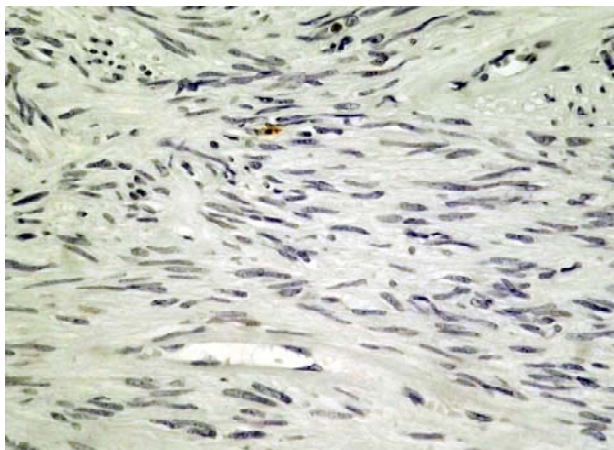**Fig. 7 – Immunohistochemical feature of the tumor: KI-67 positivity in tumor cells – positivity less than 2% (×400).**

Discussion

Solitary fibrous tumors are rare forms of the spindle-shaped cell neoplasm, typical pleural origin but also published were the forms of these tumor with extrathoracic localization such as the stomach, retroperitoneum and head and back region [12]. Most solitary fibrous tumors are benign and have a favorable prognosis, although also described in 13–23% cases of pleural localization malignant tumor forms [8].

Solitary fibrous tumors of extrathoracic localization show pronouncedly histological variability [13, 14] as a result of which the differential diagnosis takes into account numerous neoplasms, such as mesenteric tumors, gastrointestinal stromal tumors, tumors of smooth muscles, an in-

flammatory myofibroblastic tumors, inflammatory fibroid polyps, neurofibromas, and many sarcomatoid mesotheliomas and tumors of mesenchymal origin. Immunohistochemical staining in these circumstances are included in the histological diagnosis. Solitary fibrous tumors showed a marked positivity to CD34 and vimentin and negativity for cytokeratin, SMA, desmin, S100, c-kit, DOG1 and PDGFRA [12, 15]. Although the clinical features and biological behavior of these cancers have been described in several publications, precise clinical behavior of the tumor is still unpredictable. The rarely reappears in the form of local recurrence, tissue destruction in the source place spot, even in the form of remote metastasis. Recurrences can occur after several decades of surgical resection, so the long-term clinical follow-up of these patients is necessary [16].

Benign solitary fibrous tumors extrathoracic localizations are extremely rare neoplasms of mesenchymal origin, however, this does not diminish the importance of diagnosis of these neoplasms, especially as recorded their unpredictable biological behavior in the form of recurrence, local destruction at the site of the original impressions, or even in the form of distant metastases [9].

Conclusion

Considering that benign solitary fibrous tumors of extrathoracic localizations are extremely rare neoplasms with unpredictable biological behavior and the possibility of recurrence, a long-term clinical and endoscopic follow-up on the yearly basis of patients with this disease is recommended.

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C A S E   R E P O R T

Good's syndrome with increasing γδ T-lymphocyte subpopulation: A case report

Gudov sindrom praćen porastom podgrupe γδ T-limfocita

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Abstract

Introduction. Good's syndrome is a rare cause of adult-onset immunodeficiency associated with thymoma. Good's syndrome should be considered in patients older than 40 years with the history of frequent infections. An abnormal immunoglobulin profile needs further investigation and flow cytometry which is crucial for establishing the diagnosis of Good's syndrome. **Case report.** We present a 56-year-old men with Good's syndrome diagnosed after a two-year history of recurrent infections. Examination of immune status of the patient showed decreased serum levels of all immunoglobulins. Flow cytometry of peripheral blood lymphocyte revealed markedly reduced peripheral B cells, CD4 T-cell lymphopenia, inverted CD4/CD8 T-cell-ratio 0.37 (CD4 – 20.82%, CD8 – 70.7%). Analysis of the subpopulations of T-lymphocytes showed relative increasing γδ T cell receptor (TCR) T lymphocytes. Computed tomography scan of the chest showed a mediastinal mass compatible with thymoma of the diameter of 40 mm. After initiation of intravenous immunoglobulins the patient was in the good clinical condition and without bacterial complications. As the patient refused the operative treatment we continued to control the mediastinal tumor mass which did not increase during a 3-year follow-up. **Conclusion.** The presented patient had a typical immunological finding for Good's syndrome, but also the increase in γδ TCR T-lymphocyte subpopulation for which it is difficult to determine whether this is pathogenetic or secondary reactive event.

Key words:
acquired immunodeficiency syndrome; thymoma; comorbidity; adult; diagnosis, differential; flow cytometry.

Apstrakt

Uvod. Gudov sindrom je redak oblik imunodeficijencije kod odraslih udružene sa timomom. Na Gudov sindrom treba misliti kod bolesnika starijih od 40 godina sa istorijom čestih infekcija. Sniženje koncentracije imumoglobulina u serumu zahteva dalje ispitivanje uključujući protočnu citometriju koja je od ključnog značaja za postavljanje dijagnoze Gudovog sindroma. **Prikaz bolesnika.** Prikazali smo bolesnika, starog 56 godina, kod koga je postavljena dijagnoza Gudovog sindroma posle dvogodišnjeg perioda ponavljanih infekcija. Imajući u vidu snižen nivo svih imunoglobulina u serumu urađena je protočna citometrija limfocita periferne krvi. Ovom metodom utvrđeno je potpuno odsustvo B-ćelija, CD4 limfopenija, inverzan odnos CD4/CD8 T-ćelija – 0,37 (CD4 – 20.82%, CD8 – 70,7%). Analiza subpopulacija T-limfocita pokazala je relativno povećanje γδ T ćelijski receptor (TĆR) T limfocita. Kompjuterizovana tomografija (KT) grudnog koša viđena je medijastinalna tumorska masa koja odgovara timomu prečnika 40 mm. Posle započinjanja lečenja mesečnom primenom intravenskih imunoglobulina došlo je do značajnog sniženja infektivnih komplikacija. S obzirom na to da je bolesnik odbio operativno lečenje nastavljeno je KT praćenje medijastinalne tumorske mase koja se nije povećala tokom 3-godišnjeg perioda. **Zaključak.** Kod prikazanog bolesnika protočnom citometrijom utvrđen je nalaz tipičan za Gudov sindrom, ali je registrovano i povećanje subpopulacije γδ TĆR T limfocita za koje nije jasno da li je patogenetski ili reaktivni događaj.

Ključne reči:
imunitet, sindromi stečenog nedostatka; timom; komorbiditet; odrasle osobe; dijagnoza, diferencijalna; citometrija, protočna.

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Introduction

Good's syndrome (GS) is a rare cause of combined B- and T-cell immunodeficiency in adults associated with thymoma [1]. It was first described in 1954 by Good [2], who reported hypogammaglobulinemia in an adult patient with thymoma. It is a rare type of adult-onset immunodeficiency characterized by hypogamaglobulinemia, lower number or absence of peripheral blood B-cells, and variably, defects in cell-mediated immunity [3]. The patients with Good's syndrome have a bone marow defects imparing B-cell maturation and deficiencies in other cell lineages [1]. It was often considered as a subset of common variable immunodeficiency (CVID) with thymoma, weheteas nowdays this disorder is classified as a distinct entity by the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency [4].

unds showed normal breathing, with inspirium basal crackles in both sides, and there was no hepatosplenomegaly or lymphadenopathy. A complete blood count revealed leukocytosis (11×10^9/L) with neutrophilia in differential, normal erythrocyte and platelet counts. The patient's blood chemistry showed elevation of acute phase reactants of inflammation [sedimentation of erythrocytes (SE) 56/1h, C-reactive protein (CRP) 44 mg/L, fibrinogen 5.4g/L)]. Serum levels of all immunoglobulins were decreased (Table 1). Examination of bone marrow aspirate specimen revealed mild hypercellularity without pathological finding. Immunoserological analyses of rheumatic factor, antinuclear antibodies, anion gap metabolic acidosis (AGMA), and crioglobulins were negative. Antibodies to human immnunodeficiency virus were negative. Proteinuria was 0.4 g/24 h. Serum and urine immunofixation did not demonstrate any monoclonal component. Tumor markers

Table 1

Immunoglobulin levels and lymphocyte subset analysis of peripheral blood

Parameters	Results I May 2009	Results II Feb 2014	Referent ranges
IgA (g/L)	< 0.01	< 0.1	0.7–4.0
IgM (g/L)	< 0.0117	< 0.01	0.4–2.3
IgG (g/L)	3.53	4.2	7.0–16
B-Ly (%)	0	0	3–13
T-Ly (%)	79	89	65–84
T-Ly (cell/μl)	1,042	1602	1,084–2,822
CD4$^+$ T-Ly (%)	21	20	32–57
CD4$^+$ T-Ly (cell/μl)	275	360	703–1,588
CD8$^+$ T-Ly (%)	56%	43	16–38
CD8$^+$ T-Ly (cell/μl)	738	744	259–1,150
CD4$^+$/CD8$^+$ index	0.37	0.47	1.07–2.77
αβ T-Ly (% of T-Ly)	86%	70	87–94
γδ T-Ly (% of T-Ly)	11%	30	2–6
NK cells (CD3$^-$ CD16$^+$CD56$^+$)	21%	21	8–32
NK cells (CD3$^-$ CD16$^+$ CD56$^+$)/μL	271	378	208–1,097

Ig – immunoglobulin; Ly – lymphocites; NK cells – natural killer cells.

We presented the patient with typical immunological findings for Good's syndrome (hypogamaglobulinemia, few or absent B-cells, CD4+ T-cell lymphopenia and abnormal CD4+/CD8+ T- cell ratio), associated with increasing of γδ T-lymphocyte subpopulation.

Case report

A 56-year-old patient, was hospitalized for the first time in our department in April 2010. His main simptoms were prolonged cough with scanty yellowish sputum during two weeks, diarrhea, with occasionally mucous stools and weight loss. His condition started 2 years before when he had repeated outpatient visits and hospital admissions either from diarrhea or respiratory tract infections (frequent episodes of sinususitis and pneumococcal pneumonia three times). Two months before admission sinus surgery had been done because of frequent sinusitis.

The patient had never smoked, nor consumed alcohol, and his family history was noncontributory.

Physical examination on admission to our hospital revealed a fever of 38.2°C and moist skin. Auscultation of lung so-

carcinoembryonic antigen (CEA), alpha fotoprotein (AFP), cancer antigen 19–9 (CA 19-9), and prostate specific antigen (PSA), were in referent ranges. Cutaneous test of cell mediated immunity purified protein derivative (PPD3) showed anergy. Culture of trouth and nose yielded *Streptococcus pneumoniae*. Culture of sputum showed the presence of *Candida albicans*. The analysis of specimen smear for acid-fast bacilli was negative. Otorhinolaryngological finding showed chronic rhinosinusitis and recurrent endonasal polypus. Radiography of frontal and maxillar paranasal caves showed signs of chronic inflamation. The posteroanterior chest radiography showed reticulonodular changes in the lower parts both pulmonary fields. Computed tomography (CT) scan of the chest, showed a mediastinal mass compatible with thymoma of the diameter of 40 mm, bronchiectasies, and reticulonodular changes in the lower parts both pulmonary fields, that correspond to fibrosis (Figure 1). Abdominal ultrasound, gastroscopy and colonoscopic examination disclosed the normal findings. Flow cytometric immunophenotyping of peripheral blood lymphocytes revealed undetectable levels of peripheral B-cells, CD4$^+$ T-cell lymphopenia, as well as inverted CD4+/CD8+ T-cell ratio

(Table 1). Analysis of T-lymphocytes regarding the type of T-cell receptor (TCR) expression, showed relative increasing of γδ T-lymphocyte subpopulation (11% of T-cells).

Fig. 1 – Computed tomography (CT) of the chest showing mediastinal mass compatible with thymoma (diameter of 40 mm).

Bearing in mind the above findings, diagnosis of Good's syndrome was established. Thus, the patient was treated with intravenous polyclonal immunoglobulins in the four-week intervals. After initiation of intravenous immunoglobulins, the patient was in good clinical condition and without bacterial complications, but with reccurent episode of herpes zooster infection. We considered to do thymectomy, however the patient refused surgical intervention. The mediastinal tumor mass was regularly monitored by CT scan and did not incease during a 4-year follow-up. Control flow cytometric immunophenotyping of peripheral blood, after a 5-year follow-up, confirmed the persistence of absolute B-cell lymphopenia, CD4+ T-cell lymphopenia (360 cells/μL), as well as inverted CD4/CD8 T-cell ratio (0.47). Moreover, a relative increase of γδ T-lymphocyte subpopulation was detected (30% of T-cells).

Discussion

Good's syndrome, defined as thymoma associated with immunodeficiency, is a rare cause of combined B- and T-cell immunodeficiency in adults, represented with a similar frequency in male and female patients[1]. It can occur in children, although this is extremely rare[5]. Its exact prevalence is unknown but it only represents 1% to 2% of patients, which are treated by intravenous immunoglobulin (IVIG) therapy for a primary deficiency of immunoglobulins[1]. Patients with Good's syndrome usually present in the 4th or 5th decade of life. According the literature data, the mean age of initial symptoms was 56 years (range, 29–75)[3, 6]. Similarly, in the presented patient the diagnosis of Good's syndrome was established when he was 56 after a 2-year hystory of recurrent infections.

The pathogenesis of Good's syndrome is unknown, but there are two hypotheses[7]. *In vitro* studies showed defects in B-cell precursor growth and differentiation and T-lymphocyte pro-

liferation as well as interleukin-2 production[8]. It has been demonstrated that T-lymphocytes from patients with thymomas can inhibit immunoglobulin production in healthy controls[1]. Loss of B-cell function is probably due to autoimmune destruction by T-cells or autoantibodies[1, 9]. It is supported by the frequent association of Good's syndrome and various autoimmune diseases[9]. They include pure red cell aplasia, myasthenia gravis, oral lichen planus, aplastic anemia, macrocytic anemia, leucopenia, thrombocytopenia, monoclonal gammopathy and autoimmune hemolytic anemia[9].

The principal immunological findings in Good's syndrome are hypogamaglobulinemia, few or absent peripheral blood B-cells, an abnormal CD4+/CD8+ T-cell ratio, CD4+ T-cell lymphopenia, and impaired T-cell mitogenic responses[1]. Almost all patients have reduced serum IgG, IgA and IgM. Flow cytometric immunophenotyping of peripheral blood lymphocytes of our patient showed all of changes consistent with Good's syndrome. Besides, relative increasing of γδ T-lymphocyte subpopulation was noticed, which was persistent finding after a 5-year follow-up. Lymphocyte bearing the γδ TCR comprise a small proportion (5%) of the total peripheral blood lymphocytes[10]. An increased proportion of circulating γδ T-cells has been found in infections, T-cell leukemia as well as in patients with some primary immunodeficiencies, such as CVID, Wiskott-Aldrich syndrome, ataxia teleangiectasia, with or without infections at the time of evaluation[11, 12]. It is not known whether increasing of γδ T-lymphocytes is a primary event involved in the pathogenesis of the disease or a reactive event emerged as the consequence of the disease or chronic antigenic stimulation induced by bacterial or viral antigens. It has been hypothesized that γδ T-lymphocytosis may arise from dysregulation of γδ TCR gene expression in association with defects in αβ TCR gene expression[12]. It was supported by the finding of markedly reduced CD4+/CD8+ T-cell index as observed in the presented patient.

The initial clinical presentation is either a mass-lesion thymoma or a recurrent infection[13–15]. Thymoma occurs in 10% of patients with adult-onset hypogammaglobulinemia, whereas 6–11% of thymoma patients have hypogammaglobulinemia[1]. Thymoma associated with infections appear almost simultaneously in 38% cases, in other cases diagnosis tymoma preceded the diagnosis of hypogammaglobulinemia (42%), infection, or diarrhea. In 20% of cases, thymoma is diagnosed 3 months to 15 years after other clinical manifestations[8, 9]. In the presented patient thymoma and hypogammaglobulinemia were diagnosed simultaneously, but after a 2-year period of frequent infection.

The main clinical characteristics of Good's syndrome are increased susceptibility to bacterial infections, opportunistic viral and fungal infections[1, 13, 14, 16, 17]. Most patients experienced reccurent sinopulmonary infections secondary to encapsuled organisms (*Haemophilus influenzae*, *Streptococcus pneumoniae*), skin infections, bacterial diarrhea (*Giardia lamblia*, *Salmonella spp*, *Campylobacter jejuni*) and urinary tract infections. The most common virus infection is caused by cytomegalovirus. Infections caused by herpes simplex virus, human herpes virus type 8 and varicella-zoster virus are also frequent. Although systemic fungal infections are not characteristic for Good's syndrome, mucocutaneous candidiasis occur

in 24% of cases [3]. The presented patient had a tipical hystory of recurrent sinopulmonary and skin infection and diarrhea that was the reason for immunological examination.

The prognosis of Good's syndrome is worse than X-linked agammaglobulinaemia (XLA) and CVID and mortality of approximately 45% has been reported in a systematic review of 152 patients with this syndrome [9]. Thymoma itself is not belie-ved to contribute towards excess mortality in this condition [1]. The predominant causes of death are infections associated with immunodeficiency [9].

Treatment of antibody deficiency in GS requires supplementary intravenous immunoglobulin replacement to ma-intain adequate levels of immunoglobulin. Their use improves infection control, reduce hospitalization and decrease the use of antibiotics [9]. The treatment of thymoma is surgical removal or debulking of the tumor and the most important indicator of a long-term prognosis is completeness of tumor resection [15]. Thymectomy has a favorable effect on associated conditions like myasthenia gravis and pure red cell aplasia. On the contrary, GS associated with thymoma in general is not resolved by surgical treatment of thymoma [18, 19]. In some cases, it was observed that it might worsen the hypogammaglobulinemia [19]. Hypogammaglobulinemia and clinical manifestations can last for years after thymectomy [18, 19–21]. It was suggested that the hypogammaglobulinemia is not directly caused by thymoma than by an autoimmune or other immunoregulatory processes [8]. Taking this into consideration, the question arises wether we

should do thymectomy in patients with GS and benign lesions which does not increase during follow-up. As the presented patient refused surgical treatment, we do not know what would be the effect of thymectomy on the course of the disease in him. The presented patient had a significant reduction of bacterial infections after regular immunoglobulin replacement. On the other side, he still had recurrent herpes zooster as a sign of persistently decreased cellular immunity.

Conclusion

Good's syndrome is extremely rare. The presented patient is first described patient with Good's syndrome in our country. Good's syndrome should be considered in all patients older than 40 years with frequent respiratory and gastrointestinal infections. An abnormal immunoglobulin profile needs flow cytometric analysis which is the gold standard for determination of immunological defects. Increased awareness about the clinical and immunological profile of this syndrome may increase its early recognition and prevent mortality. Further studies are needed to elucidate the pathogenesis and significance of γδ T-lymphocyte subpopulation in this clinical entity.

Conflict of interests

The authors declare that they have no conflict of interests.

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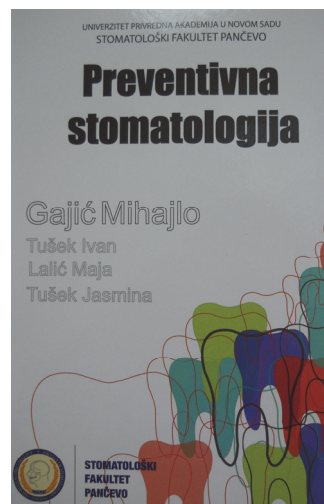
PRIKAZ KNJIGE /BOOK REVIEW

Preventivna stomatologija

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Kada kažemo preventivna stomatologija, većina stomatologa taj termin veže za dečju i preventivnu stomatologiju. Na značaj preventivne stomatologije međutim, ukazuje definicija Svetske zdravstvene organizacije, koja prevenciju stavlja na prvo mesto i glasi: „Stomatologija je nauka i veština prevencije, dijagnostike i lečenja oboljenja, anomalija i traumatskih povreda usta i zuba“. Treba istaći da autori ovog udžbenika naglašavaju da preventivna stomatologija svojim multidisciplinarnim pristupom objedinjuje aktivnosti različitih stomatoloških, medicinskih i društvenih naučnih disciplina, poput sociologije, psihologije, epidemiologije, statistike, zdravstvenog vaspitanja itd.

Udžbenik je napisan na 480 stranica, tvrdo ukoričen i sastoji se od 23 poglavlja, koja logičnim redosledom i značajem pojedinih anatomomorfoloških karakteristika usne duplje, te prisutnom mikroflorom, gde već u prvom poglavlju autori naglašavaju da stomatolozi moraju imati na umu činjenicu da su karijes i parodontopatija izrazito preventabilna oboljenja. Danas, inače, preovladava mišljenje, da radi nove strategije u prevenciji i terapiji oralnih oboljenja izazvanih mikroorganizmima dentalnog biofilma, istraživanje bi trebalo usmeriti upravo prema interakcijskim odnosima koji vladaju u mešovitom bakterijskom konzorcijumu, te su se autori u pisanju pridržavali ovih aktuelnih tendencija, o čemu svedoče brojni najnoviji podaci iz navedene literature, koji mogu biti integrisani u pisanje jednog udžbenika, a naročito u poglavljima 2 i 3, koja posebnu pažnju posvećuju oralnom biofilmu sa aspekta novih naučnih saznanja.

Posebno bih istakla „Procenu rizika od nastanka karijesa“ u poglavlju 5, s obzirom na to da Američka akademija za dečju stomatologiju poslednjih godina ukazuje na značaj procene rizika od nastanka karijesa i na osnovu toga naglašava značaj donošenja odluka o preventivnim protokolima koji su od suštinskog značaja za očuvanje oralnog zdravlja. Prema istraživanjima Ismaila i sar. iz 2008. godine, većina stomatologa ne zapaža faktore rizika od nastanka karijesa, te autori u ovom poglavlju predstavljaju testove i programske pakete za procenu nivoa rizika od nastanka i progresije karijesa, koji su zasnovani na multifaktorijalnosti ovog oboljenja. U vezi s tim, poseban problem savremenog društva predstavlja i karijes u ranom detinjstvu koji je još uvek nerešiva enigma, pre svega zbog velikog broja predisponirajućih faktora koji su taksativno navedeni i detaljno analizirani u poglavlju „Prevencija karijesa u ranom detinjstvu“.

Značajan deo udžbenika čine i tekstovi „Profilaktičke mere u prevenciji oboljenja usne duplje“. Logičnim redosledom i fotografskim zapisima visokog kvaliteta opisani su postupci i preparati, konvencionalni i savremeni, koji bi trebalo da nađu svoje mesto u svakodnevnom kliničkom radu. S obzirom na njihovu zastupljenost u većini stomatoloških udžbenika, izdvojiću i poglavlje 16 o prevenciji oralnih oboljenja kod osoba sa posebnim potrebama. Sveobuhvatnost obrađene problematike zavređuje pažnju, imajući u vidu da 8% školske dece u Srbiji pokazuje neku smetnju u razvoju. Psihološke barijere koje ima većina hendikepiranih osoba zbog različitih mentalnih, kognitivnih i emocionalnih hendikepa,

izazivaju nekontrolisan strah prema stomatološkoj ordinaciji, smanjuju broj poseta stomatologu i posledično utiču na održavanje i stanje oralnog zdravlja. Značajnu barijeru čine i stavovi koje zastupa stomatološka profesija u promociji, prevenciji i lečenju usta i zuba hendikepiranih osoba. Prema istraživanjima, samo 20% stomatologa je spremno da ih leči. Podaci o strategiji za poboljšanje oralnog zdravlja osoba sa posebnim potrebama su sistematično prikazani u ovom delu udžbenika.

Takođe, istakla bih poglavlje 19 u kojem su izneti aktuelni podaci iz svetske literature o značaju oralnog zdravlja kod ljudi trećeg životnog doba. Ako prihvatimo pretpostavku da će 2025. god. biti oko dve milijarde starijih ljudi, ova značajna promena demografske strukture stanovništva u velikoj meri će se odraziti na orijentaciju celokupne stomatološke službe, kako bi se uspešno prilagodila sve većem broju starih osoba. Dakle, uz teorijsku potporu, praktično iskustvo autora

došlo je do potpunog izražaja pri izboru građe i kompozicije ovog dela udžbenika.

I na kraju, a po značaju nikako nevažno, istakla bih poglavlje 21, o oralnim mukozitisima nakon onkoloških terapija, kojima nije posvećeno dovoljno pažnje u našim stručnim krugovima, a, nažalost, potrebe su svakodnevno sve veće i veće.

U ovom udžbeniku, autori su na savremen način kompletirali aktuelne poglede na celinu preventivne stomatologije, sa harmoničnom organizacijom poglavlja, pratećim tabelarnim i fotografskim zapisima, moderno grafički i vizuelno realizovani, te se sa zadovoljstvom može preporučiti stomatološkoj javnosti.

dr sc. stom. Radojka Delić
specijalista bolesti usta i zuba

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a) The title should be concise but informative, while subheadings should be avoided;

b) Full names of the authors signed as follows: *, †, ‡, §, ||, ¶, **, ††, ...

c) Exact names and places of department(s) and institution(s) of affiliation where the studies were performed, city and the state for any authors, clearly marked by standard footnote signs;

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The second page should carry a structured abstract (250-300 words for original articles and meta-analyses) with the title of the article. In short, clear sentences the authors should write the **Background/Aim**, major procedures – **Methods** (choice of subjects or laboratory animals; methods for observation and analysis), the obtained findings – **Results** (concrete data and their statistical significance), and the **Conclusion**. It should emphasize new and important aspects of the study or observations. A structured abstract for case reports (up to 250 words) should contain subtitles **Introduction, Case report, Conclusion**). Below the abstract **Key words** should provide 3–10 key words or short phrases that indicate the topic of the article.

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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.

Introduction. After the introductory notes, the aim of the article should be stated in brief (the reasons for the study or observation), only significant data from the literature, but not extensive, detailed consideration of the subject, nor data or conclusions from the work being reported.

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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:

Jurhar-Pavlova M, Petlichkovski A, TrajkovD, Efinska-Mladenovska O, Arsov T, Strezova A, et al. Influence of the elevated ambient temperature on immunoglobulin G and immunoglobulin G subclasses in sera of Wistar rats. Vojnosanit Pregl 2003; 60(6): 657–612.

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u desnom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fus-noti, ne u zaglavlju. Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

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