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CASE REPORTS / KAZUISTIKA

HISTORY OF MEDICINE / ISTORIJA MEDICINE

Zsuzsanna Kossuth (1817–1854), like her brother Lajos Kossuth, was a Hungarian freedom fighter in the Hungarian Revolution (the War of Independence) in 1848–1849. In April 1849, Lajos Kossuth appointed her chief nurse of all the military hospitals in Hungary giving her the responsibility to organize the entire medical military system in the country. She also sent an appeal for women to volunteer as medical nurses, and organized their work. Her birthday, February 19, the Hungarian parliament declared the Day of Health Workers in Hungary.
This year 200 years of her birthday is marked (see pp. 386–90).

Žužana Košut (1817-1854), kao i njen brat Lajoš Košut, bila je borac za slobodu Mađarske u Mađarskoj revoluciji (Rat za nezavisnost) 1848–1849. godine. U aprilu 1849, Lajoš Košut imenovao je za glavnu sestru svih vojnih bolnica u Mađarskoj dajući joj odgovornost za organizovanje celokupnog vojnog saniteta u državi. Ona je, takođe, uputila apel ženama da se dobrovoljno prijave za medicinske sestre i organizovala njihov rad.
Njen rođendan, 19. februar, mađarski parlament proglasio je za Dan zdravstvenih radnika Mađarske.
Ove godine obeležava se 200 godina od njenog rođenja (vidi str. 386–90).

ORIGINAL ARTICLES

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Unprotected autogenous bone block grafts in the anterior maxilla: resorption rates and clinical outcomes

Nezaštićeni koštani autotransplantati u prednjem segmentu gornje vilice: stepen resorpcije i klinički rezultati

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Abstract

Background/Aim. The use of autogenous bone grafts for augmentation of the resorbed alveolar ridge is still considered the gold standard in implant dentistry. The aim of this study was to analyze the resorption rate of autogenous bone block grafts from the retromolar region placed in the frontal segment of the upper jaw unprotected by barrier membranes, to assess the stability of implants placed into the grafted bone, as well as to monitor its changes during the healing period. **Methods.** The study included 18 patients with a total of 20 grafted sites. The residual alveolar ridge was measured before and after the augmentation and prior to implant placement. All implants were restored with provisional crowns within 48 hours after the placement. Implant stability was assessed using resonance frequency analysis. **Results.** The average period from ridge augmentation to reentry was 5.4 months (range 4–6 months). At reentry the healed alveolar ridge had a mean width of 6.1 ± 1.27 mm. The mean calculated width gain was 3.04 ± 1.22 mm. The overall surface resorption of block grafts was 0.68 ± 0.69 mm (18.85%). At the time of implant placement the mean value of implant stability quotient (ISQ) was 71.25 ± 5.77. The lowest ISQ values were noted after three weeks of healing, followed by a gradual increase until week 12. After 12 weeks implants showed significantly higher ISQ values compared to primary stability ($p < 0.05$ Wilcoxon signed ranks test). During the 3-years follow-up period no cases of implant loss were recorded. **Conclusion.** Despite a significant resorption of bone grafts, it was possible to place implants in all the cases and to use the immediate loading protocol without affecting implant survival rate.

Key words:
maxilla; alveolar ridge augmentation; transplantation, autologous; dental implants; bone resorption; treatment outcome.

Apstrakt

Uvod/cilj. Primena autogenih koštanih graftova (implantata) za uvećavanje smanjenog (resorbovanog) alvelarnog grebena još uvek se smatra zlatnim standardom u implantologiji. Cilj ove studije bio je analiza stepena resorpcije autolognih koštanih blok transplantata nezaštićenih barijernim mebranama, uzetih iz retromolarnog predela mandibule i postavljenih u frontalni segment gornje vilice, procena stabilnosti implantata ugrađenih u povećanu regiju, kao i praćenje promene implantne stabilnosti tokom perioda oseointegracije. **Metode.** U studiju je bilo uključeno 18 pacijenata sa ukupno 20 autotransplantata. Širina rezidualnog alveolarnog grebena merena je pre i posle postavljanja transplantata, kao i neposredno pre ugradnje implantata. Svi implantati su opterećeni privremenim nadoknadama 48 sati nakon ugradnje. Stabilnost implantata procenjivana je primenom analize rezonantne frekvencije. **Rezultati.** Srednje vreme između uvećavanja grebena i ugradnje implantata iznosilo je 5,4 (4–6) meseci. Pre ugradnje implantata srednja vrednost širine grebena iznosila je 6,1 ± 1,27 mm, a povećanja širine grebena u odnosu na vrednosti pre uvećanja 3,04 ± 1,22 mm. Površinska resoprcija grafta iznosila je 18,85% (0,68 ± 0,69 mm). Srednja vrednost koeficijenta implantne stabilnosti (ISQ) u momentu ugradnje iznosila je 71,25 ± 5,77. Najniže vrednosti ISQ zabeležene su u trećoj nedelji nakon ugradnje, što je bilo praćeno postepenim porastom do dvanaeste nedelje zarastanja. Nakon dvanaest nedelja vrednosti ISQ bile su statitički značajno više od vrednosti u momentu ugradnje ($p < 0,05$ Wilcoxon test). Tokom trogodišnjeg perioda praćenja nije bilo izgubljenih implantata. **Zaključak.** Bez obzira na značajan stepen resorpcije autotransplantata, kod svih pacijenata bilo je moguće ugraditi implantate u uvećani greben, kao i primeniti protokol ranog opterećenja bez uticaja na stepen preživljavanja implantata.

Ključne reči:
maksila; alveolni greben, podizanje; transplantacija, autologna; stomatološki implanti; kost, resorpcija; lečenje, ishod.

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Introduction

Quantity and quality of available bone for dental implants placement significantly affect final results of implant surgery. Besides other variables, long-term maintenance of esthetic results is largely dependent on thickness of labial cortex covering the implant [1]. Therefore, sufficient bone volume is among the most important factors when it comes to implant surgery in the anterior maxilla.

The use of autogenous bone grafts for augmentation of the resorbed alveolar ridge is still considered the gold standard in implant dentistry [2]. Although usage of autogenous bone block grafts from the retromolar region proved to be safe and effective procedure [3–5], there are still some issues which should be considered when planning this kind of surgery. Horizontal ridge augmentation with autogenous block grafts and a bioinert expanded polytetrafluoroethylene (ePTFE) membrane is well documented, with good clinical results [6]. The lack of this procedure is a certain risk of wound dehiscence, membrane exposure and subsequent site infection [7]. Usage of collagen membranes reduces risk of dehiscence but it seems that their barrier function is limited to a few weeks [8]. On the other hand, it has been demonstrated that mandibular bone grafts can be used for ridge augmentation without barrier membrane [9]. Still, such approach might be related to increased resorption of the graft, affecting the final result of grafting procedure [10, 11].

At last, this kind of surgery should enhance not only dimensions of residual alveolar ridge, but also should improve bone quality at the site of future implant placement. This is particularly important in the frontal segment of the upper jaw, where increased bone density and implant stability should allow for immediate loading of the implants, providing patients with esthetically acceptable restoration in a shortened period of time.

Therefore, the aim of this study was to analyze clinical outcomes of autogenous bone block grafts from the retromolar region placed in the frontal segment of the upper jaw, to assess resorption rates of grafts unprotected by barrier membranes at the time of implant placement, as well as to assess the stability of implants placed into the grafted bone and to monitor its changes during the healing period of implants restored according to immediate loading protocol, as well as to report survival rate of these implants.

Methods

The study sample included 18 patients. The patients were fully informed about the surgical procedures and treatment alternatives. The protocol of the study was approved by the institutional Ethics Committee.

Inclusion criteria comprised the American Society of Anesthesiology (ASA) physical status classification system I and II patients, aged 20 or more, missing one or more teeth in the frontal segment of the upper jaw. In all the cases the available width of the residual ridge was insufficient for placement of standard diameter implants without significant augmentation at the implant site. The minimum time allowed between tooth extraction and augmentation procedure was six weeks. When due to severe infection or trauma postextraction defects presented with less than three walls, grafts were placed before complete bone healing (less than four months post extraction).

Upon standard flap reflection, the width of alveolar ridge was measured with a caliper prior and after the augmentation in two levels, at 5 mm and 10 mm from cementoenamel junction (CEJ) of the neighboring teeth. Special ablative bur, 5 mm in diameter, was used to remove cortex at the recipient site and prepare bony bed for the future bone graft.

All grafts were harvested from the retromolar area. The osteotomy was performed with a trephine bur with 5 mm inner diameter, in a straight surgical handpiece, under copious saline irrigation. The trephine bur was graduated and preparation was performed to the depth of 10 mm. Using a thin surgical chisel and a hammer, the block graft was mobilized and prepared for the augmentation procedure. After harvesting procedure, the bone block was slightly adapted to the recipient site and secured by osteosynthesis screws of 10 mm in length in order to obtain bicortical fixation. The augmented alveolar ridge was measured again with caliper at the same reference points as before the graft placement. Voids around the block graft were filled with anorganic bovine bone matrix (ABBM) particles. A periostal-releasing incision was made and primary closure was obtained without any tension in the grafted area.

After 6 months the grafted sites were re-exposed and second surgery was performed. New measurements of ridge width were made on the grafted site, using the same caliper and reference points as during the augmentation procedure. Osteosynthesis screws were removed and the Brånemark System® Mk III implants (10 mm length, 4.0 mm diameter) were placed in an optimal position, following instructions from the manufacturer.

The primary stability of implants placed in the grafted area was measured by resonance frequency analysis (RFA). Measurements were repeated during the healing period at the postoperative weeks 3, 6, 8 and 12. The measuring devices (Smartpeg™) were attached to the implant and measurements were performed according to the manufacturer's instructions, with the probe aiming from the buccal direction. The probe was held at the distance of 2–3 mm until the instrument displayed the implant stability quotient (ISQ) value. Two ISQ values were recorded and used as a mean value for statistical analysis.

After implant placement temporary crowns were made from composite material and fixed within 48 hours after the weeks after implants installation.

Descriptive statistics was used to report the mean values and standard deviations of the reported parameters. Student´s *t*-test and Wilcoxon signed ranks test were used to analyze the differences in graft resorption rates and ISQ values. *P* values of < 0.05 were considered to be statistically significant. Calculations were performed using SPSS 10.0 statistical software.

Results

In 18 of the patients (11 males, 7 females; mean age 29 years; range 19–47 years), a total of 20 alveolar sites were

augmented (Table 1). No major intraoperative and postoperative complications were noted.

The widths of the residual alveolar ridge before and after the augmentation and also at the time of implant placement are shown in Table 2. Prior to augmentation, the mean width of 3.06 mm was insufficient to allow optimal placement of 4.0 mm diameter implants. It is interesting that the mean thickness of the graft was less than 4 mm, although a trephine bur of 5 mm diameter was used for graft harvesting, indicating that some of bony tissue was lost during graft mobilization and adaptation to the recipient site (Table 2).

The average period from ridge augmentation to reentry was 5.4 months (4–6 months). At reentry the healed alveolar ridge had the mean width of more than 6 mm, which was sufficient for implant placement. The mean calculated width gain was approximately 3 mm. The overall surface resorption of block grafts was 18.85% on 5 mm and 20.04% on 10 mm from CEJ. There was no statistically significant difference between graft resorption in these two reference points ($p > 0.05$; *t*-test) (Table 2).

Out of 20 implants 19 had primary stability higher than 60 ISQ (Figure 1). At the time of implant placement the

Table 1

Augmentation sites and demographic data

No	Age	Gender	Time of tooth loss (months ago)	Reason of tooth loss	Recipient site
1	25	M	1.5	Trauma	21
2	25	M	3	Trauma	21
3	38	F	84	Trauma	21
4	23	F	42	Trauma	11
5	24	M	4	Trauma	11
6	24	F	84	Trauma	21
7	24	M	6	Trauma	11
8	22	M	36	Trauma	11
9	22	M	36	Trauma	12
10	29	F	180	Infection	12
11	25	F	8	Trauma	11
12	25	M	120	Trauma	22
13	24	M	120	Trauma	21
14	30	M	1	Trauma	22
15	49	F	3	Trauma	21
16	34	M	18	Trauma	21
17	39	M	36	Trauma	22
18	35	M	18	Infection	22
19	35	M	19	Infection	23
20	34	F	1.5	Infection	21

M – male; F – female.

Table 2

Measurements of residual ridge width

Width		Pre-augmentation	Post-augmentation	Thickness of graft	Re-entry width	Graft gain	Amount of surface resorption
5 mm from CEJ (mean ± SD)	Mean	3.06 ± 1.55	6.78 ± 1.26	3.72 ± 0.86	6.10 ± 1.27	3.04 ± 1.22	0.68 ± 0.69
	Minimum	1.8	4.5	2	4.3	0.7	0
	Maximum	4.8	9.2	5.3	9	5	2.5
10 mm from CEJ (mean ± SD)	Mean	5.84 ± 1.69	9.47 ± 1.79	3.63 ± 1.17	8.74 ± 1.59	2.90 ± 1.11	0.73 ± 0.75
	Minimum	3.7	6	0.5	6	0.5	0
	Maximum	10.2	12.5	5.3	12	5.3	2.8

CEJ – cementoenamel junction; SD – standard deviation.

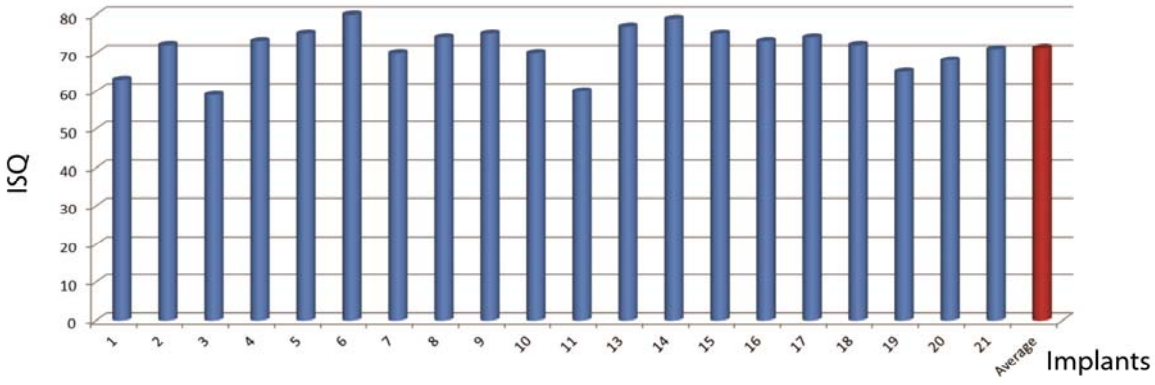

Fig. 1 – Primary implant stability quotient (ISQ) of 20 implants.

mean value of implant stability was 71.25 ± 5.77 ISQ. During the healing period, slight decrease of the mean implant stability was noted after three weeks of healing, just to be followed by another rise of ISQ values till the end of the observation period (Figure 2). There were no significant differences between the implants stability measured at the weeks 3, 6 and 8 and the primary implants stability ($p > 0.05$) but the difference between the implants stability at twelve weeks after the surgery and the primary stability was significant ($p < 0.05$ Wilcoxon signed ranks test).

During the 3-year follow-up period no cases of implant loss were recorded, constituting survival rate of 100%.

As an alternative to ePTFE membranes, it was demonstrated that resorbable collagen membranes also exert protective effect and reduce amount of graft resorption. Von Arx and Buser [13] demonstrated grafts resorption rate of 7.2% using collagen membrane and ABBM particles. However, besides the protective effect of collagen membranes it was shown that ABBM materials *per se* are able to reduce graft resorption. In the study from Maiorana et al. [14] the resorption of bone block grafts of only 9.3% for the sites treated with ABBM particles was found, whereas the sites without coverage demonstrated the resorption rate of 18.3%. Such effect might be attributed to the fact that these bone substitutes demonstrate slow and minimal resorption [15, 16].

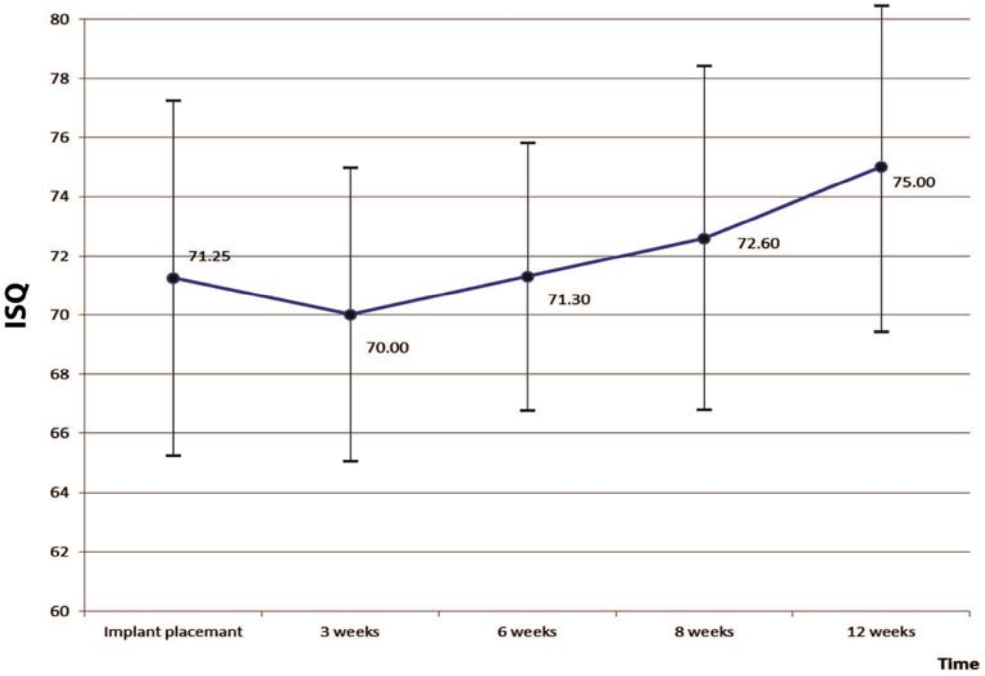

Fig. 2 – Changes of the implant stability quotient (ISQ) (mean ISQ values and standard deviations) during the healing period.

Discussion

Several techniques have been proposed for augmentation of the residual alveolar ridge, including bone blocks harvested from the mandible and positioned at the time of implant placement [12], but also bone grafts from intra-oral or extra-oral donor sites transplanted several months before the implantation [7].

However, the principal issue when discussing bone block grafts is amount of surface resorption, as this phenomenon might significantly affect final result of grafting procedure. Graft resorption is largely dependent on the usage of barrier membranes. In the study from Antoun et al. [11] grafted sites covered by non-resorbable ePTFE membrane showed the mean surface resorption of 0.3 mm (resorption rate of 7.5%) compared to the control group sites without membrane, with the mean resorption of 2.3 mm (resorption rate of 45%) [10].

Still, in several studies it was shown that autogenous bone blocks might be used for ridge augmentation without coverage by membranes and/or particulated grafting materials. Using autogenous bone grafts from chin and mandibular ramus, Cordaro et al. [9] reported 20% of graft resorption on maxillary sites. In a similar protocol, the resorption of 13.1% was reported for ramus block grafts used for augmentation of future implant sites in the upper jaw [17]. In our study the mean surface resorption of grafts from the retromolar region was 19.45%, which was similar to the results of Proussaefs et al. [18], who reported 17% of graft resorption using mandibular ramus block grafts, and particles of Bio-Oss at a periphery. In contrast to studies in which ABBM particles were used to protect graft surface, in our study this material was used only to fill the gaps around block graft and recipient bone, but it seems that such procedure does not affect the amount of graft resorption. Still, it has to be noted that the apparently signifi-

cant resorption of almost 20% of graft width actually represents the loss of only 0.7 mm in the horizontal dimensions of future implant site. From that point of view, it seems that for a significant number of cases this degree of graft resorption might be clinically acceptable.

It is of interest that monitoring of RFA results during the healing period showed similar pattern of ISQ changes as reported in previous studies. Although not statistically significant, it was evident that ISQ values decreased during the first three weeks of healing. Such a decrease, already well recognized in the literature [19–22], reflects initial phases of bone remodeling around newly placed implants and, from our results, it seems that such a process is similar both in grafted and native bone. Also, it was shown that initial fall of implant stability is followed by gradual increase of ISQ values, which were significantly higher after 12 weeks of healing.

Finally, it is of importance that initial implant stability was sufficiently high to allow immediate loading of implants placed into the grafted anterior maxilla. Such possibility is particularly important in this part of the jaws, as it provides esthetically acceptable restorations to be made in a shortened period of time. In the present study, the mean ISQ values of primary stability (71.25) were high enough to justify the immediate loading protocol [23]. Even more, although two implants demonstrated slightly lower values of the primary stability (58 and 59 ISQ) these implants were subjected to immediate loading without affecting their survival. Such a result is in agreement with studies indicating that the primary stability of ISQ *per se* has low predictive value regarding future osseointegration of implants [24].

High ISQ values in this study might be partially explained by changes in bone density following the grafting procedure. It was demonstrated that block grafts from mandibular ramus show high degrees of mineralization (68.7%) and that such a high percentage of mineral content is largely preserved during the healing period [25]. Hence, it seems reasonable to believe that grafting of the anterior maxilla by autogenous block grafts from the retromolar area improves bone density of future implant sites.

Furthermore, although there was a slight decrease in ISQ values over the first three weeks of healing, the stability of implants was still high enough not to affect the process of osseointegration, which is demonstrated by the fact that no implants were lost during the follow-up period. Several studies reported 100% survival rate of immediately loaded single implants in anterior maxilla [26–30]. The results of our study indicate that the same high survival rates might be obtained in both grafted and native sites. From this study it seems that bone augmentation of the anterior maxilla using bone blocks from the mandibular ramus is a safe and predictable procedure, with low complications rates. Although resorption of grafts unprotected by barrier membranes might be substantial, the amount of residual graft was large enough to allow optimal implant placement in most of the cases.

Conclusion

Considering the results of this study, it seems that the stability of implants placed into the grafted bone is high enough to allow the immediate loading protocol to be used, not affecting the survival rate of implants during the 3-year follow-up period.

Acknowledgments

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Conflicts of interest

The authors deny any conflicts of interest regarding this study. A set of special ablative and trephine burs for graft harvesting was produced by Hager & Meisinger, GbmH, Germany. RFA measurements were performed using the Osstell mentor™ (Integration Diagnostics AB, Göteberg, Sweden). All devices and instruments were obtained under commercial conditions.

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Prevalence and possible predictors of the occurence of denture stomatitis in patients older than 60 years

Prevalencija i mogući prediktori nastanka protetskog stomatitisa kod pacijenata starijih od 60 godina

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Abstract

Background/Aim. Denture stomatitis (DS) is one of the most common oral health problems among elderly population with removable dentures. Despite the high prevalence, etiology of the disease is not completely understood. It appears to be multifactorial, with a predominance of local factors. The aim of the study was to determine the prevalence and risk factors that contribute to the development of DS in upper removable denture wearers. **Methods**. This clinical study comprised three groups of subjects with upper removable dentures: the DS group, and the positive and negative control groups. Swab samples were taken from the tongue and palatal mucosa for microbiological examination. Data of denture age, dentures night wearing, unstimulated salivary flow rate (USFR) and saliva pH values were evaluated for all the participants. **Results**. The prevalence of DS was found to be 26.5%. Significantly higher values of overnight wearing (p = 0.000) and the mean age of dentures (p = 0.022) were found in the DS group compared to the controls. In relation to the positive mycological finding, a borderline significance difference among the groups was confirmed (p = 0.053). No significant association was found between DS and gender, age, the type of dentures, USFR, pH of saliva and bacteria findings. The patients who wore dentures at night had 26 times more chances to get DS compared to the patients who did not wear them overnight. **Conclusion**. This study confirms similar characteristics of DS prevalence in elderly population of Vojvodina compared to European. Continuous (overnight) wearing of dentures is considered to be major direct risk factor for DS development, while secondary role is attributed to denture age and oral *Candida* infection.

Key words:
dentures; stomatitis, denture; prevalence; candidiasis, oral; oral hygiene; preventive dentistry.

Apstrakt

Uvod/Cilj. Protetski stomatitis (PS) jedno je od najčešćih oboljenja usne duplje kod starijih osoba sa mobilnim protezama. Uprkos velikoj rasprostranjenosti, etiologija oboljenja nije u potpunosti razjašnjena. Ona je verovatno multifaktorijalna, sa predominacijom lokalnih faktora. Cilj rada bio je da se utvrde prevalencija i faktori rizika koji doprinose nastanku PS kod nosilaca gornjih mobilnih proteza. **Metode**. Studija je obuhvatila tri grupe ispitanika sa gornjim mobilnim protezama: grupu sa PS, te pozitivnu i negativnu kontrolnu grupu. Brisevi jezika i nepca uzeti su za mikrobiološko ispitivanje. Za sve ispitanike utvrđivani su podaci o starosti proteza, noćnom nošenju proteza, sijalometriji i pH vrednosti pljuvačke. **Rezultati**. Prevalencija PS iznosila je 26,5%. Utvrđena je statistički značajna razlika između grupe sa PS i kontrolnih grupa u odnosu na noćno nošenje proteza (p = 0,000) i prosečne starosti proteza (p = 0,022). Pozitivan mikološki nalaz u granicama statističke značajnosti (p = 0,053) utvrđen je kod pacijenata sa PS. Nije utvrđena značajna povezanost između protetskog stomatitisa i pola, uzrasta, tipa proteza, sijalometrijskog nalaza, pH vrednosti pljuvačke i bakteriološkog nalaza. Pacijenti koji nose proteze noću imali su 26 puta veću šansu da obole od PS u poređenju sa pacijentima koji ih skidaju preko noći. **Zaključak**. Ova studija je potvrdila slične karakteristike prevalencije PS između populacije starih osoba Vojvodine i Evrope. Kontinuirano (noćno) nošenje proteze smatra se glavnim direktnim faktorom rizika od nastanka PS, dok se favorizujuća uloga pripisuje starosti proteze i oralnoj kandidijazi.

Ključne reči:
zubna proteza; stomatitis; prevalenca; kandidijaza, oralna; usta, higijena; stomatologija, preventivna.

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Introduction

The most common oral health problem among the elderly population in Vojvodina is tooth loss and dentures wearing. Denture stomatitis (DS) is the clinical diagnosis of the disease that occurs in adults with removable dentures. Edema and inflammation of the mucosa covered by denture base are objective signs of the disease [1, 2]. Subjective symptoms as pain, itching and burning sensation are described, but in most patients with DS are asymptomatic [3]. Systematic review of numerous observational and experimental studies analyzing an association between mucosal lesions and wearing of removable dentures has shown that the DS prevalence ranges from 1.1% to over 36.7% [4]. According to Gendreau and Loewy [5], denture-related mucosal lesions are present in 15–75% of edentulous patients, mostly female. Moreover, some studies suggest that DS is present in two thirds of the patients using removable dentures [6]. Regardless of the large number of studies published there are controversial conclusions in relation to DS prevalence, mainly due to heterogeneity and variations in research methodology [1, 7, 8].

Despite the prevalence, the etiology of the disease is not completely understood. It appears to be multifactorial, with the predominance of local factors. Colonization of *Candida* genus yeasts, bacteria, denture trauma, poor oral and denture hygiene, continual and night-time wearing of removable dentures, denture age, reduced salivation, low pH values of mucosa and saliva are described as possible factors that contribute to DS occurrence [5, 9–12]. In most of the above mentioned studies, the differences between the groups with and those without DS were analyzed with univariate tests. Additionally, risk factors for DS should be studied with multivariable statistical techniques because most of the factors are interrelated [13].

There is a lack of evidence concerning the DS prevalence among elderly population in Vojvodina. Also, it remains unclear what are the major risk factors for denture-related mucosal lesions. The aim of this study was to determine the prevalence and risk factors for the development of DS in upper removable denture wearers.

Methods

This prospective clinical study included all the patients with upper removable dentures (complete dentures, acrylic partial dentures, cast partial dentures), treated at the Department of Oral Medicine of the Clinic for Dentistry of Vojvodina, Faculty of Medicine, Novi Sad, Serbia in the period from March 2010 to January 2014. The sample comprised 159 patients (132 females and 27 males, ranged 60–85 years, average age being 65.84 years) and 30 denture patients (25 females and 5 males of average age of 65.30 years, ranged 60–76 years) who were visiting the Clinic for Dentistry for regular check-ups. Exclusion criteria from the study were: relatively new dentures (aged less than one year), uncompleted medical documentation, oral mucosa lesions (except DS) and the presence of systemic disease (immune, hematologic, neoplastic, infectious and endocrine).

Based on the above mentioned criteria 39 patients were excluded from the study and three groups of patients created: the DS group, and the positive and negative control. The DS group comprised 50 patients following the diagnosis made by the oral medicine specialist according to the Newton classification [3]. Lesions were classified as DS if there were visible inflammatory changes under the denture and discomfort. The positive control group comprised 70 patients with removable dentures which were treated at the Department of Oral Medicine because of subjective symptoms (*glossodynia*, *glossopyrosis* and *dysgensia*) without the presence of oral lesions. Thirty patients with no subjective symptoms and oral mucosa lesions participated as the negative control group. The study was approved by the Local Ethical Committee of the Dental Clinic in Novi Sad. Each patient signed a written informed consent form prior to enrolment in the study.

Age, gender, prosthetic restoration information (prosthesis type, denture age, previous prosthesis, dentures night wearing), regular intake of medications, unstimulated salivary flow rate (USFR), saliva pH values and microbiological findings were collected in a questionnaire structured for the purposes of this study.

Swab samples from the tongue and palate (each lasting 10–15 sec) for mycological and bacteriological examination were taken in all the groups. All samples were taken in the morning (from 8 a.m. to 10 a.m.), and reached the laboratory within 2 h. All swab samples were taken by one investigator. Swab samples were plated on Sabouraud dextrose agar and incubated under aerobic conditions at 37°C for 48 h for yeasts. Colonies were subcultured and their identification included germ tube formation in bovine serum, chlamydospore formation in corn meal agar, ability to grow at 45°C and assimilation test (API 20C Aux, bioMerieux, Marcy l` Etoile, France). Bacteria inoculation was performed on blood agar, MacConkey's agar, and tioglikol media with dextrose (Difco, Detroit, MI, USA) in aerobic conditions at 37°C for 24/48 h. Inoculated culture media was observed after 48 h in order to detect the presence of clinically significant species of bacteria and yeasts in the examined material. Microbiological finding from the tongue or palatal swab sample for each patient was considered positive if yeasts or bacteria growths were detected with more than 5 colonies.

Whole saliva specimens were collected between 8 a.m. and 10 a.m. after swabs were taken. The patients were instructed not to eat, drink, smoke and perform oral hygiene at least 2 h before saliva collection. The patients were comfortably seated and trained to avoid swallowing saliva and asked to lean forward and spit all the saliva they made for 10 min into a sterile graduated glass tube through a glass funnel. Only the liquid component (not the foam) of saliva was measured. Saliva volume and the collection time were used to calculate USFR. The normal value of secreted unstimulated saliva was > 0.1 mL/min.

Saliva pH values were determined by using the standard pH paper indicator with the sensitivity of 0.5 (Neutralit, Merck, Darmstadt, Germany). Strips of paper indicator were placed into the glass tube with previously collected saliva. After 15 sec the values of pH were determined by comparing

the change in color of the paper strips in relation to the attached scale. The pH values from 6.5 to 7.0 were considered normal. According to the measured saliva pH value, the patients were classified into two groups: the group with acid pH value (pH < 6.5) and the group with normal and alkaline pH value (pH ≥ 6.5).

Data were described as frequency distribution, mean ± standard deviation (SD). One-way Anova for continuous variables and χ^2 test for attributive characteristics were applied for testing the differences between the groups. When normal distribution of numeric features was absent, Kruskal-Wallis test was used. If univariate analyses showed a *p*-value < 0.05 for the group difference, the variable was selected for multivariable logistic regression. Direct logistic regression was used to calculate odds ratios (OR) with 95% confidence intervals (CI). Statistical analysis was performed using SPSS, version 17 (WinWrap Basic, Nikiski, AK, USA).

Results

Among a total of 189 participants evaluated, DS was confirmed in 26.5%. The prevalence of DS associated with upper removable dentures was analyzed according to age, gender, the type of dentures and night wearing of dentures. In relation to age, the mean value of 65.14 ± 5.19 years was determinated in the DS group (Table 1).

The study included 150 subjects (24 male and 126 female), the average age 65.66 ± 4.8 years (range 60–85 years). In relation to the type of denture, a total of 99 (66%) were using complete dentures, 35 (23.3%) partial acrylic dentures and 16 (10.7%) cast partial dentures. According to the Newton classification, in the DS group localized slight hyperemia was diagnosed in 24 (48%) of the patients, diffuse hyperemia in 24 (24%) subjects, while 14 (28%) patients had papillary hyperplasia.

Significantly higher values of overnight wearing (p = 0.000) and the mean age of dentures (p = 0.022) were present in the group with DS compared to the control groups. In relation to the night wearing, 57 (81.4%) of the patients from the positive control group and 11 (36.7%) from the negative control group did not wear dentures at night, while in the patients with palatal lesions this factor was present in 41 subjects (82%).

The average age of dentures in the positive and negative control groups was 6.87 and 7.5 years, respectively and 9.58 years in the DS group. A positive microbiological finding on yeasts was more often confirmed in patients with DS than in the control groups. However, the significance of this difference was at borderline (p = 0.053). Only the mean rank saliva pH value in the negative controls showed significant differences compared with the mean rank saliva pH value in the DS and the positive control groups (p = 0.041). No signifi-

Table 1

Univariate tests of potential risk factors among the groups of patients

Risk factors	Positive control (n = 70)	DS group (n = 50)	Negative control (n = 30)	*p*-value
Age (years), $\bar{x}$ ± SD	66.19 ± 10.11	65.14 ± 10.19	65.30 ± 8.72	0.05[3]
Medications intake, n (%)	51 (73.3)	41 (81.4)	21 (70.0)	0.05[2]
USFR (mL/min), $\bar{x}$ ± SD	0.26 ± 0.23	0.26 ± 0.15	0.27 ± 0.13	0.05[1]
≤ 0.1, n (%)	3 (3.4)	1 (2)	0 (0%)	
0.11–0.3, n (%)	54 (77.1)	37 (74)	26 (86.7)	0.05[2]
> 0.31, n (%)	13 (18.6)	12 (24)	4 (13.3)	
Saliva (pH), $\bar{x}$ ± SD	6.34 ± 0.68	6.34 ± 0.65	6.70 ± 0.65	0.05[1]
< 6.5, n (%)	39 (55.7)	27 (54)	12 (40)	0.05[2]
≥ 6.5, n (%)	31 (44.3)	23(46)	18 (60)	
Denture age (years), $\bar{x}$ ± SD	6.87 ± 7.27	9.58 ± 7.27	7.50 ± 6.76	0.05[1]
Gender , n (%)				
male	10 (14.3)	9 (18)	5 (16.7)	
female	60 (85.7)	41 (82)	25 (83.3)	0.05[2]
Type of denture, n (%)				
complete	47 (67.2)	34 (68)	18 (60)	0.05[2]
partial acrylatic	16 (22.8)	11 (22)	8 (26.7)	
cast partial	7 (10)	5 (10)	4 (13.3)	
Overnight wearing of dentures, n (%)				
no	57 (81.4)	9 (18%)	11 (36.7)	0.05[2]
yes	13 (18.6)	41(82%)	19 (63.3)	
Yeasts swab, n (%)				
positive	33 (47.2)	32 (64%)	12 (40)	
negative	37 (52.8)	18 (36%)	18 (60)	0.05[2]
Bacteria swab, n (%)				
positive	16 (22.8)	8 (16%)	3 (10)	
negative	54 (77.2)	42 (84%)	27 (90)	0.05[2]

USFR – Unstimulated saliva flow rate; [1] Kruskal Wallis test; [2] χ^2-test; [3] Anova test; $\bar{x}$ – mean values; SD – standard deviation.

cant differences were found between the groups in relation to gender, age of patients, medications intake, USFR mean and range values, type of dentures, as well as of microbiological bacteria findings (Table 1).

The results of univariate analysis presented in Table 1, indicate that overnight wearing, age of the dentures, positive oral swab for *Candida* and acid pH value of saliva were significantly associated with the occurence of DS.

However, the results of logistic regression analysis indicate that only night wearing of dentures (OR = 26.16) was significantly associated with DS. The coefficient of determination from 0.495 showed a relatively good explanation of the dependent variable (DS) over the other independent variables. The *p*-value of Hosmer Lemeshow test showed that the model described well the original data (0.768 > 0.05). The overall success of prediction was 82.5%. According to the Wald criterion, night wearing of dentures significantly contributed to the prediction of disease ($p < 0.01$). Assuming that other variables remained unchanged, the ratio of the probability, which was 26.16, showed that the patients who wore dentures at night had 26 times more chances to manifest DS compared to the patients who removed them overnight (Table 2).

Disscusion

The etiology of DS is multifactorial. Factors associated with the development of DS are: local and systemic [2]. Pressure caused by dentures, continual wearing, reduced salivation, poor denture hygiene, type of dentures material and denture age are described as important factors causing the disease [5]. In relation to systemic impact of DS, diabetes mellitus, smoking, long-term use of antibiotics and corticosteroids, radio(chemo)therapy, nutritional factors – lack of vitamin B12, folate and iron are considered as most common factors for inducing DS [14].

Our results confirmed that the prevalence of DS in Vojvodina was 26.5%. Previous epidemiological studies showed that the prevalence of DS in patients who wore removable dentures ranged from 15% to 75% [5]. Furthermore, data obtained from studies in South America [15] and Finland [16] were similar to the results of our study. The outcome prevalence is significantly affected by the size of the study. In studies that included 100 or less subjects the prevalence ranged from 45% to 77.5% [17], while in those with 200 or more, outcome prevalence varied between 17% and 55,5% [13]. More than 80% of our sample consisted of older female subjects; in previous studies [8, 18] it was shown that the prevalence of DS was higher in older female adults. However, in our study, no statistically significant difference between the genders was noticed.

Our results indicate that continuous (overnight) dentures wearing are major predictor for DS occurrence. Kossioni [11] and Jeganathan et al. [19] also came to that conclusion. Furthermore, Barbeau et al. [9] found that night wearing is considerably more present in subjects with extended inflammation compared to those with restricted inflammation. In our study, the overnight or continuous denture wearing was observed in more than 80% of the patients with DS. The presence of dentures certainly caused changes in microenvironment between palatal mucosa and denture base, but continuous wearing further favored the development of DS.

The negative impact of night wearing was pronounced due to the absence of mechanical effects of saliva and its antimicrobial compounds (lysozyme, lactoferrin, salivary peroxidase, immunoglobulin A), which are its integral part [20]. It should be noted that reduced flow of saliva in the space between denture base and palatal mucosa, which is caused by good sealing of denture, possibly leads to dynamic changes in pH values and mechanical cleaning capability.

Kossioni [11] points out the relationship between the increased age of the current denture and pinpoint hyperemia.

Table 2

Multivariable logistic regression of potential risk factors

Risk factors	B	S.E.	Wald	df	*p*	EXP (B)	95% CI for EXP (B)	
							lower	upper
Age	0.018	0.025	0.530	1	0.466	1.018	0.970	1.069
Medications intake	0.618	0.665	0.832	1	0.655	1.754	0.358	2.554
USFR	-1.452	1.361	1.139	1	0.286	0.234	0.016	3.370
Saliva pH	0.317	0.408	0.605	1	0.437	1.373	0.617	3.054
Denture age	0.013	0.034	0.137	1	0.711	1.013	0.947	1.083
Overnight wearing	**3.264**	**0.571**	**32.633**	**1**	**0.000**	**26.161**	**8.536**	**80.177**
Yeasts swab	0.241	0.544	0.196	1	0.658	1.272	0.438	3.697
Bacteria swab	-0.273	0.644	0.180	1	0.672	0.761	0.216	2.689
Gender	0.627	0.689	0.828	1	0.363	1.872	0.485	7.226
Complete dentures	-0.280	0.887	0.099	1	0.753	0.756	0.133	4.304
Partial dentures	-0.224	0.980	0.052	1	0.820	0.800	0.117	5.460
Constant	-5,243	3.473	2.279	1	0.131	0.005		

USFR – Unstimulated saliva flow rate; *p* – significance; EXP (B) – Odd ratio; CI – confidence interval.

The age of dentures plays an important role for the occurrence of the disease; thus, in long-term wearing, maintenance of hygiene is difficult and there is tendency towards porosity of denture base. Roughness of denture surface contributes to the increased adhesion of microorganisms and reduced removing of denture plaque after a thorough cleaning [21]. Such a denture is considered as inappropriate and could lead to mucosal trauma and inflammation. In our study, the average age of dentures among the patients with palatal inflammation was significantly higher than that in the control groups. However, observing the influence of all risk factors, denture age did not substantially contribute to the prediction of the disease.

The salivary flow rate and saliva pH values showed no significant influence as DS development factor in this study, although hyposalivation (≤ 0.1 mL/min) was registered in 2% of DS patients. In contrast to that, Sakki et al. [10] suggest that reduced production of saliva is one of the DS risk factors. Olsen and Birkeland [22] point out the difference between the groups with and without DS in relation to the pH values of denture biofilms. However, the same authors did not confirm positive correlation between increased number of yeasts and acidity [23]. It should be noted that pH values in those studies were measured on denture biofilm using microelectrodes, and the yeasts were isolated from denture biofilm.

The prevalence of *Candida* genus yeasts in clinically normal mouths of healthy patients ranges from 25% to 50%. The increased number, up to 88%, is reported in denture wearers. Nevertheless, yeasts are also found in 52% among subjects without removable dentures [24]. It is well-known that dentures itself are most important predisposing factors in the development of oral candidiasis [7, 25]. *Candida* has been reported frequently by most of authors as a most common causative agent of DS [5, 13, 26, 27]. Despite this fact, we did not find *Candida* growth as a major risk factor in the occurrence of DS, which is also confirmed in our previous study [28]. Although the prevalence of positive *Candida* swab findings in subjects with DS showed borderline significant difference compared to the groups, logistic regression results did not extricate *Candida* as a meaningful predictor. According to some authors, the high prevalence of *Candida* oral colonization is explained by the fact that old age is associated with the development of systemic disease, changes in eating habits and hygiene, as well as the composition of the saliva [20, 29]. In some previous studies [26, 27, 30] colonization of *Candida* has been observed, but we evaluated the presence of oral *Candida* infection only at the current site (palatal lesion and dorsal surface of the tongue). Etiological factors, such as poor denture hygiene, continual and nighttime wearing of removable dentures, accumulation of denture plaque and poor-fitting dentures appear to increase the ability of *Candida albicans* to colonize both the denture and oral mucosal surfaces, where it acts as an opportunistic pathogen [5].

Several previous studies have pointed out the pronounced impact of poor denture hygiene in the development of palatal inflammation [7, 8, 12]. Even though we did not analyze this factor in the present study, our previous results emphasized the importance of maintaining proper denture hygiene [28].

Conclusion

The present study shows similar characteristics of DS prevalence between the population of Vojvodina and Europe. Continuous (overnight) wearing of dentures is considered to be the major direct factor in DS development, while secondary role is attributed to denture age and oral *Candida* infection. Local factors as gender, age, type of dentures, USFR, the *p*H of saliva, and microbiological findings show no significant influence. Nowadays, it seems that dentists insufficiently participate in raising patients' awareness of DS prevention. Patients should be educated that removing dentures at night, replacing worn-out dentures after 5 years, as well as periodical visiting dentist are essential in prevention of oral mucosa lesions and DS.

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The influence of hepatic steatosis on the success of antiviral therapy for chronic hepatitis C

Uticaj steatoze jetre na uspeh antivirusne terapije hroničnog hepatitisa C

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Abstract

Background/Aim. Chronic hepatitis C and liver steatosis often appear simultaneously in the same person, and steatosis can lead to worsening of liver disease and reducing the success of the treatment of chronic hepatitis C. Treatment of one disease can influence and cause favorable impact on treatment of other diseases. The aim of this study was to determine the incidence of liver steatosis in patients with chronic hepatitis C and to examine the impact of steatosis of the liver and other predictors on the success of antiviral therapy for chronic hepatitis C. **Methods.** The study included 123 patients with chronic hepatitis C treated with pegylated interferon alfa 2a in combination with ribavirin. The patients were divided into two groups based on the presence of cirrhosis: the group I consisted of 43 (34.9%) patients with steatosis and the group II consisted of 80 (65.1%) patients without liver steatosis. The success of the treatment was evaluated on the basis of the stable virological response. **Results.** The presence of steatosis was determined in 34.96% of the patients. The overall success of antiviral therapy was found in 74.79% of the patients. The success of antiviral therapy was present in 62.79% of the patients with hepatic steatosis, and in 81.25% the patients without steatosis ($p < 0.05$). The success of antiviral treatments was seen in 80.95% of the patients with hepatic steatosis and the genotype of hepatitis C virus 3. The predictors of antiviral therapy success for chronic hepatitis C in our study were patient's age, duration of infection, genotype 3, steatosis and severe fibrosis or cirrhosis. **Conclusion.** Liver steatosis is often present in patients with chronic hepatitis C. It has negative impact on the efficacy of antiviral therapy in patients with infection with genotype non-3 hepatitis C virus. Therefore, hepatic steatosis in these patients must be eliminated or treated prior to application of antiviral therapy.

Key words:
hepatitis c; fatty liver; genotype; age factors; interferon-alpha; ribavirin; treatment outcome.

Apstrakt

Uvod/Cilj. Hronični hepatitis C i steatoza jetre se često javljaju udruženo kod iste osobe, a steatoza jetre može dovesti do pogoršanja bolesti jetre i može smanjiti uspeh lečenja hroničnog hepatitisa C. Lečenjem jedne bolesti povoljno se utiče na ishod lečenja druge bolesti. Cilj ovog rada bio je da se utvrdi učestalost steatoze jetre kod bolesnika sa hroničnim hepatitisom C i da se ispita uticaj steatoze jetre i drugih prediktora na uspeh antivirusne terapije hroničnog hepatitisa C. **Metode.** U studiju su bila uključena 123 bolesnika sa hroničnim hepatitisom C, koji su lečeni pegilovanim interferonom alfa 2a u kombinaciji sa ribavirinom. Bolesnici su na osnovu prisustva steatoze jetre podeljeni u dve grupe, grupu I sa 43 bolesnika i steatozom jetre i grupu II sa 80 bolesnika bez steatoze jetre. Uspeh lečenja ocenili smo na osnovu postignutog stabilnog virusološkog odgovora. **Rezultati.** Postojanje steatoze utvrđeno je kod 34,96% bolesnika. Ukupan uspeh antivirusne terapije postignut je kod 74,79% bolesnika. Uspeh antivirusne terapije zabeležen je kod 62,79% bolesnika sa steatozom jetre i kod 81,25% bolesnika bez steatoze ($p < 0,05$). Uspeh antivirusnog lečenja postignut je kod 80,95% bolesnika sa steatozom jetre i genotipom 3 virusa hepatitisa C. Prediktori uspešnosti antivirusne terapije hroničnog hepatitisa C u našem radu bili su starost bolesnika, dužina trajanja infekcije, genotip 3, steatoza i izražena fibroza jetre. **Zaključak.** Steatoza jetre često je prisutna kod bolesnika sa hroničnim hepatitisom C i ima negativan uticaj na efikasnost antivirusne terapije kod bolesnika sa infekcijom genotipom non-3 hepatitis C virusa. Zbog toga se steatoza jetre kod ovih bolesnika mora eliminisati ili lečiti pre primene antivirusne terapije.

Ključne reči:
hepatitis c; jetra, masna infiltracija; genotip; životno doba, faktor; interferon-alfa; ribavirin; lečenje, ishod.

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Introduction

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide. The third of those who become chronically infected are predicted to develop liver cirrhosis or hepatocellular carcinoma. According to recent estimates, more than 185 million people around the world have been infected with HCV, of whom 350,000 die each year [1]. Despite of the high prevalence of this disease, most people infected with the virus are unaware of their infection. Furthermore, the HCV prevalence across Europe ranges between 0.4% and 3.5%, with a wide geographical variation and higher rates in the south and the east [2, 3].There are six genotypes of HCV having epidemiological and clinical significance. Genotype 1 dominates in European countries and also in Serbia, genotype 3 is more common among injecting drug users, and has a direct steatogenic effect on the liver [4, 5].

During the last decade, the standard of care (SOC) for chronic HCV patients consisted of pegylated interferon-alfa (PEGIFN) 2a or 2b combined with ribavirin (RBV). In patients with HCV genotype 3, the combination therapy with PEGIFN plus RBV is usually given during 24 weeks, achieving rates of stable virological response (SVR) of about 75–85%. In patients with HCV genotype 1 antiviral therapy is usually given for 48 weeks, resulting in SVR of 40–50% [6]. Recently approved boceprevir and telaprevir used in combination with PEGIFN plus RBV and polymerase inhibitors such as nucleos(t)ide inhibitors (NIs) and non-nucleoside inhibitors (NNIs) substantially improve the SVR rates in both treatment-naive and treatment experienced genotype 1 patients, but they are not available in our country. Due to the lack of efficacy and frequent side effects, the predictors of favorable virological response are studied in order to improve antiviral therapy for selected patients who are most likely to achieve SVR and healing. The predictors of favorable virological response are: white race, younger patients (< 40 years), female gender, body mass index (BMI) ≤ 25 kg/m^2, the absence of cirrhosis and severe fibrosis (F 3–4), the absence of insulin resistance and diabetes, the absence of steatosis, the absence of comorbidities such as HIV or hepatitis B coinfection, excess alcohol intake, HCV genotype 2 and 3, low HCV baseline viremia (< 600,000–800,000 IU/mL) and host interleukin 28B gene polymorphisms [7, 8].

Chronic hepatitis C and liver steatosis often appear simultaneously in the same person, and the prevalence of steatosis in chronic hepatitis C is 2–3 times higher than the prevalence of steatosis in other chronic liver diseases, ranging 40–80%, depending on the research and applied criteria [9, 10]. It has been proven that genotype 3 has a direct steatogenic effect on hepatocytes, and in patients with chronic hepatitis caused by HCV genotype 3, the prevalence of steatosis is 70–80% [11, 12]. Steatosis represents the accumulation of fatty particles, mainly of triglycerides in hepatocytes, the limit is usually set at 5% of hepatocytes affected by fatty change, < 5% is considered to be the normal state, > 5% is considered as steatosis [13]. The clinical significance of liver steatosis is reflected on faster progression to fibrosis in patients with chronic HCV infection, less success of antiviral therapy and risk for developing hepatocellular carcinoma [14–16].

Our hypothesis was that liver steatosis often occurs in patients with hepatitis C and reduces the success of antiviral therapy. The aim of this study was to determine the incidence of liver steatosis in patients with chronic hepatitis C, and the impact of steatosis and other predictors on the success of antiviral therapy.

Methods

The study included 123 patients with chronic hepatitis C, treated in the Clinic for Infectious Diseases, Clinical Center of Vojvodina in Novi Sad, Serbia, during the period from January 2010 to June 2012. All the subjects were "naive patients", not treated with antiviral therapy before. The patients were divided into two groups based on the presence of steatosis of the liver: the group I of 43 patients with hepatic steatosis and the group II of 80 patients without steatosis. The presence of steatosis was determined by histopathological examination of liver biopsy, on the basis of > 5% of hepatocytes affected by fatty changes. Histopathological examination of liver biopsy was performed in the Centre for Pathology and Histology, Clinical Center in Novi Sad, Serbia according to the Metavir score. From the research were excluded: obese patients (BMI $\geq$ 30 kg/m^2), patients who used drugs that could lead to cirrhosis and people with excessive use of alcohol (alcohol consumption of more than 40 mg *per* day for men and more than 20 mg *per* day for women six months before treatment).

All the patients were treated with combined therapy, PEGIFN alfa 2a or alfa 2b and ribavirin in standard doses. The duration of treatment depended on the genotype of hepatitis C virus, genotype 2 and 3 HCV treatment lasted 24 weeks, and for genotype 1 and 4 HCV 48 weeks. Antiviral therapy is considered successful when a sustained viral response, based on the absence of HCV RNA in serum of patients 6 months after completion of antiviral treatment, using the polymerase chain reaction (PCR) method was achived. PCR testing and genotyping was conducted at the Virology Laboratory of the Institute for Infectious and Tropical Diseases, Clinical Center of Serbia, Belgrade, with Cobas Amplicor HCV Test version 2.0 (Roche Diagnostics, Menheim), sensitivity of 50 U/mL, before treatment, during treatment and six months following the completion of treatment.

Statistical analysis was performed with the statistical package SPSS version 13.0. The descriptive statistical parameters are shown in standard statistical variables, arithmetic mean ($\bar{x}$), standard deviation (SD), interval values (maximum and minimum). Tests of statistical significance were determined by ANOVA parametric data (analysis of variance), and the non-parametric Fisher's or Mann Whitney's test. A statistically significant value was set at $p < 0.05$. In analyzing the impact of risk factors on the success of the therapy multivariate logistic regression analysis was used.

Results

From a total of 123 patients the majority (89) of the patients were male (72.36%), the average age of patients was 36.05 (SD ± 11.12) years. In the overall sample 92 (74.79%) patients were younger than 40 years. The average BMI for all the patients was 24.37 ± 2.44 kg/m^2. The most common route of transmission of HCV infection was through intravenous drug use, in 81 (65.9%) patients. The average duration of HCV infection was determined in 106 of the patients with the known route of HCV transmission, which was 11 (11.96 ± 9.36 years). Alanine aminotransferase (ALT) was elevated in 113 (91.86%) of the patients, with the average value of 106.80 ± 74.112 U/L [normal range (nr) 5–48 U/L]. Aspartate aminotransferase (AST) was elevated in 78 (63.41%) of the patients, with the average value of 57.76 ± 36.706 U/L (nr 5–37 U/L). Gamma glutamiltransferase (GGT) was elevated in 61 (50.45%) of the patients, with the average value of 71.36 ± 49.900 U/L (nr 1–64 U/L). The value of total bilirubin was normal in all the subjects and all the patients had normal albumin. They were mostly HCV genotype 1, found in 75 (60.98%) of the patients, 41 (33.33%) of the patients had genotype 3, genotype 4 was found in 5 (4.06%) of the patients and genotype 2 in two (1.63%) of the patients. There were no patients simultaneously infected with two or more genotypes of HCV. The average value of HCV RNA viral load before treatment was 3,911578,888 ± 7,869,037,777 U/mL. The vast majority of the patients,107 from 123 (86.99%), had a HCV viral load greater than 800,000 U/mL. Histopathological examination of liver biopsy found that 28 (22.76%) of the patients were without liver fibrosis (F0), with mild fibrosis (F1) were 45 (36.58%) of the patients, with moderate fibrosis (F2) were 34 (27.64%) of the patients, with severe fibrosis (F3) were 15 (12.19%) of the patients and with liver cirrhosis (F4) was one (0.81%) of the patient. In relation to the existence of mild/moderate (F0-F2) and severe fibrosis or cirrhosis (F3-F4), the majority of the patients, a total of 79 (64.22%) had no or mild to moderate fibrosis (F0-2). From a total of 123 patients included in the study it was found that 43 (34.96%) of the patients had hepatic steatosis, while 80 (65.04%) of the patients had no steatosis. Demographic, clinical and virological characteristics of the patients are shown in Table 1.

The success of antiviral therapy combination (PEGIFN+RBV) measured by the achievement of SVR was found in 92 (74.79%) of the patients. From the usual predictive factors for achieving SVR, gender of patients did not show statistically significant (Fisher test, $p = 0.292$). SVR was achieved in 69 (77.52%) of the men and 23 (64.70%) of the women. The patients who achieved SVR were significantly younger mean age 33.41 ± 10.48 years compared to those who did not achieve SVR mean 46.17 ± 12.20 years (*t*-test, $p = 0.001$). The patients who achieved SVR had a slightly lower BMI (mean 24.17 ± 2.48 kg/m^2), compared to BMI in those who did not achieve SVR (mean 25.16 ± 2.16 kg/m^2) but this difference was not statistically significant (*t*-test, $p = 0.092$). The average duration of HCV infection in the patients who achieved SVR was 9.9 ± 8.75 years, which was significantly shorter compared to the duration of infection in the patients who did not achieve SVR and it was 16.87 ± 12.79 years (*t*-test, $p = 0.004$). None of biochemical parameters monitored as potential predictors of achieving SVR, was statistically significant.

Table 1

Baseline characteristics of the patients with chronic hepatitis

Characteristics	Patients (n = 123)
Gender (male/female), n (%)	89/34 (72.36/27.64)
Age (years), $\bar{x}$ ± SD	36.05 ± 11.12
BMI(kg/m^2), $\bar{x}$ ± SD	24.37 ± 2.44
IVDUs, n (%)	81 (65.9)
Duration of HCV infection (years), $\bar{x}$ ± SD	11.96 ± 9.36
Glucose (mmol/L), $\bar{x}$ ± SD	5.05 ± 1.01
Cholesterol (mmol/L), $\bar{x}$ ± SD	4.82 ± 0.964
Triglycerides (mmol/L), $\bar{x}$ ± SD	1.32 ± 0.641
ALT (U/L), $\bar{x}$ ± SD	106.80 ± 74.112
AST (U/L), $\bar{x}$ ± SD	57.76 ± 36.706
GGT (U/L), $\bar{x}$ ± SD	71.36 ± 49.900
Alpha-fetoprotein (U/L), $\bar{x}$ ± SD	3.80 ± 4.932
HCV genotype, n (%)	
1	75 (60.98)
2	2 (1.63)
3	41 (33.33)
4	5 (4.06)
Viral load (×10^6 U/mL), $\bar{x}$ ± SD	3.9 ± 7.8
Liver fibrosis, n (%)	
F0-2	79 (64.22)
F3-4	44 (35.78)
Steatosis, n (%)	43 (34.96)

BMI – body mass index; IVDUs – intravenous drug users; HCV – hepatitis C virus; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGT – gamma glutamyltransferase; $\bar{x}$ – mean value; SD – standard deviation.

According to HCV genotype, all the patients were divided into two groups, the group I of the patients with genotype 3 and the group II of the patients with genotype non-3 (HCV genotype 1, 2 and 4). SVR in the genotype 3 group was achieved in 36 (87.80%) of the patients, and in the genotype non-3 group in 56 (68.29%) of the patients which was statistically significantly higher SVR in patients with HCV genotype 3 (χ^2 test, p = 0.049). The mean value of HCV viral load in the successfully treated patients was 2.330.839 U/mL, whereas in the patients treated unsuccessfully it was 2.735.340 U/mL, which was not statistically significantly different (p = 0.608). According to liver fibrosis the patients without fibrosis or with mild/moderate fibrosis (F0-2) had SVR in 63 (79.74%) of the cases, the patients with severe fibrosis or cirrhosis (F3-4) in only 17 (38.63%) of the cases. This difference was statistically significant (χ^2 test, p = 0.004). The impact of demographic, biochemical, virological and histopathological factors on SVR are shown in Table 2.

Finally, in the patients with hepatic steatosis SVR was achieved in 27/43 (62.79%) of the patients, whereas in the patients without steatosis SVR was achieved in 65/80 (81.25%) of the patients. The difference in achieving SVR in the patients with steatosis and the group without steatosis was statistically significant (Fisher test, p = 0.042). Analysis of all the predictive factors for achieving SVR (age, duration of infection, genotype 3, the presence of steatosis and fibrosis) observed in the multivariate logistic regression analysis, showed that two factors, namely age of the patients and the presence of fibrosis, significantly contributed to achieving SVR. Considering SVR in 21 patients with steatosis and HCV genotype 3, it was achieved in 17 (80.95%) of the patients, while in 22 patients with genotype non-3 and steatosis it was achieved in 11 (50.00%) of the patients. The SVR according to the presence of steatosis and HCV genotype is shown in Figure 1.

Discussion

Several factors have influence on the prevalence of steatosis: the number of patients infected with HCV genotype 3, which gives a higher incidence of steatosis compared to ot-

Table 2

Characteristics of patients with respect to stable virological response (SVR) rates

Characteristics	SVR (n = 92)	Without SVR (n = 31)	*p*
Gender (male/female), n	69/23	20/11	0.292
Age (years), $\bar{x}$ ± SD	33.41 ± 10.48	46.17 ± 12.20	**0.001**
BMI (kg/m^2), $\bar{x}$ ± SD	24.17 ± 2.48	25.16 ± 2.16	0.092
Duration of HCV infection (years), $\bar{x}$ ± SD	9.9 ± 8.75	16.87 ± 12.79	**0.004**
HCV genotype 3/non3, n (%)	36/56 (87.80/68.29)	5/26 (12.20/31.71)	**0.049**
HCV viral load (×10^6 U/mL)	2.33	273	0.608
Fibrosis (F0-2/F3-4), n (%)	63/17 (79.74/38.63)	16/27 (20.25/61.36)	**0.004**
Steatosis (yes/no), n (%)	27/65 (62.79/81.25)	16/15 (37.21/18.75)	**0.042**

BMI – body mass index; HCV – hepatitis C virus; $\bar{x}$ – mean value; SD – standard deviation.

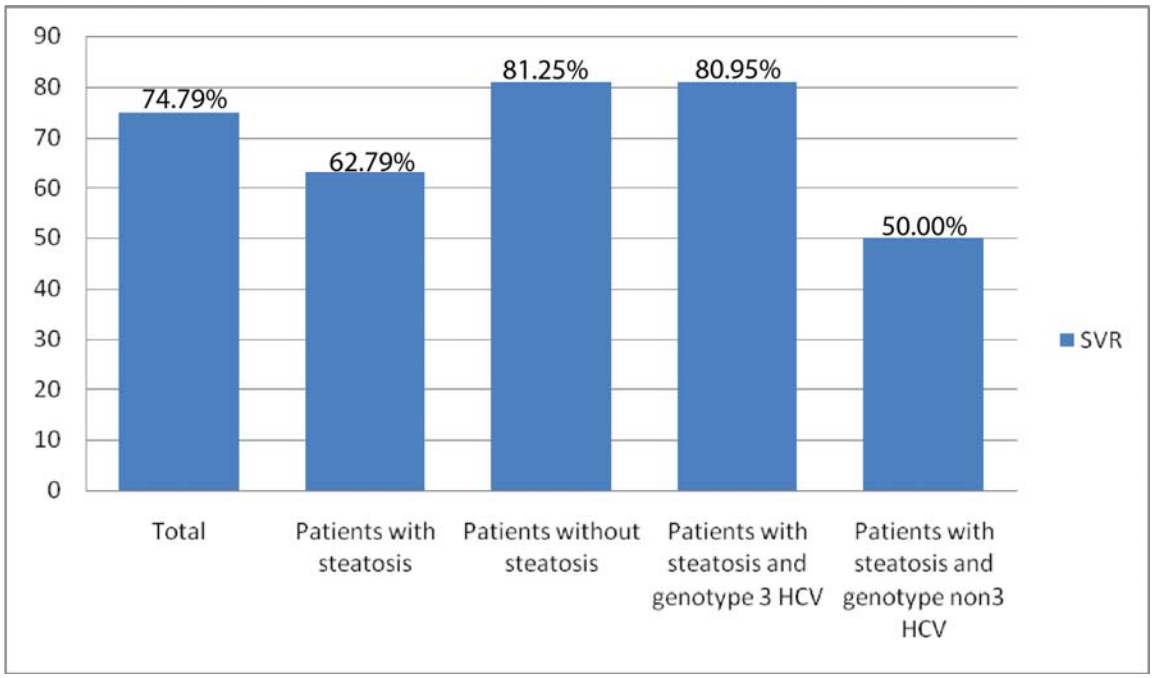

Fig. 1 – Sustained viral response (SVR) according to the presence of steatosis and hepatitis C virus (HCV) genotype.

her genotypes, the number of patients with a higher BMI, or a larger number of obese and overweight patients, patients with diabetes mellitus, patients with metabolic syndrome, and histopathological criteria for the diagnosis of steatosis (> 0% > 1%, or more than 5% of hepatocytes affected by fatty change). In our study, the prevalence of liver steatosis in the patients with chronic HCV infection was 34.96%. A similar prevalence of steatosis in patients with hepatitis C by 31.92% found Savooula et al. [17]). Fierbinteanu-Bratićevici et al. [18], found the prevalence of steatosis as 57%, but they applied lower histopathological criteria for the diagnosis of steatosis (≥ 1% of hepatocytes affected by fatty change) and they did not exclude patients with excessive alcohol consumption, which contributed to the greater prevalence of steatosis.

The overall success of antiviral therapy in the treatment of chronic hepatitis C by achieving SVR in our study was found in 74.79% of the patients. Results of studies with dual antiviral therapy (PEGIFN+RBV) show that treatment success is achieved in about 60% of cases, regardless of HCV genotype, in 50% of patients with HCV genotype 1 infection, and in 80% of patients with HCV genotypes 2 and 3 [19, 20]. This result is consistent with the results of studies in Serbia [21, 22], and better than studies from other countries [19, 23]. Better treatment success could be explained by choosing patients with predictors of good therapeutic response. In our study all the patients were "naive" patients not previously treated with antiviral therapy, the study excluded obese patients and active addicted to psychoactive substances. The predictive factors for achieving SVR in our study were age, duration of infection, HCV genotype 3, the presence of steatosis and the presence of marked fibrosis, as it was found by other authors who studied the predictive factors for successful treatment of patients with chronic hepatitis C [8, 24].

In most of previous studies steatosis is considered as a negative predictive factor of antiviral therapy [25–28]. In recent years, researchers have found that steatosis has a negative impact on the achievement of SVR only in patients with HCV genotype 1, but liver steatosis in patients with genotype 2 or 3 HCV does not have influence on the success of antiviral therapy [29–31], and achieving successful treatment of chronic HCV infection leads to the reduction in liver steatosis [15, 32]. In our study, the treatment success in the patients with hepatic steatosis was achieved in 62.79% of the patients, whereas in the patients without liver steatosis SVR was achieved in 81.25% of the patients, with statistically significant difference. Similar results were obtained Savvoula et al. [17], in patients with chronic hepatitis C and steatosis treatment success of 56.6% *vs* 76.8% in the patients without steatosis Werling et al. [33] applying the same methodology as in our study, also showed that hepatic steatosis has a negative impact on the effectiveness of antiviral therapy, e. g. 36% of patients with steatosis compared to 71% in patients without steatosis. In their study, not all patients were naive, and all had genotype 1 HCV, and this is the reason for the success of the treatment to be worse than in our study. Among the authors showing that steatosis has no negative impact on the success of antiviral therapy are Cross et al. [34], who found that only HCV genotype and obesity have negative influence on the success of antiviral treatment. Today, the prevailing opinion is that hepatic steatosis has a negative impact on the efficacy of antiviral therapy in patients with genotype 1 HCV, while in patients with hepatic steatosis and HCV infection with genotype 3 steatosis has no negative impact on the success of antiviral therapy [31, 35, 36] Negro [37], the author who studied much steatosis in patients with hepatitis C, concludes that viral steatosis induced by genotype 3 HCV does not reduce the success of antiviral therapy, whereas metabolic steatosis caused by other factors (obesity, insulin resistance, diabetes, etc), reduces the success of antiviral therapy for chronic hepatitis C. In our study, concerning the SVR in the patients with steatosis and HCV genotype 3, it was achieved in 80.95% of the patients, which is the same as in all the patients without steatosis (81.25% of the patients), which confirms the opinion that steatosis in patients with HCV genotype 3 has no influence on the success of antiviral therapy.

Conclusion

Hepatic steatosis often occurs in patients with chronic hepatitis C, and has a negative impact on the success of antiviral therapy for chronic hepatitis C. Hepatic steatosis has the strongest negative impact on the success of antiviral therapy in patients with genotype non-3 HCV, whereas in those with genotype 3 steatosis does not have influence on the success of antiviral therapy. Therefore, hepatic steatosis in patients with genotype 1 HCV should be eliminated or treated before switching to antiviral therapy, while in patients with steatosis and genotype 3 HCV antiviral therapy should be applied regardless of the degree of steatosis, because successful antiviral therapy will eliminate or reduce steatosis. Today it is believed that hepatic steatosis has a negative impact on the efficacy of antiviral therapy in patients with genotype 1 HCV, while in patients with hepatic steatosis and HCV infection with genotype 3 steatosis does not have negative impact on the success of antiviral therapy.

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Refractive errors in premature infants with retinopathy of prematurity after anti-vascular endothelial growth factor (anti-VEGF) therapy

Refrakcione greške kod prevremeno rođene dece sa prematurnom retinopatijom nakon terapije antivaskularnim endotelnim faktorom rasta (anti-VEGF)

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Abstract

Background/Aim. Retinopathy of prematurity (ROP) is a vasoproliferative retinopathy which affects the blood vessels of the retina during its development. The aim of this study was to evaluate the incidence and the degree of refractive errors in premature infants with severe ROP treated with anti-vascular endothelial growth factor (anti-VEGF) (bevacizumab). **Methods.** This prospective study included 21 patients (42 eyes) nine months old who received intravitreal injection of anti-VEGF therapy. The control group consisted of 45 patients (90 eyes) who were subjected to laser treatment. In cycloplegia each patient underwent retinoscopy, keratorefractometry, and A-scan ultrasonography. **Results.** Myopia was present in 47.62% of the eyes in the study group and in 33.33% of the eyes in the control group, but there were no statistically significant differences between these groups. Seven (16.67%) eyes in the study group and 17 (18.89%) eyes in the control group were discovered to have high myopia (SE–spherical equivalents < -3.0 D – dioptre). Clinically significant hypermetropia was higher in the study group (47.62%) than in the control group (34.44%), but with no statistically significant difference. In addition, high hypermetropia was significantly greater in the control group (15.56%) than in the study group (11.90%) ($p < 0.001$). Astigmatism was more common in the control group than in the study group (81.11% *vs* 71.43%, respectively), especially high astigmatism (56% *vs* 43%, respectively). Also the more common form of astigmatism was with the rule (WTR) both in the study and the control group (42.86% *vs* 55.56%, respectively). Anisometropia was significantly greater in the control group (24.44%) than in the study group (9.52%) ($p < 0.05$). The children from the study group had significantly greater lens thickness, and a shorter anterior chamber depth than children from the control group ($p < 0.01$). There was no significant difference in the axial length of the eye between the groups. **Conclusion.** At the 9-month follow-up myopia was present in the patients with severe ROP treated with anti-VEGF, but high myopia was present to a lesser degree than in the laser treated patients. This difference is possibly related to anterior segment development. Research into the longer-term refractive outcomes is necessary with observation of the biometric components, visual acuity, and the visual field in order to monitor the real effects of this therapy.

Key words:
retinopathy of prematurity; refraction, ocular; diagnostic techniques and procedures; myopia; astigmatism; vascular endothelial growth factors.

Apstrakt

Uvod/Cilj. Prematurna retinopatija (*retinopathy of prematurity*, ROP) je vazoproliferativna retinopatija koja deluje na krvne sudove retine tokom njenog razvoja. Cilj rada bio je proceniti učestalost i stepen refrakcionih anomalija kod prevremeno rođene dece sa teškom formom ROP, a koja su lečena anti-vaskularnim endotelnim faktorom rasta (anti-VEGF) (bevacizumab). **Metode.** Prospektivnom studijom obuhvaćeno je 21 dete (42 oka), starosti devet meseci, kod kojih je zbog teške forme ROP primenjena anti-VEGF terapija. Kontrolnu grupu činilo je 45 dece (90 očiju) kod kojih je primenjena laser fotokoagulacija. Kod svakog deteta nakon cikloplegije urađena je retinoskopija, keratorefraktometrija i A-scan ultrasonografija. **Rezultati.** Miopija je bila prisutna kod 47,62% očiju studijske grupe i kod 33,33% očiju kontrolne grupe, ali bez statistički značajne razlike između njih. Visoka miopija (stem ekvivalent – SE < -3.0 dioptrije – D) bila je zastupljena kod 16,67% očiju studijske grupe i kod 18,19% očiju kontrolne grupe. Klinički značajna hipermetropija bila je češća u studijskoj nego u kontrolnoj grupi (47,62% *vs* 34,44%). Visoka hipermetropija bila je mnogo zastupljenija u kontrolnoj grupi (15,56% *vs* 11,90%; $p < 0,001$). Astigmatizam, posebno visoki, bio je mnogo učestaliji u kontrolnoj grupi nego u studijskoj grupi. Najčešća forma astigmatizma u obe grupe bio je pravilan astigmatizam. Anisometropija bila je mnogo

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češća u kontrolnoj grupi (24,44%) nego u studijskoj grupi (9.52%) ($p < 0.05$). U studijskoj grupi bila je značajno veća debljina sočiva i plića prednja očna komora nego u kontrolnoj grupi. U dužini oka nije bilo značajne razlike između očiju ispitivanih grupa. **Zaključak.** Kod bolesnika sa ROP, kod kojih je primenjena anti-VEGF terapija, kratkovidost je bila prisutna, ali visoka kratkovidost manje nego u grupi ispitanika lečenih laserom. Ova činjenica je verovatno u vezi sa razvojem prednjeg segmenta oka, te je za praćenje uticaja anti-VEGF terapije na razvoj refrakcionog statusa oka potreban duži vremenski period i veći broj ispitanika.

Ključne reči:
retinopatija kod prematurusa; oko, refrakcija; dijagnostičke tehnike i procedure; miopija; astigmatizam; faktori rasta endotela krvnih sudova.

Introduction

Retinopathy of prematurity (ROP) is a vasoproliferative retinopathy which affects the blood vessels of the retina during its development. The consequence of this process is a disturbance in the growth and development of the retina. The final result is blindness [1, 2]. ROP develops in two phases. In the first phase, hyperoxia leads to obliteration of immature retinal blood vessels. The consequence of the obliteration of the blood vessels is hypoxia and neovascularization growth in the second phase. This ischemia induces the vascular endothelial growth factor (VEGF) that stimulates the growth of new blood vessels leading to the destruction of the retinal architecture, vitreous body affecting and consequent blindness [3, 4].

The economic development of the country determines the possibility of ROP screening and treatment and consecutive ROP induced blindness. The incidence of blindness varies between 15% in developed countries, and 60% in middle-income countries [5, 6].

The destruction of the peripheral avascular retina by laser photocoagulation is still considered the gold standard of treatment [7, 8]. Different studies have found a high incidence of myopia in patients who have been subjected to laser treatment. Ablation of the peripheral avascular retina, caused by laser treatment, leads to inflammation and scar tissue formation with a higher reported incidence of refractive errors [9–13].

Over the last couple of years the use of intravitreal anti-VEGF therapy has become common in ROP treatment, and there have been positive results [14–17].

The aim of this study was to determine the incidence and degree of refractive errors in anti-VEGF treated premature infants with severe ROP.

Methods

This prospective study was performed over six months at the Ophthalmology Clinic in the Clinical Center, Niš, Serbia. It was approved by the institutional ethics committee and all the parents signed the agreement form for their children to participate in the study.

A total of 21 premature infants (42 eyes), nine months old, with severe ROP (ROP 3+) were included in the study group. They received a single intravitreal dose (0.625 mg) of bevacizumab in both eyes. The control group included 45 patients (90 eyes) with severe form of ROP (ROP 3+) treated by laser photocoagulation. The screening program was performed according to the guidelines published by the American Academy of Ophthalmology, American Academy of Pediatrics and American Association for Pediatric Ophthalmology and Strabismus [18]. The findings were classified according to the International Classification of ROP criteria (ICROP) [19]. The treatment was carried out according the recommendations of the Early Treatment for ROP study group (ET ROP) and Bavecizumab Eliminates the Angiogenic Threat of Retinopathy of Prematurity (BEAT-ROP) Cooperative Group [14, 20].

Refractive errors were diagnosed in cycloplegia induced by topical administration of one drop of Atropin solution 0.25% twice per day for three days. The refractive error was measured in the vertical and horizontal meridian in both eyes and was recorded to the nearest dioptre (D) using streak retinoscopy. Keratorefractometry was performed by an automated keratorefractometer. Ultrasound biometry and the estimated axial length (AL), anterior chamber depth (ACD) and lens thickness (LT) were measured. The results were recorded in the form of spherical equivalents (SE, spherical plus half of the cylinder power). Clinically significant myopia was defined as SE ≤ -1.0 D, and high myopia as less than -3.0 D (in the process of emmetropization et the age of 9 months only low myopic errors tend to show a hyperopic shift, but myopic as less than -3.0 D shows a tendency to further increase). Hyperopia was clinically significant when SE is greater than +3.0 D and high when greater than +4.0 D. Anisometropia was defined as significant when the difference between the eyes was 1 D (cyl) or more, and high if 2 D or more. Astigmatism was recorded as a negative cylinder, and defined as clinically significant (1 D cyl or more) and as high (2 D cyl or more). For analysis of the axis of astigmatism, three classes were recorded: with the rule (WTR 0°–15° and 165°–180°), against the rule (ATR 75°–105°), and oblique (O 16°–74° and 106°–164°).

Continuous variables were presented as mean values, standard deviations and medians, while categorical variables were presented as frequencies and percentages. The normality of distribution of continuous variables was assessed by the Shapiro-Wilk test. Differences in continuous variables between the two independent study groups were analyzed by Student's *t*-test for independent samples in case of normal distribution of variables, and Mann-Whitney test in case of deviation from the norm. We used Kruskal-Wallis test to determine the significance of the differences in the continuous variables between the groups with regard to their deviation from the norm. The differences in the frequencies of categorical variables between the groups were compared by means of χ^2-test, and if necessary we used Fisher's correction. The statistical significance was determined at the level of $p < 0.05$. Statistical analysis was performed using SPSS 15.0.

Results

Among the examined 66 prematurely born children with severe ROP (ROP 3+), 36 (55%) were male and 30 (45%) female. Their birth history, including their gestational age (GA) and birth weight (BW) by groups is shown in the Table 1.

The mean spherical equivalent in the study group of children treated with anti-VEGF was -0.50 D (range -8.13 to 3.5 D), and in the control group of children treated with laser photocoagulation it was -0.20 D (range -12.88 to 7.88 D). Myopia (SE ≤ -1.0 D) was observed in 20 (47.62%) eyes in the study group and in 30 (33.33%) eyes in the control group, but there were no statistically significant differences between these groups. Seven eyes (16.67%) in the study group and 17 (18.89%) eyes in the control group were discovered to have high myopia (SE < -3.0 D). The incidence of emmetropia (SE ≥-1.0 D but < +3.0 D) was lower in the study group [2 (4.76%)] than in the control group [28 (31.11%)] ($p < 0.001$). Clinically significant hyperopia (SE ≥ +3.0 D) was higher in the study group (20; 47.62%) than in the control group (31; 34.44%), although there was no statistically significant difference (Table 2). Also, high hyperopia (SE > +4.0 D) was significantly lower in the study group (11.90%) than in the control group (15.56%; $p < 0.001$).

The astigmatism incidence was lower in the children with severe ROP treated with anti-VEGF [30 (71.43%)] than in those treated with laser photocoagulation [73 (81.11%)]. The more common form of astigmatism was with-the-rule (WTR) astigmatism, with no statistical significant difference between the groups [18 eyes (42.86%) *vs* 50 eyes (55.56%)]. The incidence of oblique astigmatism was lower in the study group than in the control group [7 eyes (16.67%) *vs* 19 eyes (21.11%)]. Astigmatism against-the-rule (ATR) was most frequent in the patients treated with anti-VEGF therapy [5 eyes (11.90%)] (Table 3).

The median value of astigmatism in the study group was -0.50 ± 3.59 Dcyl and in the control group it was -0.27 ± 3.90 Dcyl. High astigmatism (≥ 2.0 Dcyl) was most common in the patients of the control group [41 eyes (56%)] (Table 4).

Anisometropia has significantly lower incidence in the study group 4 (9.52%) than in the control group 22 (24.44%) ($p < 0.05$) (Table 5).

Table 1

Demographic characteristics of the 66 study patients

Characteristics of patients	LFC treated sROP	Anti-VEGF treated sROP
Patients, n	45	21
male, n (%)	23 (51)	12 (57)
female, n (%)	22 (49)	9 (43)
GA (weeks), $\bar{x}$ ± SD	30 ± 4	29 ± 4
BW (g), $\bar{x}$ ± SD	1,200 ± 500	1,175 ± 425

LFC – laser photocoagulation; sROP – severe retinopathy of prematurity; GA – gestational age; BW – birth weight; VEGF – vascular endothelial growth factor.

Table 2

Refractive errors in the patients with sROP treated with anti-VEGF and laser

Refractive errors	LFC (n = 90)	antiVEGF (n = 42)
	n (%)	n (%)
Myopia	30 (33.33)	20 (47.62)
High myopia	17 (18.89)	7 (16.67)
Emmetropia	28 (31.11)*	2 (4.76)
Hyperopia	31 (34.44)	20 (47.62)
High hyperopia	14 (15.56)*	5 (11.90)

***$p < 0.001$.**

n – number of eyes; LFC – laser photocoagulation; VEGF – vascular endothelial growth factor; sROP – severe retinopathy of prematurity.

Table 3

Types of astigmatism according to axis

Astigmatism type	LFC (n = 90)	anti-VEGF (n = 42)
	n (%)	n (%)
Astigmatism presence	73 (81.11)	30 (71.43)
WTR	50 (55.56)	18 (42.86)
ATR	4 (4.44)	5 (11.90)
Oblique	19 (21.11)	7 (16.67)

n – number of eyes; WTR – with the rule; ATR – against the rule; LFC – laser photocoagulation; VEGF – vascular endothelial growth factor.

Table 4

Distribution of astigmatism

Astigmatism distribution	LFC (n = 90)	Anti-VEGF (n = 42)
	n (%)	n (%)
From 1 Dcyl to 2 Dcyl	32 (44)	17 (57)
≥ 2 Dcyl	41 (56)	13 (43)

n – number of eyes; LFC – laser photocoagulation; Dcyl – dioptre cylinder; VEGF – vascular endothelial growth factor.

Table 5

Distribution of anisometropia

Anisometropia distribution	LFC (n = 45)	Anti-VEGF (n = 21)
	n (%)	n (%)
Anisometropia, n (%)	22 (24.44)	4 (9.52)*
≥ 1 D to < 2 D	11 (12.22)	2 (4.76)
≥ 2 D	11 (12.22)	2 (4.76)

***p < 0.05.**
n – number of eyes; LFC – laser photocoagulation;
D – dioptre; VEGF – vascular endothelial growth factor.

The biometric characteristics of the eyes of premature infants with severe ROP, treated with laser photocoagulation or anti-VEGF therapy are given in Table 6. The children from the study group had significantly greater lens thickness, and shorter anterior chamber depth than those from the control group ($p < 0.01$). The values of the other biometric characteristics did not show statistically significant differences between the study and the control groups.

Table 6

Biometric characteristics of the study and control groups

Biometric characteristics	LFC (n = 90)	Anti-VEGF (n = 42)
	$\bar{x} \pm$ SD (median)	$\bar{x} \pm$ SD (median)
ACD (mm)	2.90 ± 0.40 (3.01)	2.81 ± 0.37 (2.89*)
LT (mm)	3.96 ± 0.32 (3.90)	4.34 ± 0.66 (4.09*)
AL (mm)	19.77 ± 1.47 (19.47)	19.93 ± 1.24 (19.72)
CR (mm)	7.68 ± 0.23 (7.65)	7.80 ± 0.37 (7.69)

***p < 0.01.**
n – number of eyes; ACD – anterior chamber depth; LT – lens thickness; AL – axial length; CR – corneal curvature; LFC – laser photocoagulation; VEGF – vascular endothelial growth factor.

Discussion

Our results are somehow different from the results of several previously published studies in which the prevalence of myopia is significantly lower in eyes treated with anti-VEGF therapy [16, 17, 21–24]. In the present study, the most frequent refractive error in both groups was myopia, although high myopia (SE< -3.0 D) was more common in the laser-treated group. Hypermetropia was more common in the anti-VEGF group, but high hypermetropia was more common in the laser treated group ($p < 0.001$). In the BEAT-ROP clinical trial a greater prevalence of myopia, especially very high myopia (≤ -8.0 D), was found in the eyes that received peripheral laser therapy than in eyes that received the intravitreal anti-VEGF therapy [22]. Harder at al. [17] reported that the refractive error was less myopic in the anti-VEGF group than in the laser treated group (17% *vs* 54%, respectively), especially for high myopia (9% *vs* 42%, respectively). Chen at al. [21] recorded a lower incidence of high myopia and more frequent emmetropia in the eyes of children treated with bevacizumab than those treated with laser therapy. Furthermore, Hwang at al. [23] found anti-VEGF treatment to be associated with less myopia than panretinal photocoagulation. Martínez-Castellanos et al. [24] are also of the opinion that anti-angiogenic therapy is associated with lower myopia. The possible reasons for the somewhat different results in our study can be found primarily in the stricter border for high myopia (SE< -3.0 D) than that used by other authors (SE < -5.0 D or SE < -8.0 D), in addition to which our patients were younger, aged nine months. We used atropine solution 0.25% for cycloplegia, but in other studies, cyclopentolate solution 1% was used, or a combination with tropicamide solution 0.5%. We chose this range for high myopia because the age of our subjects was lower, they were 9 months of age opposed to other studies in which the age of subjects ranges from 2 to 2 and a half years. It is well-known that the refractive error abnormalities of ROP patients have been found to be present early in infancy and persist into adulthood [25]. Wang et al. [26] showed that the rapid progression of myopia in eyes with severe ROP has a critical period of 1.3 years and that the later progression of myopia slower. Myopia present in a 9-month-old child with the history of severe ROP is associated with long-term myopia and carries a high risk of developing into high myopia. The true nature of myopia in preterm infants, as well as its progression, is not well-understood. It is believed that it results from the influence of three etiological factors: prematurity, severity of ROP, and changes due to the different therapies applied [22]. In normal development, most eye growth takes place in the first year of life: the refractive state changes as the axial length increases and the cornea and lens flatten. However, prematurity may affect the emmetropization process of ocular development in the postnatal stage, the so-called myopia of prematurity (MOP) [22, 25]. This myopia is nonaxial, consisting of the steeper cornea, shallower anterior chamber and increased lenticular thickness. This suggests that severe ROP and/or its treatment may result in arrested development of the anterior segment. During the rapid eye growth at an early age, all these abnormal biometric parameters may come together and result in the significant progression of myopia in eyes with severe ROP [5, 25, 27]. In the present study we found significantly greater lens thickness and shorter anterior chamber depth, especially

in the group treated with anti-VEGF ($p < 0.01$), but there were no statistically significant differences in axial length and corneal curvature. Our results are similar to the results of other authors regarding the biometric characteristics of eyes with severe ROP [5, 27, 28].

One possible explanation for the progression of myopia in a severe form of ROP treated with laser is the growth signals hypothesis. It has been shown that the peripheral retina in the eyes of very preterm infants will vascularize or differentiate only to the location of the anterior termination of the endothelial cell precursors at birth. Thus, the retinal vessels may never reach the *ora serrata*. Because of this, the arrested maturation of the photoreceptors decreases the levels of the local growth factors required for signaling the pathways involved in anterior segment development. Intravitreal anti-VEGF therapy allows continued development of the retinal vessels beyond the neurovascular ridges, which is minimal or absent following laser treatment. It is possible that this development could allow continued expression of the local growth factor and the signaling pathways necessary for a more normal anterior segment with minimal myopia [5, 14, 22].

In the present study, we found that the prevalence of astigmatism was greater in the laser treated group, especially high astigmatism. Also in both groups, the most common type of astigmatism was with-the-rule (WTR). These results agree with the results of other authors [29–31]. Astigmatism has been known to be associated with ROP, but the exact mechanism of this astigmatism in prematurity and ROP is not completely understood. Corneal curvature (CR) is one of the determinant factors in refractive error. It is usually steep in newborn infants and even steeper in premature infants [31]. In our study we noted that the cornea was steeper in the laser-treated group than in the anti-VEGF group, but not statistically significant. Most of the eyes in our and other studies showed WTR astigmatism, which can usually be explained by the larger number of laser spots in the temporal part of the retina in the laser treated eyes, and which can lead to changes in the corneal curvature in the horizontal axis [28-31]. However, there is still no good explanation for frequent WTR astigmatism in eyes treated with anti-VEGF therapy.

Our results, in accordance with the results of the other authors, show that anisometropia is significantly greater in the laser treated group (12.22%), especially for high anisometropia. This can be explained by an imbalance in the severity of the disease between the eyes and/or that laser treatment may not be done in both eyes identically.

This is supported by the finding of Geloneck at al. [22] that myopia increases by -0.14 D for every 100 laser spots.

The limitations of this study are the small number of patients and its short duration.

Conclusion

Our study confirms that severe ROP treated by anti-VEGF therapy causes less incidence of high myopia (SE < -3.0 D), less value of astigmatism and anisometropia, which means the less possibility of amblyopia and strabismus incidence.

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Reconstruction of lateral attic wall in acquired cholesteatoma

Rekonstrukcija lateralnog zida atika kod stečenog holesteatoma

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Abstract

Background/Aim. Attic cholesteatoma is an epithelial cystic pseudotumor which arises in the top compartment of the middle ear. Surgery is the only therapeutic treatment for attic cholesteatoma. The aim of this study was to analyze the surgical and audiological results in tympanoplasties that use a logical application of several techniques for the management of attic cholesteatoma. Our hypothesis was that the tympanoplasty technique with cartilage/bone reconstruction of the achieve better outcome than the tympanoplasty technique with only temporal fascia reconstruction of the lateral attic wall. **Methods.** This retrospective clinical study included 80 patients, aged 16–65 years, with attic cholesteatoma undergoing canal "wall up" tympanoplasty with lateral attic wall reconstruction, under general anesthesia in the Eear, Nose and Throat Clinic, Military Medical Academy in Belgrade between 2006 and 2010. The patients were divided into two groups according to the type of lateral attic wall reconstruction: the group I of 60 patients with cartilage/bone plus temporalis fascia lateral attic wall reconstruction and the group II of 20 patients with only temporal fascia lateral attic wall reconstruction. Postoperative follow-up examinations were done at least 5 years after the surgery. The χ^2 test was used to compare postoperative sequelae for two groups of operated patients with lateral attic wall reconstruction. The independent and paired samples *t*-test of air conduction and air-bone gap were used to compare the results of preoperative and postoperative hearing tests. **Results.** The differences between hearing measurements of the two groups according to preoperative and postoperative auditory thresholds of the air conduction and the air-bone gap were considered no statistically significant. The difference between the two groups recarding to recurrent attic retraction pocket appearance and recurrence of cholesteatoma was considered statistically significant and the results were much better in the group I of the operated patients with cartilage/bone lateral attic wall reconstruction. **Conclusion.** "Wall up" tympanoplasty for attic cholesteatoma with lateral attic wall reconstruction leads to good anatomical and audiological results. A significant hearing improvement was obtained in both the types of lateral attic wall reconstructions in this study. Reconstruction with cartilage or mastoid cortex bone showed favorably long-term functional and anatomical results compared to primary tympanoplasty using only temporal fascia for lateral attic wall reconstruction in cases of attic cholesteatoma.

Key words:
cholesteatoma, middle ear; tympanoplasty; otologic surgical procedures; recurrence; hearing; treatment outcome.

Apstrakt

Uvod/Cilj. Atik holesteatom je epitelna cistična pseudotumorska izraslina u gornjem spratu bubne duplje. Hirurgija je jedino terapijsko rešenje za atik holesteatom. Cilj rada bio je da se analiziraju hirurški i audiološki rezultati nekoliko tehnika timpanoplastike koje smo koristili u hirurškom lečenju bolesnika sa atik holesteatomom. Naša hipoteza je bila da timpanoplastika sa rekonstrukcijom lateralnog zida atika uz pomoć hrskavice/kosti daje bolje rezultate nego timpanoplastika sa rekonstrukcijom lateralnog zida atika uz pomoć samo temporalne fascije. **Metode.** Ova retrospektivna studija obuhvatila je 80 bolesnika sa atik holesteatomom, starosti od 16 do 65 godina, kojima je urađena timpanoplastika sa čuvanjem zadnjeg zida zvukovoda i sa rekonstrukcijom lateralnog zida atika u opštoj endotrahealnoj anesteziji u Klinici za otorinolaringologiju Vojnomedicinske akademije u Beogradu u periodu 2006–2010. godine. Bolesnici su razvrstani u dve grupe prema tipu rekonstrukcije lateralnog zida atika i to: grupu I činilo je 60 operisanih bolesnika sa rekonstrukcijom lateralnog zida atika uz pomoć hrskavice/kosti i temporalnom fascijom; grupu II činilo je 20 operisanih bolesnika sa rekonstrukcijom lateralnog zida atika uz pomoć samo temporalne fascije. Postoperativno praćenje bolesnika sprovedeno je tokom perioda od najmanje 5 godina nakon operacije. χ^2-test je korišćen za upoređivanje postoperativnih rezultata u obe grupe bolesnika sa rekonstrukcijom lateralnog zida atika, *t*-test za vaz-

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dušnu vodljivost i vazdušno-koštanu razliku čujnosti je korišćen za upoređivanje preoperativnih i postoperativnih rezultata kod sluha svih bolesnika. **Rezultati.** Nije ustanovljena statistički značajna razlika u čujnosti bolesnika obe grupe prema preoperativnim i postoperativnim nalazima praga sluha za vazdušnu vodljivost i vazdušno-koštanu razliku čujnosti. Utvrđena je stistički značajna razlika u ponovnoj pojavi atik retrakcionog džepa i pojavi recidivantnog holesteatoma između dve grupe bolesnika, sa boljim postoperativnim rezultatom u grupi bolesnika sa rekonstrukcijom lateralnog zida atika uz pomoć hrskavice/kosti i temporalne fascije. **Zaključak.** Timpanoplastikom, tehnikom sa čuvanjem zadnjeg zida zvukovoda i rekonstrukcijom lateralnog zida atika, postižu se dobri anatomski i audiološki rezultati u operacijama atik holesteatoma. Značajno poboljšanje sluha je postignuto u oba tipa rekonstrukcije lateralnog zida atika u našoj studiji. Rekonstrukcija lateralnog zida atika uz pomoć autografta hrskavice ili kosti korteksa mastoida daje bolje dugoročne funkcionalne i anatomske rezultate nego timpanoplastika koja koristi samo temporalnu fasciju za rekonstrukciju lateralnog zida atika.

Ključne reči:
uvo, srednje, holesteatom; timpanoplastika; hirurgija, otološka, procedure; recidiv; sluh; lečenje, ishod.

Introduction

Attic cholesteatoma is keratin-producing squamous epithelium cyst (sac) in the epitympanum with or without spread in the mastoid or in the other parts of the middle ear [1]. Attic cholesteatoma is a chronic disease of the middle ear which resorbs bone. Attic cholesteatoma can damage hearing and vestibular function and sometimes leads to egzocranial and endocranial life-threatening complications. Not a single theory has been able to explain the clinical characteristics of all cholesteatoma types including attic cholesteatoma: uncoordinated hyperproliferation, invasion, migration, altered differentiation, aggressiveness and recidivism [2]. According to invagination theory of primary acquired cholesteatoma development, the pathogenesis of attic cholesteatoma has the following characteristics: Eustachian tube dysfunction; poor aeration of the epitympanic space; retraction of the *pars flaccida*; normal migratory pattern altered; accumulation of keratin, enlargement of the sac [3].

The early attic retraction pocket appearance signifies the beginning of attic cholesteatoma [1]. Mirko Tos published "Classification of the attic retraction pocket" in 1980 and it is still valid nowdays [4, 5]. Tos and Poulsen [4], and Sudhoff and Tos [5] established four stages of *pars flaccida* retraction development. The stages are: stage I (*pars flaccida* is not adherent to the malleus); stage II (*pars flaccida* is adherent to the malleus); stage III (hidden retraction pocket); stage IV (hidden retraction pocket with erosion of outer attic wall-scutum).

For all these stages in classification of the attic retraction pocket, especially for the stage IV, we can say that there is a possibility for attic cholesteatoma occurrence, known as „potential" cholesteatoma. The next step in attic cholesteatoma development is the accumulation of keratin (debris) in the attic retraction pocked with the possibility to clean it. If it is not possible to clean the debris (debridement) from the middle ear, then we can say that it is „dry" cholesteatoma. „Wet" cholesteatoma with periodical or constant otorrhea on the sick ear appears after infection with germs on „dry" cholesteatoma. How to manage early attic retraction pocket and prevent attic cholesteatoma occurrence is still an unsolvable task for otologists. Retraction pocket is a precursor for recurrence of cholesteatoma, too.

Otomicroscopy finding is almost enough to diagnosticate attic cholesteatoma. Several diagnostic procedures can also help to establish the final diagnosis of attic cholesteatoma: ear endoscopy, hearing tests, imaging – Schuller's view X ray, temporal bone computed tomography (CT), cone beam CT [6]. Surgery is still the only therapeutic treatment for attic cholesteatoma. The objectives of attic cholesteatoma surgery are to: remove the cholesteatoma for cured dry ear; restore or maintain functional capacity of the ear; maintain normal anatomy (if possible); manage complications as priority.

Each case should be treated individually according to the extent/location of cholesteatoma and preoperative counseling. Preoperative counseling with a patient about the advantages and disadvantages of various types of surgery is necessary. There are two types of attic cholesteatoma surgery: canal "wall down" tympanoplasty; canal "wall up" tympanoplasty with the reconstruction of lateral attic wall "*mur de loget*".

There are many disadvantages of canal "wall up" tympanoplasty. It is technically more difficult, staged operation is often necessary, residual cholesteatoma is harder to detect, but this type of tympanoplasty maintains normal anatomy and restores function of the operated ear without water precaution. If it is necessary to wear hearing aid, it is easier to fit it in the canal "wall up" than in the canal "wall down" operated ears.

The aim of this study was to analyze the surgical and audiological results in tympanoplasties that use a logical application of several techniques for the management of attic cholesteatoma.

Methods

This retrospective clinical study included 80 patients, aged 16–65 years, with attic cholesteatoma (Figure 1) undergoing canal "wall up" tympanoplasty with lateral attic wall reconstruction under general anesthesia in the Ear, Nose and Throat (ENT) Clinic, Military Medical Academy (MMA) in Belgrade, between 2006 and 2010. A modification of the lateral attic wall reconstruction in cases of attic cholesteatoma was accompanied by ossiculoplasty when it was necessary. A computered otologic database and patient charts were used to obtain the necessary data. There were differents kind of at

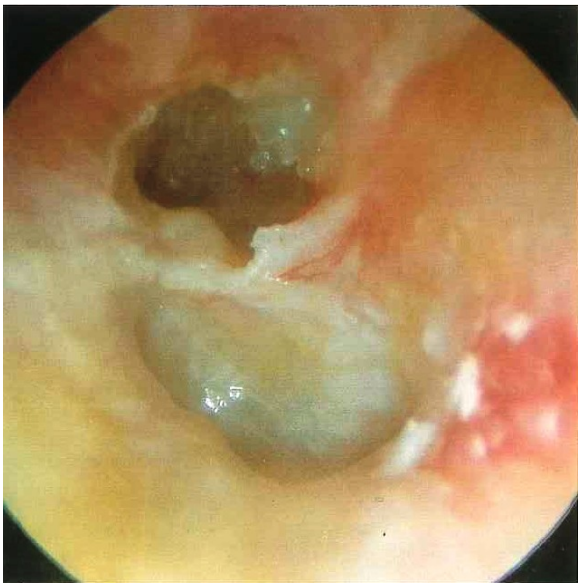

Fig. 1 – Otomicroscopy finding of attic cholesteatoma.

tic cholesteatoma extension in the middle ear. Among 80 operated patients, 19 had attic cholesteatoma localized in the epitympanic space with or without expansion in the antrum or in the Prussak's space with complete ossicular chain with or without ossicular fixation. In the other cases (61/80) attic cholesteatoma spreaded from the attic to the antrum, mastoid process or in the cavum tympani causing ossicular interruption. The most common was the damage of the long process of the incus or the damage of the other part of the incus (59/80), followed by the damage of the stapes (26/80) and the damage of the malleus (8/80). We made ossiculoplasty in the most of the operated patients (61/80) to reconstruct the sound conducting mechanism. We performed one of the types of the ossiculoplasty: incus interposition (37/80), malleostapedopexy (12/80), malleoplatinopexy (4/80), partial ossicular replacement prosthesis (PORP) (6/80) and total ossicular replacement prosthesis (TORP) (2/80). In all the cases with cholesteatoma affected incus, we used remodeling head of the malleus (4/37), interposed mastoid cortex bone (4/37), or interposed auricular cartilage (2/37) for the collumela effect instead of the incus.

The patients were divided into two groups according to the types of lateral attic wall reconstruction. Modification of one piece or the palisade technique was utilized for lateral attic wall reconstruction in cases of attic cholesteatoma (the group 1) (Figure 2): 1a) *tragus perichondrium*/cartilage island flap or; 1b) auricular cartilage with temporal fascia or 1c) mastoid cortex bone with temporalis fascia or the group 2: only temporal fascia. Postoperative follow-up examinations were done at least 5 years after the surgery. The first follow-up examination was two weeks after the surgery, then a month later and continued every three months during two years, and twice a year later on, if the postoperative period was neat. Our study was based on the otomicroscopy findings and a hearing test: audiometry with/without timpanometry and cone beam computed tomography (CT) of the temporal bones if necessary (Figure 3). Normal postoperative otomicroscopy finding or recurrent attic retraction pocket appearance or recurrence of cholesteatoma were recorded for each patient. The χ^2-test was used to compare postoperative sequelae for the two groups of the operated patients with lateral attic wall reconstruction. Hearing results were reported using four-frequency (500, 1000, 2000, 4000 Hz) pure-tone auditory thresholds and air-bone gap (PTA-ABG). The independent and paired samples *t*-test were used to compare the results of preoperative and postoperative air conduction and air-bone gap.

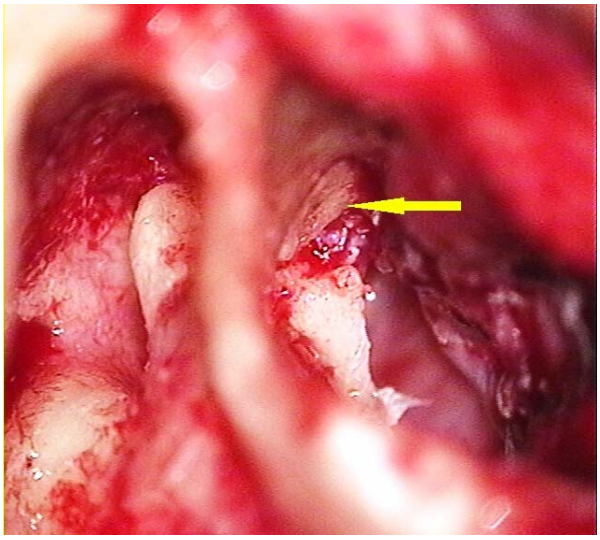

Fig. 2 – Intraoperative finding of lateral attic wall reconstruction with auricular cartilage (arrow shows shaped pice of auricular cartilage).

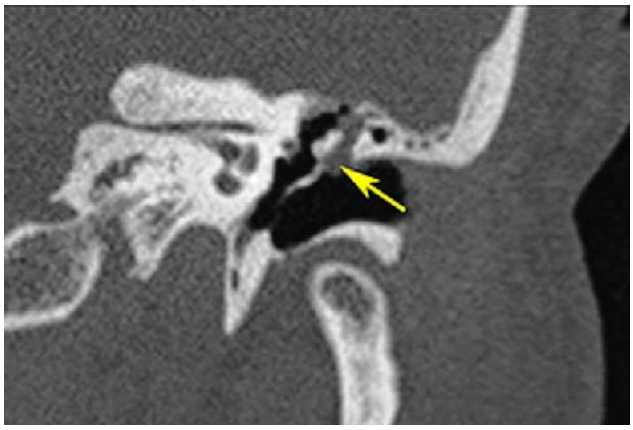

Fig. 3 – Cone beam computed tomography (CT) of temporal bone (arrow shows soft tissue of the attic cholesteatoma).

Results

We performed combined approach tympanoplasty with lateral attic wall reconstruction into 80 of the patients with histopathology verified attic cholesteatoma. The patients were divided in two groups according to the type of lateral attic wall reconstruction. The group I of 60 patients was operated with tragus perichondrium/cartilage island flap (10 patients), auricular cartilage with temporal fascia (42 patients), and mastoid cortex bone with temporal fascia (8 patients). All 60 samples show almost similar hearing benefit and postoperative sequelae (Table 1). The group II of 20 patients was operated with only temporal fascia lateral attic wall re-

Table 1

Number of recurrent retraction pockets and relapse of cholesteatoma in the operated patients with lateral attic wall reconstruction of the middle ear

Recurrent/recidivism of disease	Lateral attic wall reconstruction (number of operated patients)			
	Tragus cartilage (10)	Auricular cartilage (42)	Bone (8)	Temporalis fascia only (20)
Recurrent attic retraction pocket	2	4	2	6
Recurrent/recidivism of cholesteatoma	2	5	2	8

construction. The average duration of the follow-up period was 72 months (ranging from 60–88 months). A total of 8 (13%) cases with recurrent attic retraction pocket and 9 (15%) cases with recurrence of cholesteatomas were noted in the group I, tympanoplasty with cartilage/bone lateral attic wall reconstruction during the follow-up. A total of 6 (30%) cases with recurrent attic retraction pocked and 8 (40%) cases with recurrence of cholesteatoma were noted in the group II, tympanoplasty with only temporal fascia lateral attic wall reconstruction (Table 1). The difference between the two groups according to recurrent attic retraction appearance was considered no statistically significant ($p = 0.090$, $p > 0.05$), but the difference between the two groups according to recurrence of cholesteatoma was considered statistically significant ($p = 0.023$, $p < 0.05$). The preoperative mean air conduction was 43.75 dB and the air-bone gap was 21.24 decibels (dB) including all the patients. The postoperative mean air conduction was 22.46 dB and the air-bone gap was 7.94 dB including all the patients. The preoperative mean air conduction in the group I tympanoplasty with cartilage or bone reconstruction of lateral attic wall was 46.30 dB, SD = ±8.00 and air-bone gap was 21.72 dB, SD = ±1.93 and the postoperative results were 24.29 dB, SD = ±4.25, 8.54 dB, SD = ±0.82, and respectively. The preoperative mean air conduction in the group II tympanoplasty with only temporal fascia reconstruction of lateral attic wall was 41.20 dB, SD = ±2.28, the air-bone gap was 20.76 dB, SD = ±2.27 and the postoperative results were 20.63 dB, SD = ±1.46, and 7.33 dB, SD = ±1.13 respectively (Table 2). The difference between preoperative and postoperative mean auditory thresholds in both groups separately was considered highly statistically significant ($p = 0.0001$, $p < 0.05$). The differences between hearing measurements of the two groups according to preoperative and postoperative mean auditory thresholds of the air conduction and the air-bone gap were considered, no statistically significant ($p > 0.05$). There was no statistically significant difference in auditory improvement ($p = 0.305$) between group 1 (mean = 22.01dB, SD = ±4.07) and the group 2 (mean = 20.57dB, SD = ±1.09). There was no statistically significant difference in air-bone gap improvement ($p = 0.683$) between group 1 (mean = 13.18dB, SD = ±1.26) and the group 2 (mean = 13.43dB, SD = ±1.36).

Discussion

The incidence of recurrence of attic cholesteatoma is reported to vary (5–57%) according to data from the literature [7]. Canal "wall up" tympanoplasty with lateral attic wall reconstruction provides a good anatomical and hearing result for solving attic cholesteatoma, according to many surgeons [8, 9]. Many surgeons agree that lateral attic wall reconstruction with cartilage gives better anatomical result than lateral attic wall reconstruction with only temporal fascia according to the appearances of recurrence of attic cholesteatoma in a long period of time [10]. Actually, in our study, recurrence of cholesteatoma was rarely noted in a 5-year time in patients with cartilage lateral attic wall reconstruction (15%), contrary to only temporal fascia lateral attic wall reconstruction (40%). We used mastoid cortex bone for lateral attic wall reconstruction, except tragus or auricular cartilage, with equally good results. Whatever we use (cartilage or bone) for lateral attic wall reconstruction, the most important is to make it precisely to be fit for "*mur de loget*" reconstruction (Figure 4). There are no experiences with bone lateral attic wall reconstruction record in the "Pub Med" as we did with mastoid cortex bone.

Table 2

Preoperative and postoperative hearing measurements in both types of tympanoplasty

Group of patients	Hearing test	Air conduction (db), mean ± SD	Mean air-bone gap (db), mean ± SD	Statistical significance within the group	Statistical significance between the groups
Group I	Preoperative	46.30 ± 8.00	21.72 ± 1.93	$p < 0.000$	$p > 0.05$
	Postoperative	24.29 ± 4.25	8.54 ± 0.82		
Group II	Preoperative	41.20 ± 2.28	20.76 ± 2.27	$p < 0.000$	
	Postoperative	20.63 ± 1.46	7.33 ± 1.13		

Group I – patients with cartilage/bone lateral attic wall reconstruction; Group II – patients with only temporalis fascia lateral attic wall reconstruction.

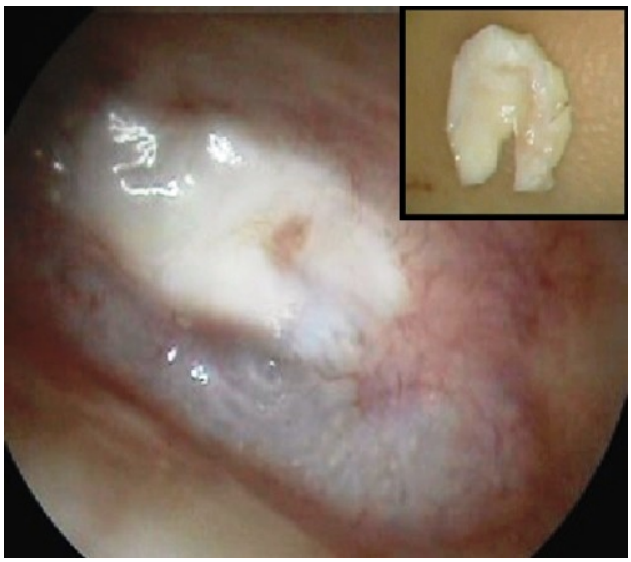

Fig. 4 – Postoperative otomicrosopy finding with a shaped pice of auricular cartilage for attic wall reconstruction of the middle ear.

A special issue is whether there is any chance to manage early attic retraction pocket before it becomes a surgery problem. Deep attic retraction pocket is an indication for surgery nowadays, according to some surgeons [10], bearing in mind the fact that attic retraction pocket eventually leads to attic cholesteatoma appearance. How to manage the attic retraction pocket?

The Eustachian tube, epitympanic compartments and the anatomy of the atticotympanic diaphragm were examined to solve the problem of attic retraction pocket occurrence [11, 12]. Eustachian tube dysfunction has been linked with the middle ear pathology and attic cholesteatoma. One of the Eustachian tube dysfunction sequelae seen is *pars flaccida* retraction of the tympanic membrane. The findings confirmed that Prussak's space has a wide connection with the mesotympanum through the posterior pouch of Troeltsch's space and may have an additional narrow passage in its roof to the lateral malleal space [13]. The lateral incudomalleal fold regularly separates the upper lateral attic from the lower lateral attic and the mesotympanum. The medial incudal fold as a rule is atrophic already at birth. The anterior tympanic isthmus thus extends from the tensor tympani tendon to the posterior incudal ligament and is the main passage for epitympanic and mastoid aeration. Openings in the tensor fold area, when present, are also important [11]. Otherwise, tensor fold resection together with the lateral incudomalleal fold can be used in the canal "wall up" tympanoplasty to improve attic aeration [14].

In some ears, the posterior tympanic isthmus may form an auxiliary narrow route for aeration *via* the incudal fossa. Concern occurs when the unregulated middle ear and mastoid aeration with Eustachian tube dysfunction and atticotympanic blockade becomes a chronic problem, leading to the attic retraction pocket followed by debris collection and fulminate attic cholesteatoma.

In order to prevent attic retraction pocket, nowadays otologists can do the following: observation (frequent control, debridement); diagnostic procedures (ear endoscopy with 0° and 30° angle, hearing tests – audiometry and tympanometry, imaging – especially cone beam CT with quite good visualisation of the temporal bones and 1,000 times less radiation dose compering to multislice computed tomography (MSCT) [15] (Figure 2); aeration tube (T-tube maybe?) with N_2O insufflations (no statistical difference of attic cholesteatoma occurence with or without implantation of aeration tube) [16]; endonasal dilatation/tuboplasty of the Eustachian tube. Preliminary results suggest that laser Eustachian tuboplasty is safe and efficient in the treatment of intractable Eustachian tube dysfunction according to some authors [16, 17]. Only a few otologists can say that a balloon Eustachian tuboplasty (BET) is a safe and effective treatment for improving Eustachian tube function and ear ventilation, but it remains to be seen in the future if it would help to prevent attic cholesteatoma occurrence [16, 18]; surgery for deep attic retraction pocket. Tympanoplasty for the correction of a retraction pocket if the *pars flaccida* can prevent further attic retraction and cholesteatoma development [8]. The question remains of is it necessary to operate deep attic retraction pocket if hearing is good and there is no otorrhea?

Conclusion

The attic cholesteatoma with a retraction pocket of the *pars flaccida* remains a difficult problem for the otologists to treat. It may lead to ossicular erosion and the interruption of ossicular chain causing difficult hearing loss. The type of tympanoplasty depends on the extent/location of cholesteatoma. A modification of the lateral attic wall reconstruction in cases of attic cholesteatoma was accompanied by ossiculoplasty when it was necessary. Tympanoplasty with lateral attic wall reconstruction leads to good anatomical and audiological results. A significant hearing improvement was accomplished in all the types of the lateral attic wall reconstructions. In attic cholesteatoma, tympanoplasty with lateral attic wall reconstruction using only one piece of cartilage or bone or the palisade technique resulted in precise reconstruction of the lateral attic wall, can prevent recurrent attic retraction development and help reduce recurrence of cholesteatoma. Reconstruction with cartilage or mastoid cortex bone showed better anatomical results compared to primary tympanoplasty using only temporal fascia for lateral attic wall reconstruction in cases of attic cholesteatoma. Postoperative hearing results were encouraging, too.

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R E F E R E N C E S

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Medicolegal characteristics of defense injuries in cases of homicides

Sudskomedicinske karakteristike odbrambenih povreda u slučajevima ubistava

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Abstract

Background/Aim. During the homicidal act, a victim usually tries to defend himself/herself, and due to these attempts he/she could sustain so-called defense injuries, mostly localized on the arms. The aim of this research was to analyze important medicolegal characteristics of defense injuries, particularly regarding their importance in forensic expertise of homicides. **Methods.** We analyzed autopsies of all homicidal cases with defense injuries in Belgrade during a three-year period. **Results.** Defensive injuries were registered in 71 victims of murder. The majority (67.61%) of victims with defense injuries were males. About 25% of victims were aged from 21 to 30 years. The majority (60) of victims were not under influence of alcohol. Homicides were mostly (90.14%) performed by mechanical weapons. The highest frequency of defense injuries was noticed in the victims with multiple homicidal injuries localized on the front side of the body. In a half (50.7%) of the cases they were present on both arms of the victim, mostly on the dorsal side of hands and forearms. Bruises were the most frequent form of defense injuries (36.61% out of 71 cases), while incisions, abrasions, gunshot injuries and stab wounds were less common. **Conclusion.** Determination of defense injuries and their medicolegal characteristics enables collecting of facts that are important for legal estimation of homicide, as well as for adequate sentence at the end of the court procedure.

Key words:
forensic medicine; homicide; defense mechanisms; serbia.

Apstrakt

Uvod/Cilj. U toku izvršenja ubistva žrtva se često brani i tada može zadobiti odbrambene povrede, uglavnom lokalizovane na rukama. Cilj rada bio je da se utvrde bitne sudskomedicinske karakteristike odbrambenih povreda, kao i da se ukaže na njihov značaj u sudskomedicinskom rešavanju slučajeva ubistava. **Metode.** Izvršena je epidemiološka retrospektivna studija svih slučajeva ubistava sa odbrambenim povredama, koja su u trogodišnjem periodu izvršena na teritoriji Beograda. **Rezultati.** Kod 71 žrtve ubistva registrovane su odbrambene povrede. Većina (67,61%) žrtava ubistava sa odbrambenim povredama bili su muškarci. Oko 25% žrtava bilo je starosti 21 do 30 godina. Najveći broj (60) žrtava nije bio u alkoholisanom stanju. U najvećem broju slučajeva (90,14%) ubistva su bila izvršena isključivo upotrebom mehaničkog oruđa. Odbrambene povrede bile su najčešće kod žrtava sa višestrukim ubilačkim povredama lokalizovanim na prednjoj strani tela. U oko polovini slučajeva (50,7%) odbrambene povreda su dijagnostikovane na obema rukama žrtve, najčešće na nadlanenoj strani šaka i podlaktica. Krvni podlivi bili su najčešća vrsta odbrambenih povreda (36,61% od 71), po učestalosti slede sekotine, oguljotine i ustreline, a najređe su ubodine. **Zaključak.** Utvrđivanjem odbrambenih povreda i njihovih karakteristika prikupljaju se činjenice koje su u sudskom postupku važne za krivičnopravnu ocenu karaktera izvršenog krivičnog dela ubistva i donošenje odgovarajuće sudske presude.

Ključne reči:
medicina, sudska; ubistvo; odbrambeni mehanizmi; srbija.

Introduction

During an incidence of murder the victim often defends himself/herself, when he/she may receive injuries that are mainly localized on the upper limbs, rarely on the feet or legs [1]. These are so-called defense injuries, which are very important from the forensic point of view, mainly for distinguishing between accidental, suicidal and homicidal act of injuring. Their presence indicates the homicidal manner of death. Furthermore, it refers to the fact that the victim was conscious in the course of committing the act of murder, at least for some time [1–8]. However, their absence does not necessarily exclude homicide, since the victim may be killed from afar by the shot from behind or there may exists a big discrepancy in power between the attacker and the victim, or the victim may be unconscious or assaulted by multiple attackers [5–9].

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There are two types of defense injuries [6–8]. The first type is defensive or passive wound, resulting from an attempt to protect the victim's head and body by protruding hands in front of himself/herself as a shield, resulting in soft tissue injuries on the dorsal side of forearms and hands, and rarely on the upper arms (Figure 1). If very intense force is applied, for example strong blows with a metal or wooden rod, besides soft tissue injuries bone fractures may occur, most commonly of the ulna. In contrast, the second type, active or offensive injuries occur due to an attempt of the victim to catch the weapon, which causes characteristic injuries on the palm of the hand. The most typical localization of the wound for murders committed with a knife is in the space between the root of the thumb and index finger, because the victim attempts to catch the blade (Figure 2) [3, 10]. In case of blows with a blunt weapon, visible injuries on the palmar side of hands, which comes in contact with the weapon, rarely occur [4, 11].

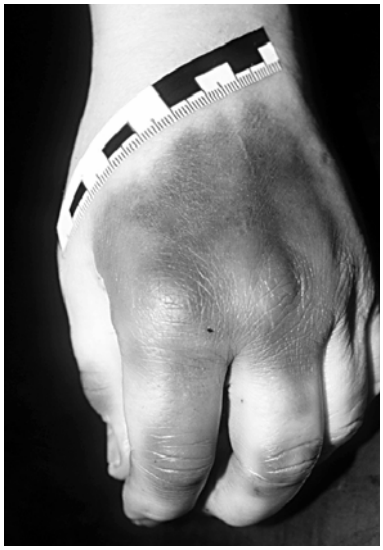

Fig. 1 – Defense bruising on the dorsal side of the left hand and fingers of the victim killed by blunt injuries.

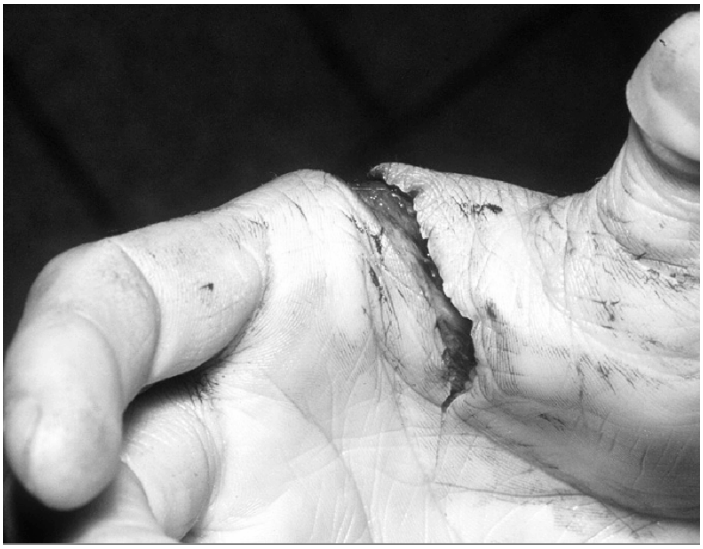

Fig. 2 – Offensively defense incision between the thumb and index finger.

In addition to the localization of defense injuries, a significant forensic characteristic is their number. Numerous defense injuries on the body of a killed person suggest that the inflicting of injuries lasted longer and that during the act of murder the victim was conscious, and hence suffered physical pains and mental suffering. In this way, the number of defense injuries in the criminal proceedings may be a significant evidence of brutal (cruel) murder, which in legal terms is characterized by intention of the attacker to inflict intense physical pains and mental suffering to the victim before committing murder.

The aim of this study was to determine the significant forensic characteristics of defense injuries, and to emphasize their importance in forensic estimation and criminal proceeding of homicides connected with their occurrence.

Methods

This paper presents a retrospective epidemiological study of intersection of all murder cases with defense injuries, that were found at autopsies performed at the Institute of Forensic Medicine in Belgrade during a three-year period. The data were obtained by studying the autopsy records, police reports and the results of toxicological analysis. We analyzed gender and age of victims, blood alcohol concentration in victims, way of committing homicide, as well as type, number and localization of defense injuries.

Complete statistical analysis of the obtained data was performed in the statistical software package IBM SPSS Statistics 19. All categorical variables were presented as percentage frequency of certain categories. For categorical variables the statistical significance of differences was examined using χ^2 test or Fisher's exact test (for small incidence of certain categories). To analyse the proportion of cases that fall into different categories of one variable and to compare the hypothetical value of these proportions, we used χ^2 test for testing the quality of correspondence. All the results were assessed by the level of significance of $p < 0.05$.

Results

The total number of homicides with defense injuries in the analyzed period was 71 (49.65% out of all 143 murders). Male victims were significantly more frequent: 48 (67.61%), women 23 (32.39%), $p = 0.003$. The victims were mostly 21–30 years old ($p = 0.125$) (Table 1). In relation to age of the victims statistically significant differences by gender were found ($p = 0.009$). Women older than 61 years were most common, while for men the highest incidence of victims aged between 21 and 30 years was found.

The high number of casualties (84.51%) was not under the influence of alcohol (Table 2), victims with the blood alcohol concentration (BAC) less than 0.50‰ were present in our sample. In 3 victims (two males and one female) the BAC was between 0.51 and 1‰. There were no statistically significant differences in the degree of intoxication in relation to gender ($p = 0.114$) and age ($p = 0.216$).

Table 1

Gender and age distribution of the victims of homicides with defense injuries

Age (years)	Male, n (%)	Female, n (%)	Total, n (%)
11–20	5 (10.42)	1 (4.35)	6 (8.45)
21–30	16 (33.33)	2 (8.70)	18 (25.35)
31–40	6 (12.50)	1 (4.35)	7 (9.86)
41–50	6 (12.50)	5 (21.72)	11 (15.49)
51–60	7 (14.58)	2 (8.70)	9 (12.68)
61–70	1 (2.09)	6 (26.09)	7 (9.86)
≥71	7 (14.58)	6 (26.09)	13 (18.31)
Total	48 (100)	23 (100)	71 (100)

Table 2

Drunken state in the victims of homicides with defense injures

Blood alcohol concentration (‰)	Male, n (%)	Female, n (%)	Total, n (%)
Negative result	38 (79.17)	22 (95.65)	60 (84.51)
< 0.50	8 (16.67)	–	8 (11.27)
0.51–1.00	2 (4.16)	1 (4.35)	3 (4.22)
Total	48 (100)	23 (100)	71 (100)

Murders with defense injuries in most cases were carried out solely by mechanical tools (64–90.14%), rarely as a combination of mechanical injuries and asphyxia (3–4.23%) the violent mechanical asphyxia only (4–5.63%) (Table 3). The largest number of homicides in the analyzed sample was performed by using a firearm (27 cases), which was usually the only way of killing followed by blunt force injuries, stab wounds and incisions (Table 3). There was a statistically significant gender difference regarding the way of committing murder ($p = 0.032$), while the differences in the age of the victim were not noticed ($p = 0.287$). Men were usually killed by firearms, while women mostly sustained injuries inflicted with blunt, pointed and sharp mechanical weapons.

In the majority (36–50.71%) of victims with defense injuries, 5 or more murderous injuries were diagnosed at autopsy, and that was significantly more frequent than other groups with a smaller number of homicidal wounds per one victim ($p < 0.001$) (Figure 3). On the other hand, only one homicidal injury was diagnosed in 14 (19.72%) victims. There was no significant difference in the number of murderous wounds in relation to gender ($p = 0.305$) and age of the victim ($p = 0.782$).

In the majority (44–61.97%) of victims homicidal injuries were localized only on the front side of the body, and it was significantly more frequent comparing to the localization of murderous injuries only on the back side (7–9.86%) or both on the back and the front side of the body (20–28.17%) ($p < 0.001$). There was no statistically significant difference in the localization of homicidal injuries with respect to gender ($p = 0.542$), age ($p = 0.349$), the type of murderous harm ($p = 0.067$) and the number of homicidal wounds ($p = 0.834$).

Table 3

Distribution of homicides with defense injures by the type of murderous violation

Type of murder	Male, n (%)	Female, n (%)	Total, n (%)
Firearm injury	20 (41.67)	3 (13.04)	23 (32.39)
Blunt injury	9 (18.75)	5 (21.74)	14 (19.72)
Stab wounds and incisions	7 (14.59)	6 (26.09)	13 (18.31)
Stab wounds	6 (12.51)	–	6 (8.43)
Manual strangulation	1 (2.08)	3 (13.04)	4 (5.64)
Incisions	–	2 (8.69)	2 (2.82)
Firearm injury and blunt force	1 (2.08)	1 (4.35)	2 (2.82)
Manual strangulation and blunt force	1 (2.08)	–	1 (1.41)
Stab wounds and blunt force	–	1 (4.35)	1 (1.41)
Ligature strangulation and blunt force	1 (2.08)	–	1 (1.41)
Firearm injury and incisions	1 (2.08)	–	1 (1.41)
Firearm injury, stab wounds and incisions	–	1 (4.35)	1 (1.41)
Incisions, manual strangulation and blunt force	–	1 (4.35)	1 (1.41)
Stab wounds, incisions and blunt force	1 (2.08)	–	1 (1.41)
Total	48 (100)	23 (100)	71 (100)

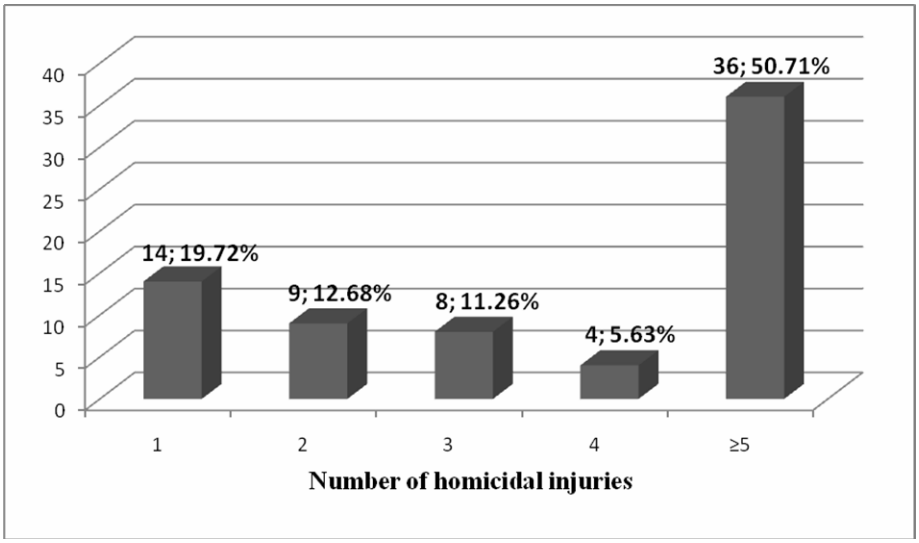

Fig. 3 – Distribution of the victims of homicides with defense injures according to the number of murderous injuries on one victim.

In more than half of the victims with defense injuries (36–50.7% of 71), they were diagnosed on both hands of the victim, which was statistically significantly more frequent than the localization of defensive injuries only on the right (19–26.8% of 71) or on the left hand (16–22.5% from 71) (p = 0.006). There was no difference in gender (p = 0.757), age (p = 0.880), the number of murderous wounds (p = 0.170) and the type of murderous wounds (p = 0.140).

In most cases, defense injuries were localized on the dorsal side of forearms (62–87.33%) and hands (41–57.76%), while they were less frequently registered on the palmar side of hands (23–32.4%) and on the upper arms (13–17%). There was no difference in gender (p = 0.695), age (p = 0.664), the number of homicidal wounds (p = 0.543) and the type of murderous harm (p = 0.343).

There was a statistically significant difference in the type of defense injuries found on murder victims (p < 0.001). Bruises were the most common type of defense injuries (26–36.61%), either as individual or combined with other types of defensive injuries. In most cases (53–74.65% from 71) only one type of defense injuries was found at autopsy, mostly bruises, while a small number of victims (18) showed the simultaneous presence of two different types of defense injuries (Table 4). There was a statistically significant difference between the type of defense injuries in males and females (p = 0.024). Firearm injuries were most common in men and bruises in women (Table 4). In regard to age (p = 0.101), number of murderous wounds (p = 0.288), and the localization of defense injuries (p = 0.678), no statistically significant differences were found. However, there were statistically significant differences in relation to the side of the body affected with murderous injury (p < 0.001) (Table 4). In the victims with murderous injuries only on the front side of the body, the most common of defense injuries were cuts, bruises and abrasions (32 victims). In the victims with murderous injuries on the back side of the body, the most common of defense injuries were cuts and stabbings (6 victims), while in the victims with murderous injuries on both sides of the body, the most common of defense injuries were bruises (7 victims).

Discussion

By studying the analyzed sample it was found that defense injuries represented frequent and significant findings at autopsy of the victims of homicide. They were found in al-

Table 4

Distribution of different types of defense injuries in relation to the total number of homicides with defense injuries and gender of victims

Type of defense injures	Male, n (%)	Female, n (%)	Total, n (%)
Brusies	7 (14.58)	10 (43.48)	17 (23.94)
Incisions	8 (16.67)	5 (21.72)	13 (18.31)
Firearm injuries	10 (20.83)	2 (8.70)	12 (16.90)
Abrasions	8 (16.67)	2 (8.70)	10 (14.08)
Brusies and abrasions	7 (14.58)	–	7 (9.86)
Incisions and stab wounds	4 (8.33)	2 (8.70)	6 (8.45)
Firearm injuries and abrasions	3 (6.25)	–	3 (4.23)
Stab wounds	–	1 (4.35)	1 (1.41)
Brusies and incisions	–	1 (4.35)	1 (1.41)
Firearm injuries and brusies	1 (2.09)	–	1 (1.41)
Total	48 (100)	23 (100)	71 (100)

most half of the murdered individuals from our sample, which is in accordance with results of one earlier study of homicides caused by penetrating forces [12], but it is significantly different from the data of Indian authors, that showed the incidence of murder victims with defense injuries of only 33.3% [13]. During the year 2005 a decline in the number of murders with defensive injuries was registered, and this tendency of decrease in the number of murders, as well as homicides with defensive injuries in the Belgrade population after 2000 has been already demonstrated by earlier researches in our population [14, 15], and can be explained by gradual stabilizing of the social situation in the post-war period.

Male victims were dominant in the sample, which can be explained by the greater criminogenic potential of men and more frequent participation of males in interpersonal conflicts driven by different motives [5, 11, 13, 16–19].

The age of victims was an important factor that determined the appearance of defensive injuries, and the obtained result that victims with defensive injuries were mostly between 21 and 30 years of age, is in accordance with the literature [11, 13, 18, 19]. The highest incidence of defensive injuries in young men is associated with the greatest physical strength of these individuals, as well as the most criminogenic potential in this period of life, as opposed to females who usually suffer from their former or current marriage/common-law partner or the intruder [16].

Analysis of the BAC in the homicidal victims did not support the hypothesis about a possible significant impact of heavy inebriation of victim to its inability to defend against attackers. Katkici et al. [11] have come to similar conclusions. In our analyzed sample 15.49% of the victims with defensive injuries had positive alcohol in blood, while Katkici et al. [11] have found a similarly result (12.31%). Therefore, if attackers estimate that the victim can defend itself, then they resort to the method of committing a murder from a distance (e.g. firearms), and if attackers assess that the victim is unable to defend (e.g. intoxicated, motionless, helpless old people, etc.), then they resort to the method of proximity, especially knife or blunt object.

This study confirmed the results of earlier studies that the use of firearms was important feature of murders in the Belgrade population after 1991 [14, 15]. It is very different from most of other European countries where possession of firearms is strictly controlled and regulated by law, and murders in these countries are mostly committed with sharp mechanical weapons [5]. Murders of women often include close contact between the victim and the attacker (stabbings and cuts, mechanical asphyxia), and it allows occurrence of defensive injuries. On the other hand, men are more often the victims of homicides committed with a firearm, in which defensive injuries usually occur during the previous physical contact, which is followed by the use of firearms [15].

The largest number of murder victims with defensive injuries suffered 5 or more murderous injuries, which is consistent with the literature [11]. With the increasing number of murderous injuries the probability of the presence of defensive injuries constantly increases, due to the prolonged homicidal act. Namely, inflicting of multiple injuries requires a longer time interval and it is typically accompanied with attempts of the victim to defend himself/herself if the consciousness is maintained. Alcohol intoxication may significantly influence the number of both homicidal and defense wounds, because sober victims have a better ability to defend themselves, so they have a larger number of murderous wounds, because the longer period is needed for their disabling, and therefore there is the greater possibility for the occurrence of defensive injuries [17].

The highest incidence of defense injuries in the group of victims with homicidal wounds localized only on the front side of the body can be explained by the fact that such localization of injuries is usually a consequence of the face to face position between the murderer and the victim, which enables the victim to make defensive hand movements toward the attacker. In the forensic sense, the existence of murderous wounds solely on the back side of the body of the victim with defensive injuries may indicate that an attacker approached the victim from behind, but the murder act was not instantaneously fatal, so there was a chance for the victim to defended himself/herself, at least for some time. From the legal point of view, such a situation can be interpreted as a murder committed in insidious way. It is also possible that there were two or more attackers, and that one of them inflicted fatal injuries to the back of the body.

In contrast to the previous studies which showed that defensive injuries were mostly localized on the left hand [11–13], our study shows that about a half of the victims had simultaneous existence of defensive injuries on both hands, which is explained by the intention to defend from murderer in any way.

Defensive injuries can be inflicted by the same weapon that was used for inflicting homicidal wounds to the victim, but they can also be produced by other tools. This often happens in murders committed by multiple attackers using different tools, when at one victim there may be many defensive injuries of various types [13]. In our analyzed sample, the most murder victims sustained only one type of defensive injuries. The bruises were most commonly registered, mostly localized on the dorsal side of the hands and forearms, which is explained by the way of defense of the victim from an attack protruding hands in front of himself/herself as a shield [11]. As previously indicated, defensive blunt injuries can be found in the victim killed by other types of homicidal injuries, for example stabbings, cuts or gunshot wounds. Such blunt injuries typically occur during the fight that precedes the fatal injuring. When blunt tool is used, defensive injuries are usually in the form of numerous contusions and bruises, which are primarily localized on the dorsal side of the forearms and hands [20].

In cases of firearm homicide in which defensive injuries were the gunshot wounds (in 12 cases as isolated, and in 4 combined with abrasions and bruises), they were created by the victim protruding hands in front of himself/herself either in an attempt to catch the attacker's firearm or to instinctively protect the head or the body. These defense wounds were typically localized on the forearms.

In cases of murders committed with a knife, as opposed to more frequent deadly murderous stabbings, most defensive injuries were cuts in comparing to stabbings [20]. The cuts

on the hands arise in the attempt to capture a tool or the arm of the attacker [20]. Similar to our sample, Turkish study shows that most of the victims had passive defensive injuries inflicted by the knife of the attacker [11].

Conclusion

Murders with defense injuries are mostly recorded in men aged from 21 to 30, who are killed in a sober state. The use of firearms is the essential feature of murders of men, while women are typically killed with sharp and pointed weapons. Defensive injuries are usually registered in murders with homicidal wounds predominantly localized on the front of the body. In our analyzed sample, the most murder victims sustained only one type of defensive injuries. The bruises were most commonly registered, mostly localized on the dorsal side of the hands and forearms.

Diagnosing defensive injuries at autopsy, exact determination of their number and accurate description of their appearance, with the obligatory photo-documentation, is of great importance for forensic reconstruction of the course of homicide, its duration, identification of homicidal weapon or weapons, and assessment of physical and mental condition of victims before and during murder. Future researches should indicate whether there is a tendency of maintaining or modifying the characteristics of homicides associated with defensive injuries.

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Assessment of Lyme disease risk by using the ecological risk index in the parks of Belgrade

Procena rizika od lajmske bolesti primenom ekološkog indeksa rizika u parkovima Beograda

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Abstract

Background/Aim. Factors determining the risk of Lyme disease (LD) may be followed in terms of the type of habitat. The evaluation of the risk of *Borrelia burgdorferi (B. burgdorferi)* transmission in humans on *Ixodes ricinus* (*I. ricinus)* tick habitats is done by means of the ecological risk index, which determines the tick habitat, abundance and infection rate. The aim of this paper was to determine the value of ecological risk index (potential and actual risk – PR and AR) on green areas in 9 parks in Belgrade and establish the correlation of this index with tick bites in humans. **Methods.** Ticks were collected in parks by means of the flag hour method and examined for the presence of LD cause in dark-field microscopic analysis. Point values were assigned to certain parameters and potential and actual risk index evaluated for each habitat. The data on tick bites from the surveyed habitats were obtained from the Protocol of patients bitten by ticks of the Sector for Preventive Medicine, Institute of Epidemiology, Military Medical Academy in Belgrade. Analysis of variance (ANOVA), Tukey test and Pearson´s coefficient were used in statistical analysis of data. **Results**. In Belgrade´s park habitats a high PR of *B. burgdorferi* transmission was determined, while AR for 4 habitats was categorised as PR, and limited for other 5 habitats. Statistically, in terms of AR values the following habitats were significantly different ($p < 0.05$): Hajd Park and Tašmajdan; Hajd Park and Kalemegdan; Hajd Park and Pionirski Park; Hajd Park and Banovo Brdo; Topčider and Tašmajdan; Topčider and Kalemegdan; Topčider and Pionirski Park; Topčider and Banovo Brdo Park. A statistically significant correlation ($p < 0.05$) between bites of adults and the number of bites of infected adults with the AR value was established. **Conclusion.** In parks of Belgrade, there is a limited AR of *B. burgdorferi* transmission on the average. The AR values vary from limited to the potential, depending on the ecological features of habitat, the number of collected ticks and their infection rate. In view of the correlation of AR with the bites in humans, this index is significant for assessing LD risk.

Key words:
ticks; risk assessment; borelia burgdorferi; disease vectors; serbia.

Apstrakt

Uvod/Cilj. Faktori koji određuju rizik od lajmske bolesti (LB) mogu se pratiti u odnosu na tip staništa. Procena rizika od prenošenja *Borrelia burgdorferi (B. burgdorferi)* kod ljudi na staništima krpelja *Ixodes ricinus* (*I. ricinus)* vrši se preko ekološkog indeksa rizika, koji određuje stanište, brojnost i zaraženost krpelja. Cilj rada bio je da se odredi vrednost ekološkog indeksa rizika [potencijalnog rizika (PR) i aktuelnog rizika (AR)] na zelenim površinama devet parkova Beograda i utvrdi korelacija ovog indeksa sa ubodima krpelja kod ljudi. **Metode.** Krpelji su u parkovima sakupljani metodom *flag* časa i pregledani na prisustvo uzročnika LB u tamnom vlažnom polju mikroskopa. Bodovanjem određenih parametara po odgovarajućim skalama, procenjivani su potencijalni i aktuelni indeks rizika za svako stanište. Podaci o ubodima krpelja sa ispitivanih staništa dobijeni su iz Protokola pacijenata sa ubodom krpelja Instituta za epidemiologiju Sektora za preventivnu medicinu Vojnomedicinske akademije u Beogradu. Pri statističkoj obradi podataka, korišćeni su analiza varijanse (ANOVA), Tukey test i Pearsonov koeficijent. **Rezultati**. Na staništima parkova Beograda ustanovljen je visok PR transmisije *B. burgdorferi*, dok je AR 4 staništa svrstan u kategoriju mogućeg rizika, a na ostalih 5 staništa procenjen je kao ograničen. Po vrednostima AR statistički značajno su se razlikovala ($p < 0,05$) staništa: Hajd park i Tašmajdan; Hajd park i Kalemegdan; Hajd park i Pionirski park; Hajd park i Banovo brdo; Topčider i Tašmajdan; Topčider i Kalemegdan; Topčider i Pionirski park; Topčider i Park Banovo brdo. Utvrđena je statistički značajna povezanost ($p < 0,05$) broja

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uboda odraslih i broja uboda zaraženih odraslih jedinki, sa vrednošću AR. **Zaključak.** U parkovima Beograda, u proseku postoji ograničen AR transmisije *B. burgdorferi*. Vrednosti AR variraju od ograničenog do mogućeg, zavisno od ekoloških karakteristika staništa, broja sakupljenih krpelja i njihove inficiranosti. S obzirom na to da postoji korelacija AR sa ubodima kod ljudi, ovaj indeks je od značaja za procenu rizika od LB.

Ključne reči:
krpelji; rizik, procena; borrelia burgdorferi; bolest, prenosioci; srbija.

Introduction

Lyme disease (LD) has been present in Serbia since 1987, with the constant increase in the number of infected persons [1]. The ticks of *Ixodes* species play a primary role in transmitting the disease, and in our environment specifically *Ixodes ricinus (I. ricinus)* whose bite can infect and make people contract this disease [1–3]. Based on surveys conducted in Belgrade, a number of habitats were marked as natural foci of LD. These habitats carry the risk of bites by infected ticks, especially during the season of their activity, from March until October [4]. This risk increases with the length of exposure to ticks' habitats. People whose regular professional activities are carried out in nature are especially at risk (hunters, forestry workers, workers maintaining green spaces, farmers, veterinarians, soldiers, etc.), as well as those who temporarily visit nature for recreational purposes or walking. In addition to this, the risk of being infected with the spirochete *B. burgdorferi* depends on the abundance of ticks and the rate of infection with the cause of LD in ticks [5, 6]. Past surveys in Belgrade used the entomologic risk index (ERI) [7] to evaluate the risk of infection with *B. burgdorferi*. Ecological risk index was also determined for a number of habitats, but no correlation of the actual risk (AR) index with bites in humans on these habitats was found [8].

The aim of this study was to determine the ecological risk index of transmitting *B. burgdorferi* in green spaces of nine parks in Belgrade, to prove the correlation of this index with bites in humans and based on all this to evaluate the risk of infection with LD.

Methods

The survey encompassed 9 park spaces in Belgrade, with the most frequent occurrence of bites in patients. Ticks were collected monthly by using a 1 m^2 flannel cloth (flag-hour method) for 1 h. It was checked every 20 m and attached ticks were removed, counted and placed in humidified vials and transported to a laboratory for further investigations. Tick density was expressed by the flag hour value (f/h): number of ticks sampled *per* 1 hour [9]. This survey covered adult forms and nymphs [10, 11]. Identification and determination of ticks in laboratory was performed in accordance with taxonomic keys and species descriptions. The presence of *B. burgdorferi* in ticks was detected by dark field microscopy with the magnification rate of × 400 [12].

Ecological index of *B. burgdorferi* transmission was evaluated in the following 9 habitats: Hajd Park, Bele Vode Park, Ušće, Šumice, Kalemegdan, Topčider, Tašmajdan, Banovo Brdo Park and Pionirski Park. The surveyed habitats belonging to the ecological category of parks are characterised by certain ecological features and influenced by a degree of anthropogenic factor, thus differentiating them from other ecological categories (forests, forest parks).

Hajd Park (140 m above sea level, surface area of 7.3 ha) – beside perennial vegetation [plane-tree, oak, hazel, lime-tree, thorny bushes, beech, turkey (bitter) oak, willow, *forsythia*], numerous annual green plants are also present. The park is organised for recreation purposes (trim-trails, exercise equipment). It is surrounded by roads, intercepted with streets linking it with the residential area and the city center. Apart from plants, several types of mouse-like rodents, squirrels and great many birds have been registered in the park. Stray dogs, as well as pets taken out by citizens can be also found there.

Bele Vode Park (139 m above sea level, surface area of 8.0 ha) is divided into two parts by one street. "Ibarska magistrala" main road is located on the upper side of the park. A part of the park is rather derelict, uncared for, covered in shrub vegetation. The remaining part is organised for recreation purposes (playgrounds, children's park with equipment). Perennial woody plants are present, such as: walnut tree, locust tree, chestnut, pine, birch, and *forsythia* of shrub vegetation. Of annual plants there are many sorts of grass from the *Gramineae* family. The presence of mouse-like rodents, squirrels and many birds has been registered in the park.

Ušće Park (89 m above sea level, together with the Park Prijateljstva having 150 ha surface area). Over 90% of the habitat is planned. Ušće is connected with roads to all city parts and facilities on water. There are many recreational trails in Ušće (a bicycle track particularly stands out), great many restaurants, shopping and cultural facilities, and a part of the space is used for public events (concerts). This habitat is comprised of the following vegetation: a poplar tree, pine-tree, weeping willow, *forsythia*, birch-tree, thorny bushes and lime-tree, as well as annual species, mostly grass of the *Gramineae* family. Ušće abounds with animals such as mouse-like rodents, squirrels, as well as numerous birds. Walking the pets is allowed in Ušće, but limited in terms of time. Ušće is an area visited a lot by people.

Šumice Park (130 m above sea level, surface area of 1.0 ha) is mainly under perennial vegetation (plane-tree, common lilac, hazel, oak, *forsythia*, plum). Annual plants from the *Gramineae* family are mainly present, and as for animals: mouse-like rodents, squirrels, pets, stray dogs and many birds. The park is surrounded by roads. Recreational trails are built inside the park, as well.

Banovo Brdo Park (97 m above sea level, surface area of 4.0 ha) is intercepted with numerous trails, with many exercise devices especially tailored for recreational purposes. The park is

comprised of the following vegetation: hazel, pine, chestnut, weeping willow and *forsythia*, and grass of the annual vegetation. The following animals are present: mouse-like rodents, squirrels, stray dogs, pets and many species of birds. The park has good connections with the main road and residential area. There are many hospitality facilities on the outskirts of the park.

Kalemegdan (125.5 m above sea level, surface area of 57 ha), has good connections with all city roads. A fortress is on one side and the Zoo on the other side. Vegetation is comprised of the perennial woody plants: plane-tree, beech, common oak, Turkish hazel, many sorts of conifer trees and bushy vegetation, and various annual plants from the *Gramnineae* family. Beside many fortress visitors, there are many people engaged in sports (basketball, tennis grounds), recreation and guests (pubs, zoo garden). Of animals there are many kinds of birds, squirrels and stray dogs in Kalemegdan.

Topčider (78 m above sea level, surface area of 35 ha) – one half of this space is dedicated to an organised park space and the other half to forests. The perennial vegetation is comprised of: turkey oak, spruce tree, silver fir, oak, plane-tree, lime-tree and cypress, and of bushy vegetation such as: hazel, locust-tree and hawthorn. Annual grass, bushy trees and flowers can be found in the part belonging to the park space. This space is organised with trails, benches and hospitality facilities, thus good many people visit it. Numerous birds, squirrels, hedgehogs, mouse-like rodents live in the park and forest parts.

Tašmajdan (136 m above sea level, surface area of 6 ha) – the vegetation is comprised of perennial woody and bushy plants, as well as annual grass. Besides plane-tree and common oak, horse chestnut is also present. This space is intertwined with walking trails, furnished with benches to relaxation, and the outskirts of park are dedicated for hospitality facilities. It is highly frequented by people during the year.

Pionirski Park (126 m above sea level, surface area of 3.6 ha) – this habitat is comprised of great many common oaks, nettle trees (*celtis australis*), gingko trees, horse chestnuts, annual grass and flowers. The park is organised for relaxation and recreation, with great many trails, benches and street lights. A part of park is cultivated (with flowers), and the other part is under grass. Apart from numerous birds, squirrels and pets, the presence of mouse-like rodents was registered in the park.

In order to evaluate the ecological risk index, a value of potential (PR) and AR of *B. burgdorferi* [13] transmission was determined for each selected habitat of infected *I. ricinus* ticks. The suitability, amount and accessibility of habitat are parameters given point values of 1 to 5 according to the previously prepared chart. The resulting score of those ecological parameters represents the value of potential risk for a certain habitat. PR categories are divided into three groups: I, high risk (11–15 points); II, moderate risk (6–10 points); III, low risk (less than 6 points).

In order to evaluate the AR of *B. burgdorferi* transmission, two more parameters were established: the amount of *I. ricinus* ticks collected in 1 hour, as well as the rate of infection found in them, rated according to the same principle. The total points of all five parameters for one habitat results in the key value of AR of *B. burgdorferi* [13] transmission. The habitats can be divided into five groups according to the number of points: habitats with a definite risk, from 21 to 25 points; habitats with a possible (potential) risk, from 16 to 20 points; habitats with a limited risk, from 11 to 15 points; habitats having no present risk, from 6 to 10 points; habitats having no risk likely, less than 6 points.

The Protocol of patients bitten by ticks from the Institute of Epidemiology, Sector for Preventive Medicine of the Military Medical Academy in Belgrade was used as a source of data on the number of tick bites in people on the surveyed habitats. The protocol keeps records on removed tick bites from patients who were treated in the Institute of Epidemiology, and who were bitten by ticks on the surveyed habitats in Belgrade.

Statistical analysis

Analysis of variance (ANOVA) was conducted to compare average tick densities and average tick infection rates. Secondary analysis was performed by using Tukey test. Correlations between AR values of each locality with the number of tick-bitten humans were assessed by using Pearson′s correlation coefficient. Each p value of < 0.05 was considered statistically significant.

Results

A total of 856 ticks (adult and nymphal forms) were collected from the surveyed Belgrade´s parks during one season of ticks' activity, from March until October. Table 1 shows the average value and standard deviation of the number of collected ticks. There was almost two and a half times greater number of collected adult ticks (11.06 ± 8.64) than nymphs (4.67 ± 1.83).

The average value of flag-hour (f/h) for adult ticks amounted to 11.7 f/h in the surveyed habitats, and 4.68 f/h in case of nymphs. The greatest numbers of adult ticks (30.63 f/h) were collected in Topčider Park, while the number of nymphs was almost seven times smaller (4.50 f/h). In Pionirski Park habitat, flag-hour of nymphs (1.38 f/h) and adult ticks (1.75 f/h) marked the lowest values. The number of adult ticks was approximately two times greater in several park habitats: Banovo Brdo, Kalemegdan, Šumice, Ušće (Table 2).

Table 1

Number of collected ticks in the selected parks

Forms of tics	$\bar{x} \pm SD$
Both forms of ticks	14.9 ± 9.15
Adults	11.06 ± 8.64
Nymphs	4.67 ± 1.83

Table 2

Value of flag-hour (f/h) in parks of Belgrade

Parks in Belgrade	Adults ticks (f/ h)	Nymphs (f/ h)
Hajd park	16.38	6.88
Park Bele vode	11.13	7.38
Ušće	10.25	5.13
Šumice	12.75	5.50
Kalemegdan	6.88	3.75
Topčider	30.63	4.50
Tašmajdan	3.50	4.13
Park Banovo Brdo	6.38	3.38
Pionirski park	1.75	1.38
The average value	11.07	4.68

The ticks collected in the surveyed parks were infected 19.0% on the average. The greatest number of infected ticks, 33.2%, was registered in Hajd Park, and the least one in Šumice (14.1%) and Banovo Brdo (13.8%) (Table 3). Bele Vode and Kalemegdan parks had a similar percentage of infected ticks (21.5% and 22.3%, respectively). Almost the same percentage of infected ticks was registered in Pionirski Park (15.7%) and Tašmajdan (15.9%) habitats, just like Topčider and Ušće that had almost equal representation of infected ticks (16.7% and 17.8%, respectively).

The average value of PR was classified as a high risk category (11.66 ± 1.32) in the surveyed park habitats. The PR value ranged between 10 in Kalemegdan and Banovo Brdo parks to 13 in Bele Vode Park. In Hajd Park, Topčider and Tašmajdan, PR value was rated with 11, while Ušće and Šumice had one point more (Table 3). The average value of AR in all surveyed parks classifies them as limited risk of transmitting LD cause (14.83 ± 2.41). The AR values in habitats were obtained by including the values of the number of collected ticks *per* flag/hour and their infection rate (Table 3). Four habitats (Hajd Park, Bele Vode Park, Šumice, Topčider) were assigned with the possible AR, while other five parks were classified as habitats with limited AR (Ušće, Kalemegdan, Tašmajdan, Banovo Brdo Park, Pionirski Park). The lowest evaluated AR in parks was demonstrated in Kalemegdan (12.13). In Tašmajdan and Banovo Brdo the AR values (12.25) were equal, almost identical to the previous habitat. Slightly higher AR was demonstrated in Pionirski Park (13.5). Ušće Park was rated with 15, while Bele Vode and Šumice were assigned with some more points (16.13). The greatest risk of transmitting the cause of LD was demonstrated in Hajd Park (17.88) and Topčider (18.25).

In terms of the months observed in park habitats, AR value rises from March (Figure 1) and reaches its peak value (19.4) as early as April, then starting to gradually decline without a significant variation in the trend and ultimately reaching the lowest value in October (12.3).

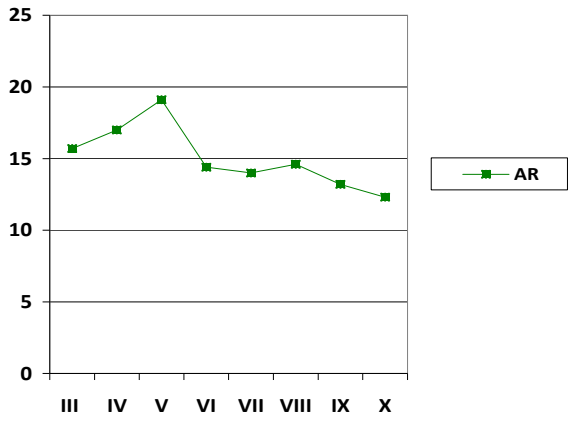

Fig. 1 – The values of actual risk (AR) by months.

Table 3

Number of collected adult ticks (f/ h), percent of infected adult ticks, potential risk and actual risk in the parks of Belgrade

Parks in Belgrade	Number of collected adult ticks (f/ h)	Percent of infected adult ticks (%)	Potential risk	Actual risk
Hajd park	16.38	33.2%	11	17.88
Park Bele vode	11.13	21.5%	13	16.13
Ušće	10.25	17.8%	12	15.00
Šumice	12.75	14.1%	12	16.13
Kalemegdan	6.88	22.3%	10	12.13
Topčider	30.63	16.7%	11	18.25
Tašmajdan	3.50	15.9%	11	12.25
Park Banovo brdo	6.38	13.8%	10	12.25
Pionirski park	1.75	15.7%	12	13.50

f/h – flag-hour.

Of the park habitats, the following habitats were statistically significantly different in terms of AR values: Hajd Park and Tašmajdan ($p < 0.05$); Hajd Park and Kalemegdan ($p < 0.05$); Hajd Park and Pionirski Park ($p < 0.05$); Hajd Park and Banovo Brdo ($p < 0.05$); Topčider and Tašmajdan ($p < 0.05$); Topčider and Kalemegdan ($p < 0.05$); Topčider and Pionirski Park ($p < 0.05$); Topčider and Banovo Brdo Park ($p < 0.05$). This comparison is shown in Table 4. The greatest AR was estimated in Topčider (18.25) and Hajd Park (17.88).

By comparing the AR values between the surveyed park habitats, we came to the conclusion that the values for Hajd Park and Topčider statistically differ ($p < 0.05$) from the values obtained for Tašmajdan (12.25), Kalemegdan (12.13) and Banovo Brdo (12.25), which represent the lowest AR values in the group of the surveyed parks.

On the surveyed habitats, 89 adult tick bites (Table 5) were reported in patients within the period March-October. The greatest number of adult tick bites was reported in Topčider (32), and the lowest number in Pionirski Park (1). In Hajd Park (17) and Ušće (16), the number of tick bites was almost the same, and two times less frequent than in Topčider. In terms of the number of infected adult ticks removed from humans, Hajd Park (7) and Topčider (6) were the leaders, while the ticks sampled in Šumice, Kalemegdan and Pionirski Park were not infected with *B. burgdorferi*.

By applying Pearson's correlation coefficient, a statistically significant correlation between the number of adult tick bites ($p < 0.05$) and the number of infected adult tick bites ($p < 0.05$), with the value of AR (Figure 2) was established.

Table 4

Comparison of actual risk value between localities of parks

Parameters	ANOVA		Tukey-test
	F	*p*	*p*
Actual Risk	6.16	< 0.001	
Hajd Park : Tašmajdan			< 0.05
Hajd Park : Kalemegdan			< 0.05
Hajd Park : Pionirski Park			< 0.05
Hajd Park: Park Banovo brdo			< 0.05
Topcider : Tašmajdan			< 0.05
Topcider : Kalemegdan			< 0.05
Topcider : Pionirski Park			< 0.05
Topcider : Park Banovo brdo			< 0.05

Table 5

Frequency of tick bites in the selected parks of Belgrade

Parks in Belgrade	Number of tick bites (adults ticks)	Number (%) of tick bites (infected adults ticks)
Hajd park	17	7 (41.2)
Park Bele vode	8	1 (12.5)
Ušće	16	3 (18.7)
Šumice	5	0 (0)
Kalemegdan	3	0 (0)
Topčider	32	6 (18.7)
Tašmajdan	2	1 (50.0)
Banovo Brdo	5	2 (40.0)
Pionirski park	1	0 (0)
Total	89	20 (22.5)

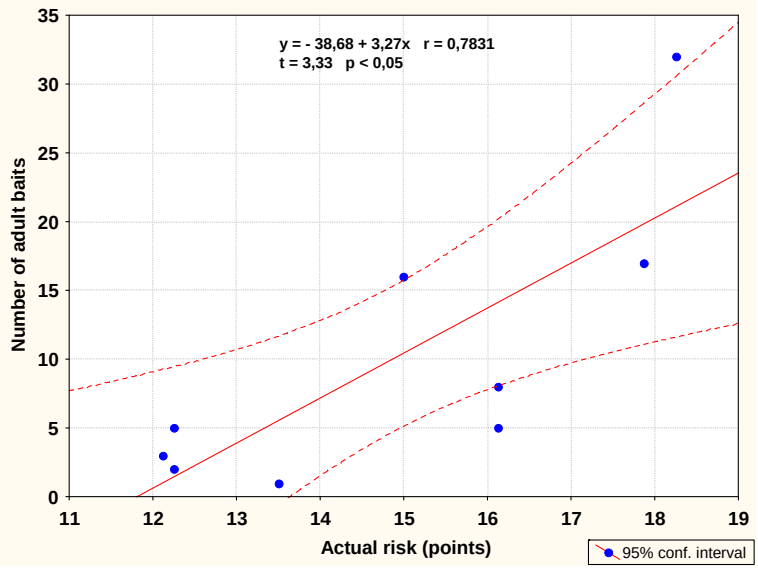

Fig. 2 – Correlation between tick bites of adults ticks and actual risk (AR) in parks.

Discussion

Factors determining the risk of LD may be followed in terms of habitat type, which was the subject of research for many authors [14]. Some scientists apply entomologic risk index [15, 16] to evaluate the risk of infection with *B. burgdorferi* in humans, while others use ecological risk evaluation, *ie* evaluation of PR and AR on Lyme disease vector habitats [14]. The method of evaluating ecological risk considers key ecological parameters (accessibility and the amount of habitat, the composition of habitat in terms of vegetation, suitability of habitat), varying from habitat to habitat, depending on their ecological category, as well as the abundance and the rate of infected adult *I. ricinus* ticks. The assessment of risk by using this method was used in our country in previous surveys made in Belgrade [8] and in the area of Vojvodina [17]. In addition to the assessment of risk index, our survey, unlike past surveys, conducted the comparison of risk index with tick bites in humans in the surveyed habitats.

Abundance of ticks in the specific habitat is one of the factors determining the risk of LD. Abundance of ticks depends on the type of habitat, *ie* ecological category (park, forest-park, forest). Some authors [18] have pointed at the greater abundance of ticks in habitats covered with richer vegetation, with lesser or minimized impact of anthropogenic factors (forest-parks and forests). The category of parks is identified in terms of its ecological features as having mainly annual vegetation, small wild animals, stray dogs, cats, pets taken out for walk by their owners. The human factor has a greater influence on these habitats in terms of a better management of vegetation and the presence of concrete tracks, sport grounds, hospitality facilities, and thus the density of ticks is smaller [19]. Adult ticks are taken into account for the assessment of AR. In the course of our survey, two and a half times greater number of adult forms (11.06 ± 8.64) than nymphal ones (4.67 ± 1.83) was collected. An average value of flag/hour was 11.07 f/h, and it ranged from the minimum value in Pionirski Park of 1.75 f/h to the highest value in Topčider Park of 30.63 f/h. In the survey conducted in Belgrade green areas in 2004, the values of flag/hour were somewhat lower ranging from 1.6–23.4 f/h, with 9.2 f/h. [8] as an average value. During the survey of the risk of infection with LD cause in workers maintaining green areas in Belgrade, the average value of flag/hour in park habitats was even lower, 8.7 f/h on the average, ranging between 8.2 and 9.2 f/h [7]. In 2010 Čekanac et al. [20] obtained higher values of flag/hour, 17.9, in the territory of Belgrade, while in surveyed green areas habitats in Vojvodina, Potkonjak, et al. [17] calculated flag/hour values of 2–80.0 f/h, while flag-hour values in the park next to the railway station (3.0 f/h) and in Kamenički Park (15.0 f/h) were close to our surveys conducted in Tašmajdan (3.50 f/h) and Hajd Park (16.38 f/h).

In our survey, the average rate of infection in *I. ricinus* ticks with *B. burgdorferi* spirochete amounted to 19.0% on the average, ranging in habitats from 13.8% in Banovo Brdo Park, to the highest of 33.2% in Hajd Park. In surveys made by Krstić and Stajković [8], the infection rate in Hajd Park was almost twice lower (19.8%), and four times greater in Tašmajdan (4.0%) compared to our results. These data point to the fact that the tick infection rate on a habitat is not a constant value, but prone to changes depending on the season, presence and diversity of reservoirs and other ecological factors [20–23]. During the survey conducted in 2007 [7], ticks in Belgrade´s parks were infected 13.65% on the average, while Potkonjak, et al. [17] published the infection prevalence in ticks from 0–33.1% in the territory of Vojvodina. Stajković, et al. [4] found the infection prevalence in *I. ricinus* of 27.0–31.7% in Belgrade´s green areas. Estonian authors [24] detected lower values of *B. burgdorferi* prevalence (9.7 %), while Romanian researchers [25] registered the infection in ticks on 183 sites ranging from 0.75% to 18.8%.

Determination of ecological risk encompasses the assessment of PR and AR in one habitat. By rating the appropriate parameters that determine PR (habitat suitability, amount and accessibility) we obtained PR values for each surveyed park. In view of the fact that PR values of 9 Belgrade´s parks were not mutually significantly varied, based on their average value, these habitats were categorised as having high risk (11.66 ± 1.32). PR ranged from the lowest value in Kalemegdan and Banovo Brdo parks (10) to the highest in Bele Vode Park (13). Similarly, in the previous survey in 2004 conducted on the majority of Belgrade´s habitats the PR was classified as high, as 13 localities had high PR, and only 3 moderate. In line with our results, Potkonjak, et al. [17] registered also high PR of LD cause transmission in Vojvodina in 8 of 12 surveyed habitats in 2011.

Aimed at assessing the AR of *B. burgdorferi* transmission, beside ecological parameters (suitability, amount, accessibility of habitat) we rated two more parameters: abundance of *I. ricinus* collected during 1 h and their infection rate with *B. burgdorferi* [14]. A total of points for all the mentioned parameters resulted in the AR value of the specific habitat. Four park habitats in Belgrade (Hajd Park, Bele Vode Park, Šumice, Topčider) were classified as potential AR, while other five (Ušće, Kalemegdan, Tašmajdan, Banovo Brdo Park, Pionirski Park) were evaluated as habitats with a limited AR of LD cause transmission. Since ecological parameters are specific for each habitat, and the abundance and tick infection rate vary depending on the season, climate factors, the presence, type and the number of *B. burgdorferi* reservoirs, our AR values are different for each habitat, and the months in the season of tick activity. The average AR value in Belgrade´s park habitats starts to rise at the beginning of season – March, and reaches its maximum value in April, then gradually starts to decline with some variation until October. The parks with the highest AR values are: Hajd Park with 17.88 and Topčider with 18.25, they are in terms of statistics significantly different ($p < 0.05$) than the parks of Kalemegdan (12.13), Tašmajdan (12.25), Banovo Brdo (12.25) and Pionirski Park (13.50), where the lowest number of *I. ricinus* ticks was collected. Similarly, the authors from Vojvodina [17] determined a limited AR for the majority of surveyed habitats (six of twelve), while evaluated a definite AR of *B. burgdorferi* transmission in three habitats, unlike our survey that did not detect habitats with the definite

AR. In past were surveys conducted in the territory of Belgrade (2004) [8], AR ranged from the limited to the definite in surveyed parks and varied depending on the month of survey.

The number of tick bites in patients from the surveyed habitats was used to evaluate the importance of AR determination in habitats. During the season of tick activity, 89 bites by adult ticks were registered in patients, with the *B. burdorferi* prevalence of 22.5%. Almost the same infection rate in removed ticks in Belgrade territory (2010) was detected by Mladenović et al. [26] (21.25%). The greatest number (32) of bites was registered in Topčider, where the AR value was the highest. The removed tick infection rate was the highest in ticks from Hajd Park (7) and Topčider (6), which, in terms of AR value, were statistically significantly different from other parks. In order to evaluate the LD risk, some authors have determined a correlation between the density or prevalence of ticks with seropositivity or the appearance of LB symptoms [27–30], while some determine the correlation of entomologic risk with the Lyme disease incidence [31]. We compared the number of adult tick bites and the number of infected adult tick bites with the AR values on the same habitats of bite occurrence. The correlation between tick bites and AR of *B. burgdorferi* transmission ($p < 0.05$) suggests that the method of assessing ecological risk is useful in LD risk assessment.

Conclusion

In Belgrade´s parks there is a limited AR of *B. burgdorferi* transmission on the average. AR values vary in parks from limited to possible, depending on the ecological features of habitats, the number of collected ticks and their infection rate. In view of the correlation of AR with bites in humans, this index is a significant one for the assessment of LD risk.

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The importance of training and education in performing total mesorectal excision in rectal cancer surgery

Značaj obuke i obrazovanja u izvođenju totalne mezorektalne ekscizije u hirurgiji karcinoma rektuma

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Abstract

Background/Aim. In the last two decades there has been a significant progress in rectal cancer surgery. Preoperative radiotherapy, the introduction of staplers and largely improved surgical techniques have greatly contributed to better treatment outcomes, primarily by reducing the frequency of early surgical complications and the rate of local recurrence. The aim of this study was to compare operative and postoperative results in the treatment of rectal cancer between the two groups of surgeons – those who are closely engaged in colorectal surgery and those who deal with these issues sporadically. **Methods**. This retrospective study included 146 patients who had underwent rectal cancer surgery at the Institute of Oncology of Vojvodina in the period from January 1, 2008 to December 31, 2010. The patients were divided into two groups, the group N1 of 101 patients operated on by trained colorectal surgeons, and the group N2 of 45 patients operated on by surgeons without training in totalmesorectal excision (TME). **Results**. Preoperative chemoradiotherapy was received by 49 (33.56%) of the patients. A statistically significant difference between the two groups was noted in the duration of surgery and the need for blood transfusion during surgery. Anastomotic leakage occurred in 3 patients from the group N1 and in 10 patients from the group N2. Seven (4.79%) of the patients developed local recurrence after surgical treatment. There were significant differences in local recurrence rate and anastomotic leakage rate between the compared groups. **Conclusion**. It is necessary to continue education and training in surgery for rectal cancer to master new technologies and surgical techniques and to improve the results of surgical treatment.

Key words:
rectal neoplasms; carcinoma; digestive system surgical procedures; surgeons; education, professional; treatment outcome.

Apstrakt

Uvod/Cilj. U poslednje dve decenije postignut je značajan napredak u hirurgiji karcinoma rektuma. Preoperativna radioterapija, uvođenje staplera kopči i ponajviše unapređena hirurška tehnika umnogome su doprineli boljim rezultatima lečenja, pre svega smanjivanjem učestalosti ranih hirurških komplikacija i stope lokalnih recidiva. Cilj ovog istraživanja bio je da se uporede operativni i postoperativni rezultati u lečenju karcinoma rektuma između dve grupe hirurga – onih koji se usko bave kolorektalnom hirurgijom i onih koji se ovom problematikom bave sporadično. **Metode**. Ova retrospektivna studija obuhvatila je 146 bolesnika koji su operisani na Institutu za onkologiju Vojvodine u periodu od 1. 1. 2008. do 31. 12. 2010. godine. Bolesnici su bili podeljeni u dve grupe. U prvoj grupi N1 bio je 101 bolesnik koje su operisali visoko edukovani hirurzi za totalnu mezorektalnu eksciziju (TME), a u drugoj N2 bilo je 45 bolesnika koje su operisali hirurzi bez edukacije iz TME. **Rezultati**. Preoperativnu hemioiradijaciju primilo je 49 (33,56%) bolesnika. Utvrđena je statistički značajna razlika između dve grupe u trajanju operacije i potrebi za krvnim derivatima tokom operacije. Dehiscenciju anastomoze indentifikovali smo kod 3 bolesnika iz N1 grupe i kod 10 bolesnika iz N2 grupe. Sedam (4,79%) bolesnika razvilo je lokalni recidiv nakon operativnog lečenja. Statistički značajna razlika ustanovljena je u broju lokalnih recidiva i dehiscencije anastomoze između dve upoređivane grupe. **Zaključak**. Neophodno je kontinuirano obrazovanje i obuka u hirurgiji karcinoma rektuma kako bi se savladale nove tehnologije i hirurške tehnike, te unapredili rezultati hirurškog lečenja.

Ključne reči:
rektum, neoplazme; karcinomi; hirurgija digestivnog sistema, procedure; hirurzi; edukacija, profesionalna; lečenje, ishod.

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Introduction

Preoperative staging, use and timing of neoadjuvant and adjuvant chemioradiotherapy (CRT), surgical technique, reconstructive options and protocols in the management of rectal cancer have evolved over the past two decades [1–5].

As a result, the management of patients with rectal cancer has become highly complex, so it is essential that surgeons acquire and maintain knowledge of rectal cancer treatment issues [6–9].

The importance of surgeon knowledge and training was illustrated in a study of Richardson et al. [6] in which patients with rectal cancer were more likely to receive sphincter-preserving surgery and were less likely to experience local recurrence if they were treated by a surgeon with greater knowledge of rectal cancer care.

The aim of the study was to compare operative and postoperative results of rectal cancer surgical treatment performed by two groups of surgeons, the first one of highly skilled and educated colorectal surgeons, and the second one of surgeons performing colorectal operations sporadically.

Methods

This retrospective study included 146 patients, operated on at Institute of Oncology of Vojvodina in the period from January 1, 2008 to December 31, 2010. The patients were divided into two groups, the group N1 of 101 (69.18%) patients operated on by high educated surgeons in rectal cancer surgery, and in the second group N2 of 45 (30.82%) patients operated on by non- colorectal surgeons (general surgeons without special training in rectal cancer surgery). The avreage age of the patients in the group N1 was 66.15 (range 43–84), and in the group N2 63.71 (range 39-84). In both groups there were 82 (56.16%) male and 64 (43.84%) female patients.

Preoperative staging included clinical examination, endorectal ultrasonography (ERUS), computed tomography (CT) of the abdomen and pelvic magnetic resonance imaging (MRI) in all the cases.

The presence of operable secondary deposits in the liver and/or lungs did not exclude patients from the study.

Rectal adenocarcinoma (3–18 cm from the anal verge), after colonoscopy and histopathological (HP) examination of the tumor, was verified in all the patients (146, 100.00%).

Preoperative chemoradiation therapy (CRT) was performed with the total dose of 50.4 Gray (Gy) divided into 25 fractions, with the daily dose of 1.8 Gy. Chemotherapy was carried out with radiation therapy in order to increase the sensitivity of tumor tissue to radiation. The patients received calcium 5-fluorouracil and leucovorin (5-FU/LV), on the day 1, 2, 10, 11, 20 and 21 of radiotherapy. Surgery was performed 8 to 10 weeks after the completion of CRT.

We performed low anterior resection (LAR) or high anterior resection (HAR), both with total mesorectal excision (TME) and by using single or double stapler technique for creation of colorectal anastomosis.

Software SPSS V.16. was used for the purposes of statistical analysis. All the data were statistically analyzed (percentage, average value, range) and presented in tables. Both Fischer's exact tests and χ^2 tests were used to compare the data between the groups. Values of $p < 0.05$ were considered as statistically significant.

Results

The average distance from the anal verge in the group N1 was 8.72 cm, while in the group N2 it was 9.16 cm. Tumor in the distal rectum (3–7 cm) was present in 52 (35.62%) of the patients. Among the total number of patients, distant metastases were found in 17 (11.64%) of the patients, in the liver (14 patients) and in the lungs (3 patients). A certain number of patients were classified as ASA 2 (49.32%).

Histopathological analysis showed a moderately differentiated tumor (GII) in most of the patients (71.92%). The majority of patients (82 (56.16%)) had no metastases in the lymph nodes (Table 1).

Anasthomosis was performed with double stapler technique in 110 (75.34%) of the patients, and by single stapler technique in 36 (24.66%) of the patients in both groups. For the group N1, the mean operation time was 104 min, and in the group N2 136 min (a statistically significant difference with $p = 0.000001$). Fifty-seven of the patients needed blood transfusion, from the group N1 21, and from the group N2 36 ($p = 0.00003$).

Protective transversostomy was performed in 27 of the patients from the group N1 and in 10 patients from the group N2.

Preoperative CRT was received by 49 (33.56%) of the patients, 42 in the N1 and 7 in the group N2 (Table 2).

Anasthomotic leakage was noticed in 3 of the patients from the group N1 and in 10 from the group N2. This difference was statistically significant ($p = 0.0004$).

Seven (4.79%) of the patients (2 from the N1 and 5 from the group N2) developed a local recurrence, which is a statistically significant difference between the two groups in the local recurrence rate. Due to the postoperative complications, 6 of the patients died a month after the operation (Table 3).

Discusion

Improved screening, surgical techniques, a more effective chemotherapy, radiation therapy and improved imaging have lead to better results in rectal cancer treatment.

Many authors have shown improved outcomes among patients with rectal cancer who were treated by surgeons with subspecialty training (colorectal surgeons) [6–8]. This includes increased use of sphincter-preserving surgery, decreased local recurrence [8], decreased anastomotic leakage [9], decreased postoperative mortality and improved survival [10]. This variation in the outcome may reflect on the differences in surgical technique, especially in the technique of TME [11–13].

Anastomosis distance from the anal verge and preoperative CRT are one of the most important risk factors for anastomotic leakage [1, 2, 10, 14–16].

In their study on 1,014 patients, Vignali et al. [15] determined clinical signs of anastomosis leakage in 2.9% of cases

Table 1

Tumor characteristics

Characteristics	N_1	N_2	Total $N_1 + N_2$
Tumor distance from the anal verge, (cm) mean (range)	8.72 (3–18)	9.16 (3–16)	8.86 (3–18)
3–7 cm, n (%)	39 (26.71)	13 (8.90)	52 (35.62)
8–16 cm, n (%)	62 (42.47)	32 (21.92)	94 (64.38)
Pathological tumour stage, n (%)			
I	15 (10.27)	5 (3.42)	20 (13.70)
II	73 (50.00)	36 (24.66)	109 (74.66)
III	13 (8.90)	4 (2.74)	17 (11.64)
T – tumor, n (%)			
0	9 (6.16)	1 (0.68)	10 (6.85)
1	10 (6.85)	3 (2.05)	13 (8.90)
2	24 (16.44)	8 (5.48)	32 (21.92)
3	55 (37.67)	30 (20.55)	85 (59.22)
4	3 (2.05)	3 (2.05)	6 (4.11)
N – nodes, n (%)			
0	55 (37.67)	27 (18.49)	82 (56.16)
1	24 (16.44)	11 (7.53)	35 (23.97)
2	22 (15.07)	7 (4.79)	29 (19.86)
M – metastases, n (%)			
0	88 (60.27)	41 (28.08)	
1	13 (8.09)	4 (2.74)	
liver	10 (6.85)	4 (2.74)	
lungs	3 (2.05)	0 (0.00)	
Avreage number of extirpated lymphnodes, mean (range)	11.26 (0–40)	10.82 (0–28)	11.12 (0–40)
1–3, n (%)	31 (21.23)	11 (7.53)	42 (28.77)
≤ 3, n (%)	15 (10.27)	7 (4.79)	22 (15.07)

N_1 – group of patients operated on by trained colorectal surgeon;
N_2 – group of patients operated on by general surgeon;
n – number of patients.

Table 2

Preoperative, operative and postoperative data

Parameters	N1	N2	Total N1+N2	*p*
Type of stapler anasthomosis, n (%)				
single	11 (7.53)	25 (17.12)	36 (24.66)	2.788
double	90 (61.64)	20 (13.70)	110 (75.34)	
Mean operation time (min)	103.91	135.67	113.7	0.000001
Protective colostomy, n (%)				
yes	27 (18.49)	10 (6.85)	37 (25.34)	0.36
no	74 (50.68)	35 (23.97)	109 (74.66)	
ASA classification, n (%)				
1	6 (4.11)	4 (2.74)	10 (6.85)	
2	50 (34.25)	22 (15.07)	72 (49.32)	
3	45 (30.82)	19 (13.01)	64 (43.84)	
Blood transfusion, n (%)	21 (14.38)	36 (24.66)	57 (39.00)	0.00003
Mean hospital stay in days (range)	15.9 (10–28)	15.3 (9–50)	15.7 (9–50)	0.684
Preoprative CRT, n (%)				
yes	42 (28.77)	7 (4.79)	49 (33.56)	0.0014
no	59 (40.41)	38 (26.03)	97 (66.44)	

ASA – American Society of Anesthesiology; CRT – chemioradiation therapy;
N_1 – group of patients operated on by trained colorectal surgeon;
N_2 – group of patients operated on by general surgeon;
n – number of patients.

Table 3

Postoperative complications, local relapses and early postoperative mortality

Postoperative complications	N1 n (%)	N2 n (%)	Total n (%)	*p*
Anasthomosis leakage				
yes	3 (2.05)	10 (6.85)	13 (8.90)	0.0004
no	98 (67.12)	35 (23.97)	133 (91.06)	0.029
Local relapses	2 (1.37)	5 (3.42)	7 (4.79)	
Early postoperative mortality	2 (1.37)	4 (2.74)	6 (4.11)	0.072

N_1 – group of patients operated on by trained colorectal surgeon;
N_2 – group of patients operated on by general surgeon;
Total – N1 + N2; n – number of patients.

in the entire group. However, for tumors localized less than 7 cm from the anal verge, clinical signs of anastomosis leakage had 7.7%, and for tumors localized in the proximal portion of the rectum in 1% of cases [15]. Our results are compatible to the results of other authors [10, 13–17].

Anastomotic leakage rate in the group N1 of the patients was low, despite the fact that they were far more radiated than the patients in the group N2 (42% *vs* 15%). Therefore, hypothetically speaking the group N2 had pointed to the more inferior results in the rate of anastomotic leakage than the group N1.

The importance of training and education in TME is particularly reflected on the duration time of surgery. We found a statistically significant difference in the mean time of the operation between the two groups of surgeons. Other authors came to the same conclusions [6–10, 18]. This can also be used when the need for intraoperative blood transfusion is concerned [6–10].

The single and double stapling techniques are equally safe. Radovanovic et al. [16] in their study found no significant difference in the anastomotic leakage rate between these techiques [16]. However, double stapling technique allows the anastomosis to be performed very low in the pelvis and operative time is shortened than in single stapling technique [15, 16].

Protective stomas do not prevent anastomosis leakage. However, stomas reduce the consequences of complications in terms of reoperations. Also, they reduce the clinical manifestations of anastomotic leakage. Norwegian multicentric randomized study, Rectal Cancer Trial On Defunctioning Stoma (REKTODES) has clearly demonstrated that protective stoma significantly reduces the incidence of symptoms of anastomotic leakage [17]. We believe that we should create protective stoma in the following cases: at very low rectal resection, when rings of staples are incomplete after resection, when the water test is positive and in patients with severe general condition.

According to our institutional protocol for rectal cancer treatment, all patients with locally advanced tumors should receive preoperative CRT. Preoperative CRT can improve local control of the disease [1–5]. In a Swedish rectal cancer trial, the reduction in the rate of local recurrence from 27% in the surgery-only group, to 11% in the radiotherapy-plus-surgery group. Also, the rate of overall survival is improved from 48% in the surgery-only group to 58% in the combined-treatment group [19]. Local recurrences after surgery only performed by general surgeons vary widely from 15% to 45%, and by contrast, surgeons who specialize in TME (dedicated colorectal surgeons) report local-recurrence rates of 7% or less [6–12, 18, 20]. Accordingly, our study shows 3.42% *vs* 1.37% local recurrence rate, respectively.

Postoperative mortality did not increase with preoperative CRT [1, 2, 19]. In our study, there was no difference in early postoperative mortality between the two observed groups of patients. Other authors also have similar results [6–13].

Conclusion

It is necessary to continue professional development in rectal cancer surgery in order to maintain existing, and also to acquire new knowledge. Therefore, it is essential to be familiar with new technologies and surgery techniques to offer maximum quality of surgical treatment to rectal cancer patients.

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GENERAL REVIEW

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In vivo methodology in behavioural pharmacology – Where are we now?

In vivo metodologija u bihejvioralnoj farmakologiji – gde smo sada?

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Introduction

Behaviour includes all the actions and responses of an organism, related to the external environment, but also the functioning of internal factors. It includes any activity that can be observed directly, through senses, or indirectly, using technical devices. Central and peripheral nervous system, and also hormones, have an essential role in the control of behaviour. Numerous researches and experts, such as pharmacologists, psychiatrists, neurologists, psychologists, and other scientists, make a huge effort in uncovering all the peculiarities of how drugs affect behaviour. Some authors, accepting the risk to wrong, defined that a drug has an influence on behaviour, if it leads to changes in mood, communication, circadian rhythms, and cognitive processes [1]. However, some external circumstances, situations, and individual characteristics can lead to completely different and paradoxical effects and impacts on behaviour, even when administering agents with well known pharmacological profile. Finally, the characteristics of a drug can affect behaviour, such as drug dosage and routes of administration, frequency of administration and interactions.

Modern medicine has made a great progress in neuroscience, from gene and receptor cloning to the revised classifications and introduction of a new group of drugs. Undoubtedly, research work represents a critical, but necessary component of progress in biomedical sciences, and especially in psychotropic drugs research field, this process is very specific and sensitive. It is the complexity of neurological and psychiatric pathology that requires a special research approach in behavioural pharmacology, as from the aspect of scientific research work methodology, so from the aspect of ethical principles [1]. The methodology of experimental work in neuropsychopharmacology includes the complementary application of many different *in vitro* and *in vivo* techniques, whereas *in vitro* methodology applied techniques of molecular biology and computer technology, while *in vivo* studies involve the use of different preclinical models and tests in the study and monitoring of behaviour.

In vivo methodology is a basis of behavioural pharmacology, which developed as a synthesis of two disciplines, experimental analysis of behaviour and pharmacology. Behaviour represents the entire activity of an individual, especially one segment of activity that is the subject of observation. Behavioural tests and models are based on monitoring of certain parameters/index, that is selected segments of individual activities, implemented by engaging skeletal musculature − somatic manifestations, and/or smooth muscles and glands − vegetative manifestations.

There are two basic types of behavioural research: research based on examination of unconditioned or spontaneous animal behavior, and research based on conditioned behaviour, when establishing a correlation between the percep-

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tion of certain stimuli (conditioned stimulus) and rewards or punishments (unconditioned stimulus).

Behavioural results are usually expressed in terms of motor activity parameters (e.g. locomotor activity and sniffing) and rarely through non-motor parameters (e.g. ultrasonic vocalization). However when possible, non-motor parameters should be monitored, because they increase the quality and specificity of research. Each individual behavioural parameter should theoretically provide information on various aspects of nervous system function, while the research work effectively monitors whether, under the influence of some treatments, it will come to an increase or decrease in the value of a parameter when compared to control values. In addition, the interpretation of the value of the monitored parameter is a particular challenge in behavioural pharmacology. Experimental, i.e. animal models of neuropsychiatric diseases try to include different aspects of human characteristics, from the physiological and behavioural parameters to etiology of disease and therapeutic effects. By definition, animal models are developed in one species with the aim of studying characteristics that occur in other species [2]. When it comes to experimental models for studying human psychophysiology and/or pathology, the essence lies in the development of the syndrome in animals that are, in a way, similar to those developed in humans.

In general, the ideal experimental model for testing any clinical condition must meet three criteria of validity: the possibility of qualitative assessment of therapeutic efficacy of the new treatment (predictive validity), similarity of behavioural signs and symptoms in humans and experimental animals (face validity) and strong correlation between the findings at neural and behavioural level in animal model, compared to the findings in patients (construct validity). It is certainly not easy to meet all three criteria of validity in experimental models, especially in the field of neuropsychopharmacology. Furthermore, a model in behavioural pharmacology is described as an experimental setup developed in a non-human species replicating human features, with the purpose of measuring physiological, pathophysiological and behavioral responses [2]. The protocols applied to record these experimental data and to measure behavioural parameters are often called tests. Here we focus on methodology, animal tests and models, used in our laboratory settings: animal methods for testing anxiolytic and antidepressant effects of drugs (elevated plus maze and forced swimming test); animal methods for testing drug effects on cognitive function (active avoidance test and Morris water maze); animal models of schizophrenia and other neuropsychiatric disorders; and genetic approach in designing psychopharmacological studies.

Animal methods for testing anxiolytic and antidepressant effects of drugs

In recent decades various experimental procedures were developed in order to broaden preclinical studies in behavioural pharmacology of anxiety and depression. Furthermore, the benzodiazepines discovery about 60 years ago strongly stimulated the development of these behavioural procedures. Using the animal models in the study of anxiety- and depression-like behaviour in humans is possible, although there is no complete evidence that rodents, the most commonly used in these experiments, experience anxiety and depression the same way like humans do. However, behavioural and physiological studies show a sufficient analogy, if not homology, between anxiety and depression in humans and rodents [3].

Elevated plus maze

Nowadays most studies use anxiety tests based on unconditioned responses during spontaneous behaviour in animals, and here we report elevated plus maze, as one of the most often used. Elevated plus maze, introduced in 1984, soon became one of the most used animal models of anxiety. A widespread application of this test is primarily for practical reasons, because this method allows a quick search of potential anxiolytic effects of drugs, without animal trainings and complex procedures [4]. This type of behaviour is based on exploration phenomenon − exploration as a behavioural activity encouraged by unknown environment [5]. If unknown environment contains contrast (light/dark, open/closed space), another basic biological process is activated: the separation of attractive and repulsive parts in unfamiliar space. In this situation, the animal is faced with two opposing motivation conflicts: to explore the available parts of space in order to find exit, or to avoid aversive parts of unfamiliar space.

Practically, this model consists of four arms: two open and two closed arms. When an animal is placed into the maze, it starts exploring different areas. It is being measured the number of entrances into the open/closed arms, as well as time spent inside of each arm. Due to thigmotaxis phenomenon (the response of an organism to physical contact or touch), i.e. innate choice of enclosed space, rodents enter more often and spend time in enclosed maze arms. Time spent in open maze arms is followed by increased plasma corticosterone concentrations, more frequent motor activity immobility responses (immobility reaction, freezing), defecation, hormonal and behavioural changes referring to elevated anxiety levels. Anxiolytics help the animal to overcome fear of open maze arms, while aversive agents inhibit the entrance into the open maze arms [5].

Forced swimming test

Forced swimming test (FS) is a standard test used to evaluate antidepressant features of substances. During the test, animal is being placed into enclosed space filled with water, thus making the aversive situation that provokes reactions of despair and hopelessness. If the substance has antidepressant potential, it will extend the time an animal spends in finding the way out of a cylinder, i.e. it will reduce the immobility time [6, 7].

However, in recent years, a more complex psychophysiological basis of forced swim reaction has been more and more pointed out to. Repeated forced swim test becomes a model for studying the process of motivation and contextual memory formation in an aversive situation, analogous to

aversive avoidance reaction, which represents the process of instrumental learning through aversive stimuli. Under the influence of aversive situation, in an early phase of forced swimming, defensive reaction is provoked by which an animal attempts to leave the aversive space. Over time, and after many unsuccessful attempts, animal enters into the stillness mode, i.e. immobility. To what extent an animal will continue to fight mostly depends on the degree of motivation. This way, the reaction of forced swimming allows for monitoring antidepressant effects, which are largely shaped by the degree of motivation, referring to motivation as the point of contact in terms of positive reinforcement factors in learning the reaction of aversive stimuli avoidance and successful conflict resolution followed by depressive symptoms [8, 9].

Animal methods for testing drug effects on cognitive function

When examining the potential effect of substances on cognitive function, one of the main issues is how these drugs influence cognition in healthy subjects, compared to those with experimentally-induced cognitive impairment [10]. There are numerous examples where the use of different substances improved certain types of memory in normal, healthy animals. On the other hand, there are examples where pro-cognitive effects of some drugs were noticed only in animals with low basal performances, while in healthy animals no measurable behavioural effect was noticed. For example, some antipsychotics (clozapine, sulpiride), reduce cognitive impairments that occur after the disruption of prefrontal cortex, and the same drugs impair learning and memory when applied to healthy subjects [11]. In this sense, study of pro-cognitive drugs on healthy animals may be a useful screening, and the lack of effect does not necessarily mean that they will not manifest in any other disturbed system. In support of using healthy subjects in the process of discovering pro-cognitive drugs is the fact that the pathophysiological basis, which would be of great importance for creating animal model of disease, is often not sufficiently known or it is usually difficult to achieve satisfactory level of analogy in animal model. Finally, normal, healthy animals usually manifest characteristic responses qualitatively identical to those manifested by animals with certain disease [11].

Active avoidance test

Active avoidance reaction has been used in behavioural experiments for many years, in order to study different memory processes. Devices that are used are different, but they down to the fact that certain activity of an animal, which can be registered, is a way to stop the aversive stimulus. If a passivity is required from an animal during aversive stimulus, then it is passive avoidance response. If certain animal activity is a way to avoid aversive stimulus, it is the reaction of active avoidance [8]. Passive avoidance test is, at the same time, a test of evaluation of anxiolytic drug effects, and also evaluation of drug effects on cognitive function [12]. Amygdaloid complex is critically involved in modulation of memory with emotionally arousing experiences, such as electrical stimulation of the paws in this test. Animal is placed into the lit compartment of the cage, where the cage has a lit and a dark compartment. During transition to the dark compartment, which is a usual reaction of rodents being animals of the dark, they are given unavoidable electric shock. That is the basis for conflict development in animals, which can be mitigated with anxiolytics, i.e. latency time of reentrance into the dark compartment, in which the animal received unavoidable electric shock the other day, can be reduced. However, drug effects on cognitive function should be always kept in mind.

Active avoidance test represents primarily a test for evaluation of cognitive functions. Active avoidance reaction (AA) is a behaviour that is easily learned, but hard to forget, therefore this way high reproducibility of results is provided when investigating drug effects on learning this reaction. AA is a complex acquired behaviour that manifests itself through aversive stimulus avoidance under strictly controlled conditions of exposing an animal to this stimulus. Fear and escape represent two decisive moments in learning this reaction. In the first learning phase, associating conditioned stimulus (light, sound) and unconditioned stimulus (electric shock), in accordance with classical principles of Pavlovian conditioning, results in conditioned fear response. This kind of fear, in further process of learning AA reaction, causes locomotor escape response from the site of aversive stimulus. Animal does not escape because of aversive stimulus, but because of the tendency to resolve fear. In second learning phase, fear represents psychophysiological precondition for further learning AA reaction. Second phase begins when animal escapes from conditioned stimulus, which means that it avoids unconditioned stimulus. This way, conditioned stimulus is a precondition for fear development, which is then the basic motive for active avoidance reaction occurrence. The disappearance of fear is conditioned by an adequate motor response, i.e. by practical avoidance in occurrence of next conditioned stimuli. This way, fear is essential for active avoidance reaction development, while fear disappearance in this reaction performance is an important precondition for its learning [8, 12].

Morris water maze

Next, a very often used laboratory task in behavioural neuroscience is Morris water maze (MWM). Morris water maze is used for studying neurobiology and neuropharmacology of spatial learning in experimental rats and mice, and there is almost no psychoactive compound that has not been tested in this task. Spatial memory is defined as capacity of orientation learning in a new environment [13]. The basic apparatus consists of a circular pool, 1.5−2 meters in diameter, containing room temperature water, and a platform placed at a pool quadrant, is 1.5 cm below the water surface [14].

Animals learn, over sequence of trials, i.e. training, after being placed into the pool, to find a way to the platform in a more efficient and faster way. Reduction of time and path-length to the platform during training refers to the successful task mastering. It is recommended that animals swim

4−6 times per day, over 5−10 days, or until escape to the platform latency reaches asymptomatic level [15]. Over time, as the popularity of MWM grew, a number of methodological approaches developed, some of which significantly expanded the area of its application [16]. The main advantage of Morris water maze, compared with other tasks used in the study of neurobiology of memory and learning, is its performance simplicity and the ability to separate memory deficits from deficits in motor and sensory processes [17]. Submerging the experimental animal into the water can be unpleasant and trigger certain stress level, but this is how significant aversive stimuli are avoided [18].

MWM does not require previous training, and testing can be performed over a couple of days with a relatively small number of animals. Because of the water environment, the influence of olfactory traces is avoided, and using a visible platform version of the test potential visual impairments can be identified. Memory impairment induced by applying treatment is independent from locomotor effects, since an increase or decrease in locomotor activity does not necessarily affect the swimming speed. Since the success in platform discovering does not depend on entire activity or body weight of an animal, this test is suitable for a number of disease models in which cognitive impairment is present [16].

Animal models of schizophrenia

Schizophrenia is a burning issue in animal modeling, because of its still unknown etiology and unique psychopathology. Attempts to replicate delusions, hallucinations and other mental disorders in an animal model seem to be very challenging. The course of schizophrenia is very variable, and environmental and genetic susceptibility factors must also be considered [19, 20]. Therefore, pharmacological animal models of schizophrenia are basically developed on current knowledge of the alterations in different neurotransmitter systems [21].

The models which mimic positive symptoms of schizophrenia, such as delusion and hallucinations, are dominantly based on dysfunction in central dopamine, serotonin and glutamate neurotransmission. Therapeutically challenging are especially negative symptoms and rodent models of affective flattening, anhedonia and diminished social interaction were already introduced. Cognitive deficits are mainly assessed in rodent models of learning/memory (Morris water maze and object and social recognition) and in rodent models of attention (prepulse inhibition - PPI). At first, the stereotypes seemed to have the most face validity. Now, it appears that these symptoms are more closely linked to drug side effects, so diverse extrapyramidal symptoms were explored, such as acute dystonia (purposeless chewing in rodents, dystonia in monkeys), parkinsonism (catalepsy in rodents), akathisia (defecation in rodents) and tardive dyskinesia (long-term antipsychotic treatment in rodents and monkeys) [22, 23]. The advantages of these models are appropriate predictive validity, with some degree of construct validity, but limited face validity.

Lipska [19] discussed dysfunctional glutamate neurotransmission in schizophrenia [19]. Namely, ketamine and phencyclidine, as non-competitive antagonists of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors, produce symptoms similar to acute schizophrenia. When an acute subanesthetic dose of NMDA antagonists were introduced in rodents, it creates schizophrenic symptoms, such as enhanced stereotyped behaviour, hyperlocomotion, cognitive deficits, and impaired social interactions. Phencyclidine and other NMDA antagonists perform their action in the prefrontal cortex, by acutely increasing extracellular levels of glutamate and dopamine, as well as norepinephrine and acetylcholine, and altering firing patterns of dopaminergic neurons. Taken together, face and predictive validity for this model are satisfying, although construct validity remains limited. However, long-term phencyclidine administration produces different effects compared with those of single injection: decrease in stereotyped locomotion and an increase in exploring behaviour, tolerance, immobility time in forced swim test and depressive symptoms. The pharmacological effects of phencyclidine as well as other NMDA antagonists are mostly not mediated by increased dopamine transmission; therefore their behavioural effects are not blocked by typical antipsychotics but reduced by atypical antipsychotics, such as clozapine [24]. NMDA antagonist model can be convenient for testing the efficacy of novel antipsychotic drugs, and the advantage of this model versus dopamine-based model is its strong construct validity in exploring the cognitive dysfunctions in patients with schizophrenia. Further, targeting the modulatory co-agonist glycine-B site of NMDA receptors has been shown as a promising approach to ameliorate NMDA receptors hypofunction and reduce severe side effects of NMDA receptors agonists [25].

The pharmacological properties of the class of agents, represented by lysergic acid diethylamide (LSD), called psychotomimetics or psychotogens, suggest a hallucinogen model of schizophrenia [26]. It has been showed that LSD, acting through direct activation of serotonin 5-HT_{2A} receptors and antagonism of 5-HT_3 receptors, attenuates the behavioural hyperactivity caused by amphetamine, as well as phencyclidine application. It has to be pointed out, however, that the tolerance was rapidly developed to the effects of LSD, whereas the symptoms of schizophrenia are persistent for a lifetime. Furthermore unlike schizophrenia, hallucinations as a consequence of LSD consumption are typically visual rather than auditory [21]. Therefore, these findings somehow impair predictive validity of the hallucinogen model of schizophrenia.

Finally, injection of picrotoxin, a gamma aminobutyric acid (GABA) receptor antagonist, into the medial prefrontal cortex causes reduction of PPI in rats [20]. Pretreatment with the dopamine antagonist haloperidol antagonized this effect, pointing out that blockade of GABA receptors in prefrontal cortex impairs sensorimotor gating in a dopamine-dependent way. However, other GABA-induced behavioural deficits, linked to schizophrenic symptoms, remain to be further elucidated for the predictive and face validity of this model.

Animal models of other neuropsychiatric disorders

An animal model of neuropsychiatric disorder is a challenging attempt to capture the essence of the condition, but it

does not claim to reproduce the complete human condition in an animal. Animal models of schizophrenia are widely used and evaluated; however, there are numerous attempts to develop animal models of other mental illnesses. The most frequent used and robust rodent models of other neuropsychiatric disorders, such as Parkinson's disease (PD), Alzheimer's disease and obsessive-compulsive disorder (OCD) are presented in Table 1.

Finally, the animal modeling of obsessive compulsive disorder (OCD) is certainly one of the most complex approaches in behavioural pharmacology. Attempts to replicate obsessions (intrusive recurrent thoughts) and compulsions (repetitive aberrant behavior) seem to be very challenging. Research has been focused on developing models for compulsivity, as the obsessions cannot be effectively approximated in animal models [31]. Among a few animal models of

Table 1

The reference animal models of other neuropsychiatric disorders

Disorder	Animal model
Parkinson's disease	Neurotoxin-based model
Alzheimer's disease	Scopalamine-induced amnesia model
Obsessive-compulsive disorder	Quinpirole-induced model

An ideal animal model of PD should consist of pathophysiological and clinical features, involving central and peripheral nervous systems, dopaminergic and non-dopaminergic neurotransmissions, as well as motor and non-motor symptoms. In addition, the progressive characteristics of the disease and age-dependent onset should be reflected [27]. Neurotoxin-based model represents a robust animal model for PD, in which toxic molecules are mainly used to lesion the nigrostriatal dopaminergic pathway. Among the different types of neurotoxin-based models, the 6-hydroxydopamine (6-OHDA) model has been validated and well established in rodents [28]. Basically, 6-OHDA is capable of inducing degeneration of both dopaminergic and noradrenergic neurons in the brain. These neurons are highly sensitive to 6-OHDA, since their plasma membrane transporters possess high affinity for this molecule. Once taken up into neurons, 6-OHDA accumulates in the cytoplasmic matrix, producing oxidative stress-related cytotoxicity. The model scores well in the face and predictive validity; however, its construct validity remains to be further elucidated. Although not ideal, neurotoxin-based animal models have significantly contributed to our understanding of the pathophysiology of PD and potential pharmacological targets.

In the field of behavioural pharmacology scopolamine has been widely used as a reference agent for inducing dementia and age-related decline in cognitive functions in animals. The use of scopolamine, as a pharmacological model of dementia, is based on the hypothesis that the age-related cognitive deficits are predominantly related to a decrease in cholinergic signaling. Since scopolamine-induced amnesia is most likely caused by blocking of the cholinergic neurotransmission, it is used to model cognitive dysfunctions associated with aging and Alzheimer's dementia [29]. Moreover, another important issue in which scopolamine is often used is the preclinical testing of new compounds to treat cognitive deficits. If the substance possesses potential procognitive properties, it will reverse the scopolamine-induced cognitive dysfunctions in animals. Thus, scopolamine-induced model provides a quick and simple way for drug testing on cognition-enhancing properties. On the other hand, this model has a limited predictive validity, since it is associated with a high number of false positive results [30].

OCD, the model induced by quinpirole, the selective dopamine D2/D3 agonist, has been widely used and evaluated [32]. The quinpirole model is based on the hypothesis that dopaminergic system is predominantly involved in controlling compulsive behaviour. Sesia et al. [32] showed that chronic exposure to quinpirole induced the clear increase in compulsive behaviour in rats, which further supported the face validity of this model. Furthermore, they found that dopamine neuron activity corresponded to behavioural outcomes, confirming the findings that compulsive behaviour likely reflects, at least partially, a disruption of dopaminergic pathways. Although with an excellent face validity, the quinpirole-induced model of OCD still lacks pharmacological predictive validity.

Genetic approach in designing psychopharmacological studies

In study of genetic basis of neuropsychiatric diseases, two approaches are primarily used: bidirectional breeding of phenotype extremes principle and genetically engineered models, with a possible combination of these two principles [33, 34]. Bidirectional breeding of phenotype extremes principle is based on determination of variability within wild mice or rats lines, where animals are selected on the basis of specific criteria [1, 34]. By using selection, two extremely different lines in terms of behaviour are produced. Animals produced with predisposition for certain behaviour, are ideal for further genetic research and genetic manipulation. By using this approach, in studying anxiety in elevated plus maze, two lines of Wistar rats are produced: rats with low anxiety-related behaviour and rats with high anxiety-related behaviour [35]. The anxiety phenotype in these two lines is further used in behavioural and pharmacological researches. Genetic engineering produced the models of genetically conditioned anxiety in mice with targeted mutations in certain genes. Discovery of genes, that provide synthesis of key proteins for certain structural and regulatory functions, is the most specific, and at the same time subtlest approach in the study of pathological forms of anxiety. For this purpose in behavioural pharmacology we can analyze natural variations in genotype, elicit ran-

dom mutations using chemical mutagens, and, the most up to date, use the possibility of gene technology.

According to the mentioned approach, genetic modification can be achieved in two ways: by developing transgenic and targeted mutant animals [35]. Transgenic technology is applicable in mice, rats, and other animal species, and consists of microinjection of selected DNA sequence into fertilized egg cell. In accordance with the law of probability principle, DNA is integrated into genome, so that the tissue distribution and expression level of transgenic constructs varies from one to another animal line. The result of such approach is mostly the excessive expression of function determined by foreign gene, but it can also result in the loss of the monitored function. In targeted mutation approach targeted vectors are generated, which are specifically, by homogenic recombination, integrated into a desired location in genome in mice embryonic stem cells. Cells modified in this way are then injected into blastocysts, by which, mutation is transferred into strain line *via* embryo in development. In the case of gene inactivation experiment, targeted gene is inactivated by introducing neomycin resistance markers and/or knocking out a part of gene, which is marked as knockout technology. Knock-out mutations can be monitored in heterozygous state (evaluation of phenotype expression potential) and homozygous state (zero phenotype analysis). Changes noticed in phenotype serve as the basis for conclusion on normal function of the examined genes in the so-called wild, unmodified animals. Targeted mutations are not confined to ablations: essentially every desired change, such as point mutations, gene switching in mice, or switching of a mice gene with homologous human gene can be inferred into genome, and these subtle changes are marked as knock-in technology. Embryonic stem cells technology is currently applicable only in mice.

Several experimental models with genetic mutations on serotonin genes and certain $GABA_A$ receptor complex subunits have been created so far [35, 36]. Serotonin neurotransmitter takes a central place in pathology of many neuropsychiatric diseases. There is a number of pharmacologically different serotonin receptor types involved in a number of central and peripheral functions. Pharmacology and neuroanatomy point to serotonin receptor role in regulation of anxiety-depression expression, especially type 5-HT_{1A}. The results of recent studies in mice with 5-HT_{1A} receptor gene deletion confirm these findings. Several mice lines with single $GABA_A$ subunit inactivated gene have been produced so far. Knock-out of γ subunits had a lethal effect, and the inactivation of α6 subunit did not lead to phenotype manifestations. The lack of β subunit produced mice with epileptic phenotype, while ablation δ subunits reduced the sensitivity to neuroactive steroids. Mice lacking α1 subunit, normally present in about 50% of $GABA_A$ receptors, show intention tremor and increased sensitivity to convulsive action of bicuculline [35]. Still, the biggest drawback of this approach is the possibility of compensatory changes in development and function of the brain, caused by an absolute lack of these genes.

Preclinical/clinical interface

More and more discoveries of psychotropic substances speed up the tempo of clinical researches. However, it is obvious that preclinical studies run faster than clinical researches. Besides, over the last 10−15 years hundreds of compounds, on the basis of promising preclinical data, have been in clinical trial phase, but the majority of them have been dismissed due to lacking clinical efficacy. The main methodological problem in preclinical researches presents inexistence of an adequate animal model of neurological and psychiatric diseases, which could be completely applicable to human. Therefore, in behavioural pharmacological research, because of the different mechanisms of behaviour control and the processes of higher nervous activity regulation, there is no possibility for translating research results from an animal to the human. Special contribution have profiled behavioural research on genetically modified animal strains, as well as the use of the whole battery of tests, which is certainly going to provide the development of new drugs with higher specificity of action. Thus it is necessary to integrate the knowledge from all derived animal models and consider possible drug side effects.

Furthermore, it is always questioned which parameters of neurotransmission one should examine in an animal model to obtain the most appropriate characterization of behavioural, as well as neurochemical changes. There are certainly many biochemical parameters that describe dopaminergic, serotonergic and noradrenergic neurotransmission (synthesis of neurotransmitters, density and functionality of different receptors and transporter, alteration in the second messenger signaling), but only a few of them can be studied at any time. It should be taken into account, however, when compared to alteration of monoaminergic systems, modulation of inhibitory/excitatory amino acid neurotransmission, such as GABA and glutamate, has much more potent and rapid behavioural effects.

Conclusion

The animal preclinical methodology represents the so-called “bottleneck” in psychotropic drugs development. The use of animal tests and models as screening techniques seems to be slow when compared with the chemical synthesis speed and fast techniques of gene sequencing. However, such *in vivo* methodology are certainly necessary in order to obtain the initial evaluation of new compounds pharmacological effects, because it is not realistic nor ethical to go directly from the test tube into the clinic.

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CASE REPORT

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Autoimmune pancreatitis type 1 and type 2: A report on two cases

Autoimunski pankreatitis tipa 1 i 2

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Abstract

Introduction. Autoimmune pancreatitis is a disease associated with autoimmune mechanisms, clinically manifested mostly as obstructive icterus with or with no entire or partial enlargement of the pancreas, histological lymphoplas-mocytic infiltration, fibrosis or granulocytic epithelial lesions with a favourable therapeutic response to the application of corticosteroids. Type 1 autoimmune pancreatitis is a systemic disease befalling the group of IgG4-related diseases in contrast to type 2 which is specific for pancreas disease. **Case report.** We presented two cases. The first one was a 64-year-old male patient with autoimmune pancreatitis complaining of abdominal pain, weight loss, weakness and exhaustion. Clinical examination showed a rare IgG4 autoimmune pancreatitis. The second one was a 37-year-old male patient complaining of abdominal pain with diarrhea. The diagnosis made revealed the presence of type 2 autoimmune pancreatitis. Following the diagnosis, immunosuppressive therapy was administered to both patients leading to the improvement of their general condition. **Conclusion.** Autoimmune pancreatitis is a rare disease, sometimes not easy to differ from pancreatic tumor or bile duct tumor with poor prognosis. Thus, early recognition of the disease is very important, since adequate treatment significantly increases the course and the outcomes of the disease.

Key words:
pancreatitis; autoimmune diseases; diagnostic techniques and procedures; diagnosis, differential; drug therapy.

Apstrakt

Uvod. Autoimunski pankreatitis (AP) je oboljenje čiji nastanak se povezuje sa autoimunskim mehanizmima i klinički se najčešće manifestuje opstruktivnim ikterusom sa ili bez uvećanja čitavog ili dela pankreasa, histološki limfoplazmocitnom infiltracijom, fibrozom ili granulocitno-epitelnim lezijama uz povoljan terapijski odgovor na primenu kortikosteroida. AP tipa 1 je sistemsko oboljenje koje pripada grupi IgG4 udruženih bolesti. **Prikaz bolesnika.** Prikazali smo dva bolesnika. Prvi bolesnik, star 64 godine, sa AP tipa 1, žalio se na bolove u trbuhu, gubitak telesne mase, slabost i malaksalost. Kliničko ispitivanje pokazalo je da se radi o retkom IgG4 AP. Drugi bolesnik, star 37 godina, na prijemu imao je stomačne bolove u predelu pojasa i tečne stolice. Postavljena dijagnoza otkrila je prisustvo autoimunskog pankreatitisa tipa 2. Oba bolesnika lečena su imunosupresivnom terapijom koja je popravila njihovo opšte stanje. **Zaključak.** AP predstavlja retko oboljenje koje je nekada teško razlikovati od tumora pankreasa ili bilijarnog trakta koji ima lošu prognozu. Stoga, veoma je važna rana dijagnoza pošto adekvatno lečenje značajno poboljšava tok i ishod bolesti.

Ključne reči:
pankreatitis; autoimunske bolesti; dijagnostičke tehnike i procedure; dijagnoza, diferencijalna; lečenje lekovima.

Introduction

Autoimmune pancreatitis (AIP) is a chronic fibro-inflammatory autoimmune disease of the pancreas that still has the cause not known completely [1]. The disease was firstly described by Sarles et al. [2] in 1961 when they noticed the presence of pancreatitis followed by hypergammaglobulinemia and sclerosis. It is supposed today that its prevalence in patients with chronic pancreatitis is 5.3% in Japan, and 11% in the USA [3]. Also, 2–3% of pancreatoduodenectomies are performed in patients with AIP due to the wrong diagnosis of pancreatic carcinoma [4]. There are two types of the disease. Type 1 AIP, a prototype

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of IgG4-related systemic diseases, is a multiple-organ disease associated with the increase of IgG4 in serum and IgG4 positive plasma cells in pancreatic biopsies (more than 10 cells in the field of view) with obliterative phlebitis and storiform fibrosis. The disease could be related to IgG4 sclerosing cholangitis, sialo- and dacryoadenitis, retroperitoneal fibrosis, tubulointerstitial nephritis, chronic sclerosing aortitis and periaortitis, and Riedel's thyroiditis [5]. There is almost no organ that could not be affected by this disease. Type 2 AIP with granulocytic epithelial lesions (idiopathic duct-centric pancreatitis) has a few or no IgG4 positive plasma cells with the presence of neutrophil infiltration. Type 2 AIP appears more often in Europe and the USA, mainly not associated with affecting the other organs, except for a little bit higher frequency of inflammatory bowel disease (IBD) in these patients. This type of the disease usually does not relapse.

Clinically, AIP could be asymptomatic, but it could be manifested as acute pancreatitis, sometimes followed by the other organs damage [6–11]. It is characterized by diffuse or focal enlargement of the pancreas that sometimes is not easy to differ from pancreatic cancer [7]. The international criteria for AIP used today, established back in 2011, significantly help in recognizing and starting adequate treatment of this disease. During the past decades various diagnostic criteria for AIP were suggested on many occassons [12]. The International Consensus Diagnostic Criteria (ICDC) for AIP, and its Japanese Amendment developed by the Japanese Pancreas Society (JPS 2011) in 2011 are used today [13]. The major difference between the ICDC and JPS 2011 is in that the Japanese criteria are more focused on type 1 AIP and require the application of endoscopic retrograde pancreatocholangiography (ERCP) when imaging methods for the diagnosis are not defined [14]. The diagnosis of type 1 AIP can be definitive and probable, and is made on the basis of radiological and ERCP findings, serology, pathohistological finding of pancreatic biopsy tissue, other organs affected, and positive response to corticosteroid therapy [15]. It is also possible to diagnose it in 70% of cases with no invasive method [16]. The diagnosis of type 2 AIP no metter it is definite or probable was made on histopathology [17].

The aim of this report was to present two patients with type 1 and type 2 AIP never registered before in our institution.

Case report

Case 1

A 64-year old patient presented to the Clinic for Gastroenterology and Hepathology, Military Medical Academy, Belgrade, Serbia, due to weakness, exhaustion, weight loss of 16 kg, occasional abdominal pain. Otherwise, the patient suffered from insuline-depended diabetes mellitus. Physical examination revealed a painful sensitivity of epigastrium. The values of laboratory parameters [sedimentation (SE), C-reactive protein (CRP), blood analysis, electrolytes, urea, creatinine, total proteins, albumin, total bilirubin, aspartate aminotransferase (AST), alamine aminotransferase (ALT), gamma-glutamyl trasferase (GGT), alkaline phospahatase (ALP), amylase, lipase, protein electrophoresis] were normal except for glycemic values (5.3–10.4 mmol/L). Colonoscopy and esophagogastroduodenoscopy were normal. Abdominal ultrasound discovered a diffusely enlarged hypoechogenic pancreas (Figure 1). It was confirmed with endoscopic ultrasonography (EUS) (Figure 2).

The patient was then submitted to multislice computer tomography (MSCT) of the abdomen that showed the enlarged, hypodense pancreas bordered by a thin capsule ("sausage-like pancreas") with the presence of *ductus pancreaticus* penetration through the tissue of the organ (Figure 3).

The increase of IgG was confirmed in serum, while IgG4 subclass analysis suggested the increased value of IgG4 of 9.8 g/L. Ultrasound-guided biopsy of the pancreas was performed (Figure 4). The pathohystological finding indicated severe intracinous fibrosis of focal storiform pattern and multiplied connective fibers next to the periductal lymphoplasmacytic infiltration. The immunohystochemical finding confirmed the presence of more than 10 IgG4 positive plasma cells under high magnification microscope.

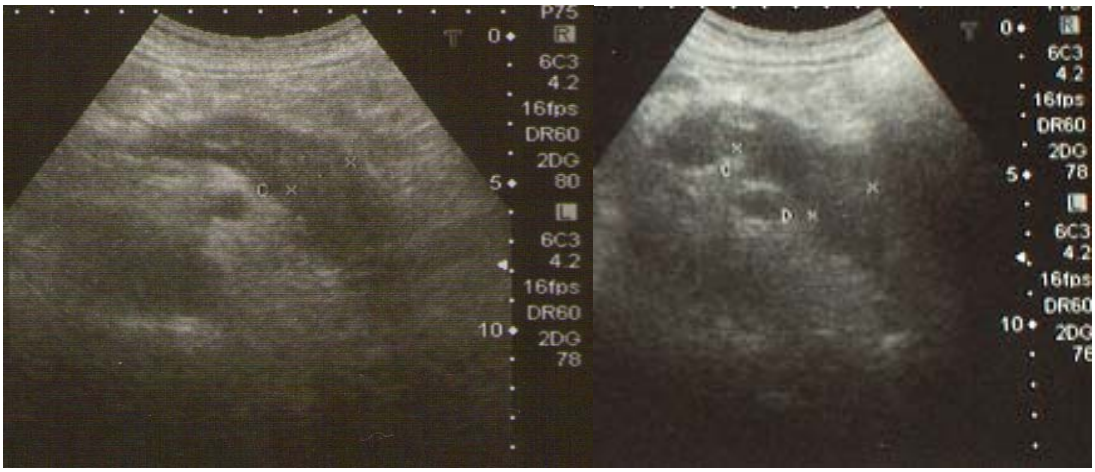

Fig. 1 – Ultrasound of the abdomen showing the voluminous hypoechogenic pancreas.

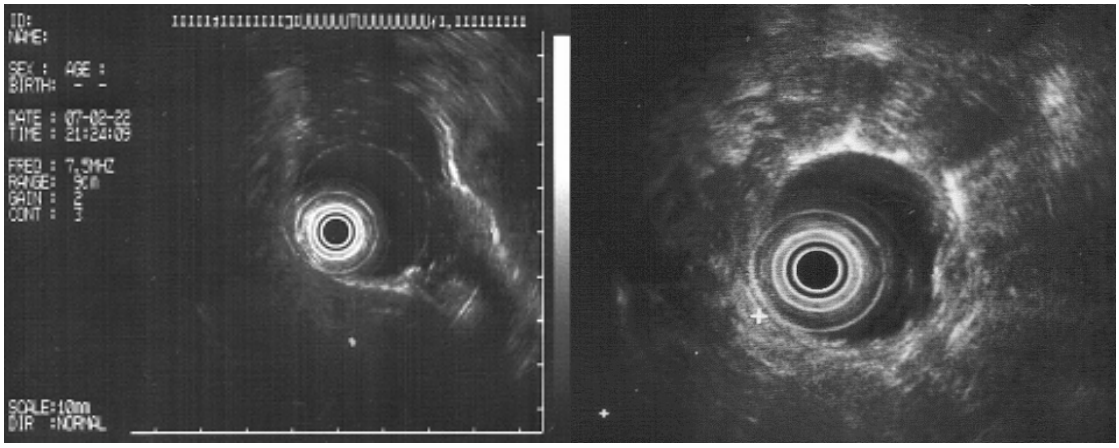

Fig. 2 – Endoscopic ultrasonography shows the enlarged hypoechogenic pancreas with no focal changes.

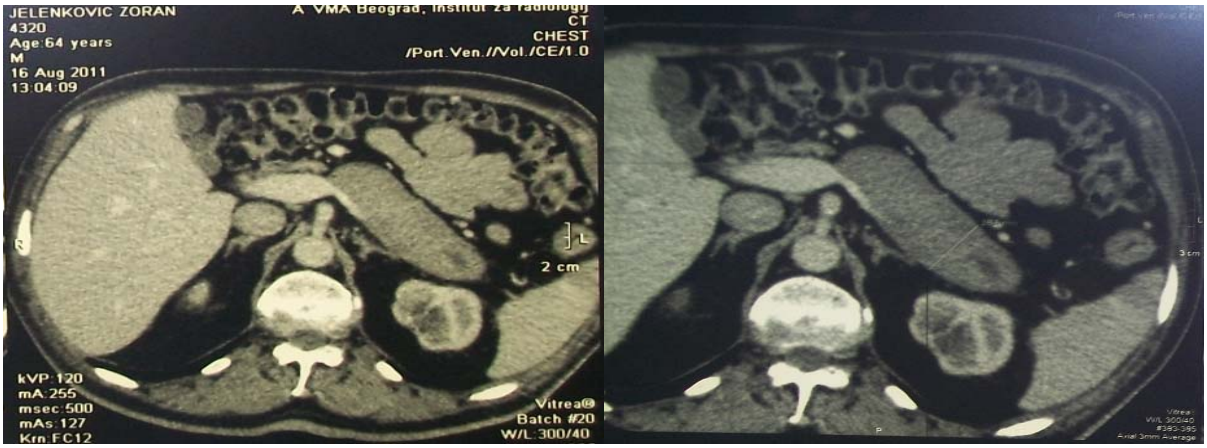

Fig. 3 – Multislice computed tomography of the pancreas with type 1 autoimmune pancreatitis.

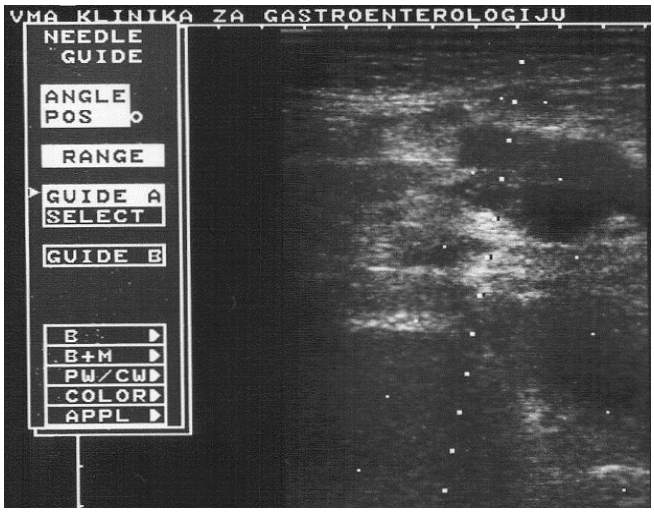

Fig. 4 – Ultrasound image of guided biopsy of the pancreas.

Following confirmation of type 1 AIP in the patient, corticosteroid therapy was administered. The patient was given prednisone 40 mg/daily within the first month. On the day 3, abdominal pain vanished, so the dose was reducing *per* 5 mg to 2 weeks upto the dose of 10 mg/daily to maintain. The control values of IgG4 were within the referent ranges (0.801 g/L). Ultrasound examination of the abdomen was normal. Two months following the beginning of the therapy, control MSCT of the abdomen was made showing the normal size of the pancreas. However, in spite of the therapy correction with insulin (the patient had type 1 diabetes mellitus) within a year there was no acceptable regulation of glycemia, thus proednisone was replaced with azathioprine 100 mg/day (recommended 1–2 mg/kg/day). The therapy caused no recurrence, so it was stopped after two years. Three years later there was no recurrence of the disease.

Case 2

A 37-year-old patient presented to the Clinic for Infectious and Tropical Diseases, Military Medical Academy, Belgrade, Serbia due to weakness, diarrhea, abdominal pain, and fever up to 38.5°C. Laboratory findings showed increased factors of inflammation [SE 138, CRP 212.33 mg/L, leucocytes (Le) 11.47 × 10^9], hyposideremic anemia (iron 3.6 μmol/L; normal range 8.9–26.8 μmol/L), normal values of biochemical parameters and serum enzymes (urea, creatinine, protein, albumin, total bilirubin, electrolytes, cholesterol, triglycerides, transaminases, GGT, ALP, amylase and lipase), immunoglobulin, chromogranin A and thyroid hormones. There was a rise in serum glucose (glucose 9.9 mg/dL) and amylase in urine (2,195 IU/h). Esophagogastroduodenoscopy was normal. Colonoscopy showed easily narrowed Bouchinis valves with patchy mucosal petechiae of the right colon, but pathohistological findings confirmed no presence of inflammatory bowel disease (Figure 5). Abdominal ultrasound revealed a diffusely enlarged hypoechogenic pancreas of lobular structure, with a smaller amount of ascites (Figure 6). That was confirmed by endoscopic ultrasound and MSCT examination of the abdomen. Biopsy was also performed. Hystopathological findings confirmed the presence of advanced autoimune pancreatitis type 2 – sclerosing lymphoplasmacytic infiltration as a sign of chronicity and characteristic ductocentric inflammation with focal granulocyte epithelial lesions (GEL).

The patient was initially treated with antibiotics (ciprofloxacin, metronidazole), proton pump inhibitor (pantoprazole 40 mg) and *per os* pancreatic enzymes (Kreon). Subjectivelly, the patient felt better, and laboratory tests showed a decrease in parameters of inflammation. After receiving pathohistological findings, the patient was submitted to the treatment with prednisone 40 mg/day within the first 14 days, while gradually reducing the dose of 5 mg for 7 days up to a maintenance dose of 10 mg/day. Laboratory control of inflammation factors, blood count and biochemistry of the enzymes revealed normal values. Two months following the start of the therapy, the patient underwent abdominal ultrasound – the pancreas was of normal size, lobular, with more hyperechogenic material, and the results regarding other parenchymatous organs were normal (Figure 7). The patient had no new attack of the disease the previous year.

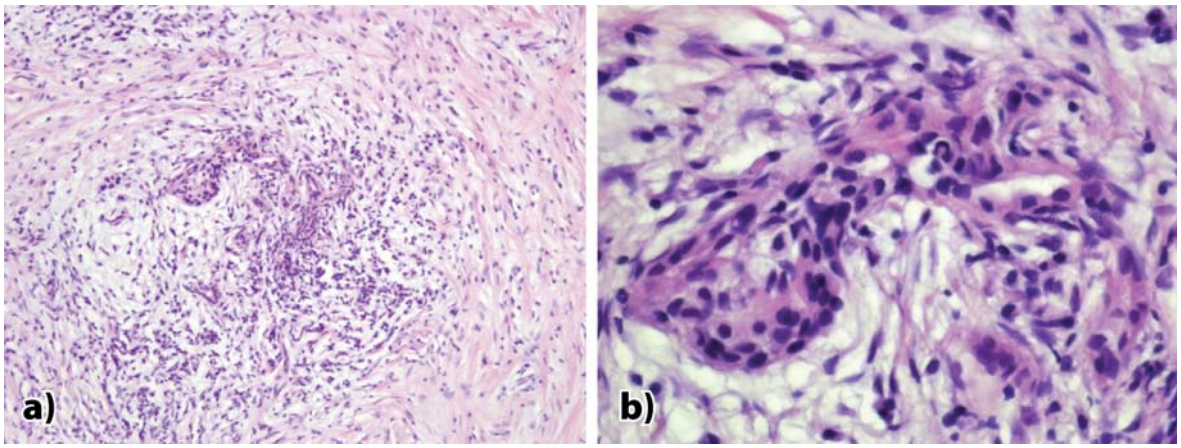

Fig. 5 – Histopathological finding of biopsy done on the pancreatic tissue. (Immunohistochemistry IgG4: a) ×100; b) ×200).

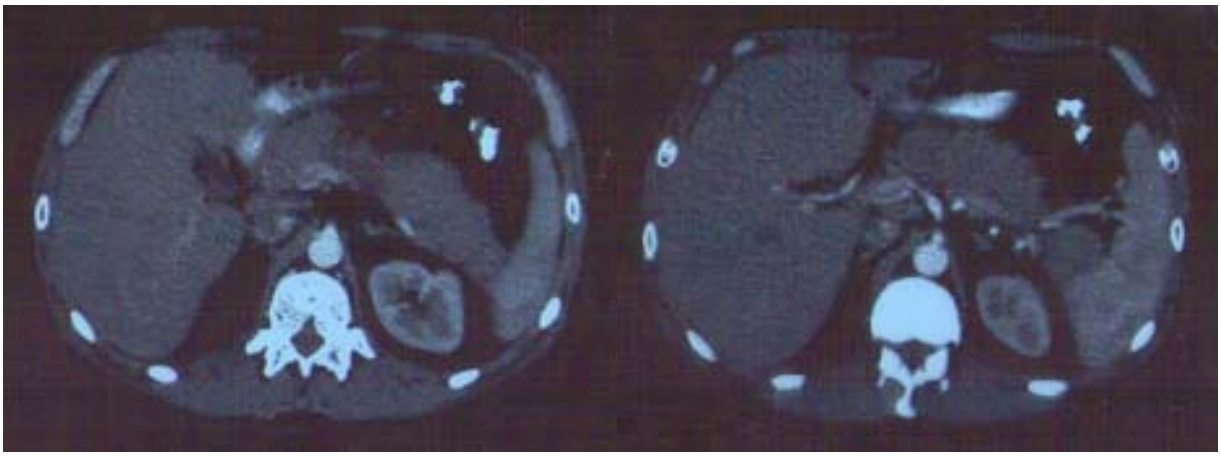

Fig. 6 – Type 2 autoimune pancreas: multislice computed tomography (enlarged, hypodense pancreas bordered by a thin capsule, "sausage-like pancreas" and a smaller amount of ascites).

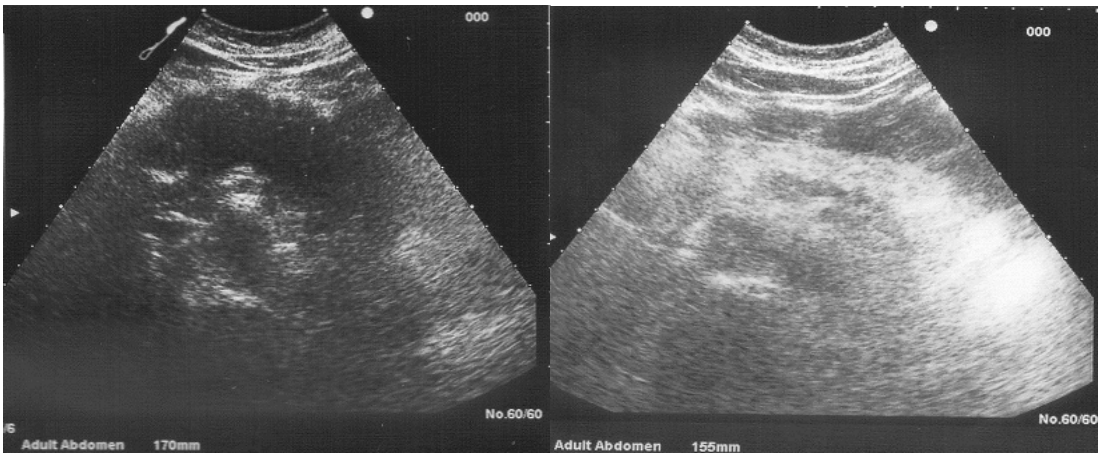

Fig. 7 – Ultrasound of the pancreas before (left) and after the therapy with corticosteroids (right).

Discussion

Autoimmune pancreatitis is a relatively new entity, the name of which was published for the first time in 1995 by the Joshida et al. [8]. Type 1 AIP (IgG4 AIP) is the best example for IgG4-associated diseases. It is featured by lymphoplasmacytic infiltration with IgG4 positive plasma cells, increase of IgG4 in serum, and good therapy response to the applied corticosteroids. Type 2 AIP is not a systemic disease, and usually occurs in younger patients. The most common radiographic presentation includes a focal change in the pancreas. Histopathologically, granulocyte-epithelial lesions were observed in intraluminal and intraepithelial neutrophil infiltration. IgG4 positive plasma cells were either not present, or present in a very small numbers [9]. AIP clinical picture includes obstructive icterus (35–75%), abdominal and back pain (32–70%), weight loss (15%), weakness, exhaustion (9%), diabetes mellitus (43–83%), other disorders (dry mouth, etc), while 15% of patients remain with no complaint [10]. It usually occurs in 70s, presented with focal (60%), and diffuse (40%) pancreatic enlargement. The image of acute pancreatitis appears in 15% of patients only [11].

The therapy for AIP includes corticosteroids (prednisone 30–40 mg/day) gradually reduced up to a maintenance dose of the drug [17]. Therapy stoppage is applied depending on the disease activity within 3 years of its beginning. Complete remission implies symptoms disappearance, as well as the loss of radiological and serological characteristics of the disease [18]. Spontaneous remission with no use of corticosteroids has also been reported in the literature. Indications for corticosteroid therapy include icterus appearance, pain or extrapancreatic AIP manifestation. Relapse commonly appears within the first 3 years of the disease (relapse within the maintenance therapy appears in 26% of cases, with no therapy in 54% of cases) [19]. Re-acutization of the disease is more often occurred if initial enlargement is more than 1/3 of the pancreas and in the presence of icterus, in commorbidity with extrapancreatic lesions (IgG4 sclerosing pancreatitis associated with AIP, proximal extra- and intrahepatic structures), incomplete remissions, as well as in the presence of genetic factors (haplotype HLA DQb1 57) [20]. Disease relapse requires application of corticosteroids, azathioprine, mycophenolate mofetil, methotraxate or 6-mercaptopurine, and currently anti-CD20 antibodies (rituximab). Immunoregulatory therapy is used in frequent relapses, in cases of resistence or pronounced adverse effects of corticosteroids [21].

Our patients were treated according to the protocol for the treatment of autoimmune pancreatitis. They did not have a relapse of the underlying disease, even after discontinuation of the therapy

It is sometimes hard to distinguish the focal form of AIP from pancreatic cancer in spite of clear criteria, since inflammatory cells could be found around cancer tissue in biopsy material, as well as IgG4 positive plasma cells, and, as we know, corticosteroids could be applied only when malignity is excluded [22]. It is known, also, that chronic pancreatitis and older age are risk factors for pancreatic cancer development. Prolonged use of corticosteroids leads to immunosuppression and could contribute to tumor appearance. So, it is necessary to control patients with AIP at regular intervals as well as to determine their tumor marker Ca 19.9. There are articles showing frequent appearance of pancreatic cancer many years after disease beginning, sometimes even at the same time with AIP [23, 24].

Conclusion

Autoimmune pancreatitis is a relatively new disease that is recognized more and more frequently today. The long-term prognosis is uncertain. The course of the disease could be affected by frequent relapses, exocrine and endocrine dysfunction of the pancreas, condition of the other affected organs, and comorbidity with the malignancy.

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CASE REPORT

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Abdominal localization of unicentric form of Castleman disease − A case report

Unicentrična forma Kastlemanove bolesti lokalizovane u stomaku

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Abstract

Introduction. Castleman disease is a rare disease of the unknown etiology, occuring in two clinical forms: unicentric or multicentric. It is characterized by the hyperplasia of lymph glands. In literature the four pathohistological forms were described: hyaline vascular type, plasma cell type, mixed type and a recently recognized plasmablastic type. The most frequent changes are localized in the mediastinum, while the abdominal localization is with significatly rare occurrence, and that was the motive for presentation of this case. **Case report.** In a 41-year old male magnetic resonance (MR) enterography showed a change in the ileocecal area without the presence of subjective symptoms of digestive tract and without loss of body mass. Due to the suspicion of stromal tumor, surgical intervention was indicated. Pathohistological findings showed Castleman *lymphadenopathia reactiva mesenterii* (plasma cell type) which was in the unicentric form. There were present only anaemia and the increased value of sedimentation from the laboratory analyses. **Conclusion.** Abdominal localization of unicentric plasma cell form occurs rarely and the surgical method of treatment presents the golden standard as it was shown in the presented case.

Key words:
giant lymph node hyperplasia; cecum; diagnostic techniques and procedures; diagnosis, differential; lymph nodes; digestive system surgical procedures.

Apstrakt

Uvod. Kastlemanova bolest je retko oboljenje nepoznate etiologije koje se javlja u dve kliničke forme: unicentrična ili multicentrična. Odlikuje se hiperplazijom limfnih žlezda. U literaturi se opisuju četiri patohistološka oblika: hijalinovaskularni tip, plazmocitni tip, mešoviti tip i skorije prepoznat plazmablastični tip. Promene su najčešće lokalizovane u medijastinumu, dok se abdominalna lokalizacija znatno ređe javlja, što je i bio razlog za ovaj prikaz slučaja. **Prikaz bolesnika.** Kod muškarca, starog 41 godinu, na magnetnoj rezonantnoj (MR) enterografiji uočena je promena u ileocekalnoj regiji bez prisustva subjektivnih simptoma iz digestivnog trakta kao i bez gubitka telesne mase. Zbog sumnje na stromalni tumor, indikovana je hirurška intervencija. Patohistološki nalaz je pokazao Kastlemanovu *lymphadenopathia reactiva mesenterii* (plazma-ćelijski tip) u unicentričnoj formi. Od laboratorijskih analiza jedino su bile prisutne anemija i ubrzana sedimentacija eritrocita. **Zaključak.** Abdominalna lokalizacija unicentrične plazmocitne forme Kastlemanove bolesti javlja se retko, a hirurška metoda lečenja predstavlja zlatni standard što je potvrđeno ovim prikazom.

Ključne reči:
kastlemanova bolest; cekum; dijagnostičke tehnike i procedure; dijagnoza, diferencijalna; limfne žlezde; hirurgija digestivnog sistema, procedure.

Introduction

Castleman disease (CD) is a rare, benign disease characterized by the lymph node hyperplasia. It was described for the first time by Castleman and Towne [1] in 1954, but its etiology has not been completely solved yet. In literature other names for this disease were mentioned: giant lymph node hyperplasia, angiofollicular lymph node hyperplasia, lymph no-

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de hamartoma, and benign lymph node lymphoma [2]. CD occurs in four pathohistological forms: hyaline-vascular type, placma-cell type, mixed type and recently recognized plasmablastic type [3]. Depending on the lymph node degree of affection, it could have two clinical forms: unicentric or multicentric. Changes are often localized in the mediastinum (65%) while in other parts their incidence is less: in the neck (16%), abdomen (12%), axilla (3%) [4, 5]. This paper presented a patient with abdominal localization of unicentric form of CD, analysis of clinical picture, diagnostic procedures and treatment.

Case report

A 41-year-old male submitted to diagnostic procedures for the last two years due to sideropenic anaemia, was hospitalized at the Clinic for Gastroenterology and Hematology, Military Medical Academy, Belgrade, for the clarification of changes in the area of terminal ileum, seen at magnetic resonance (MR) enterography. The patient denied the presence of pain in abdomen, loss of body mass, as well as difficulties of the upper parts of digestive tract. By laboratory analyses the following values were determined: sedimentation rate of erythrocytes (SE) – 51 mm/h, C-reactive protein (CRP) – 24.9 mg/L; fibrinogen – 5.6 mg/dL, leukocytes (Le) – 6.41 $\times 10^9$/L; hematocrit (Ht) – 0.42, thrombocytes (Tr) – 287 $\times 10^9$/L. Physical findings of the abdomen were without pathological changes. Auscultatory findings in lungs with no abnormalities detected. There were no signs of peripheral lymphadenopathy. On MR imaging there was a circular, clearly limited, well vascularized solid change in the ileocecal area, 34 × 37 mm in diameter which was not clearly separated from the ileum gyri. Other procedures performed were scintigraphy on Meckel diverticulum, colonoscopy, esophagogastroduodenoscopy (EGD) and radiography (RTG) of the lungs, showing no pathological changes. Echo of the abdomen showed calculosis of gallbladder. Due to the suspicion of stromal tumor of the abdomen, surgical intervention was indicated. After preoperative preparation, surgical intervention was performed: *Resectio intestini ilei terminaliss cum ileo-caeco anastomosis latero-lateralis*, cholecystectomia, appendectomia. Intraoperative findings was as follows: in the area of *meso* terminal ileum on roughly 35 cm from the valvula Bauchini, a ball-like, solid tumefaction, 7 × 6 cm in diameter, was found, performing compession to the part of ileum wall and narrowing its lumen. Tumor change on one side elevates the peritoneum and protrudes beneath the *meso* terminal ileum surface, on the other side unclearly and restrictedly compesses it (Figures 1 and 2).

Directly under this tumor change the enlarged lymph gland was noticed, which together with the tumefaction was sent to pathohistological analysis. Postoperative course was normal, with the wound which healed *per primam* and established an intestinal passage.

Pathohistological findings indicated Castleman *lymphadenopathia reactiva mesenterii* (plasma cell type). Tumor-like enlarged lymph nodus in *meso* ileum of the small intestine was thick with numerous follicles, irregularly large, reactive germinal centers (Bcl2), with the presence of IgD mantle cell zone, as well as many thiny follicles of the involutition appereance, of the concentric thiny cell mantle zone, without visible hyalinosis of blood vessels in these germinal centers (except one) (Figures 3 and 4).

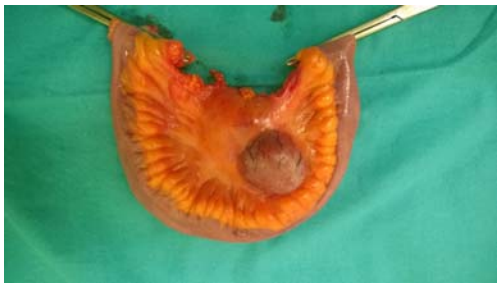

Fig. 1 − A removed part of the terminal ileum with a tumor *in meso* ileum – front view.

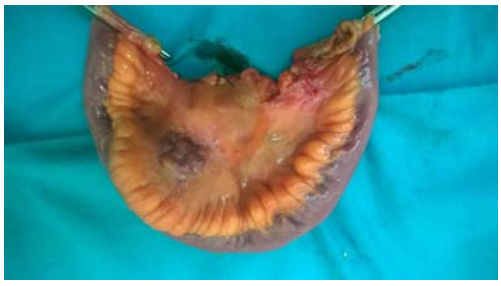

Fig. 2 – A removed part of the terminal ileum with a tumor *in meso* ileum – rear side.

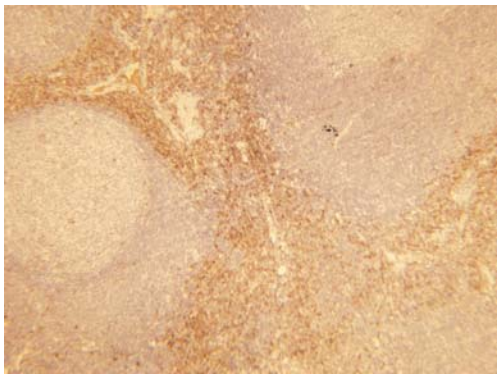

Fig. 3 – CD 138, plasma cells interfollicular (CD 138 immunostain, ×40).

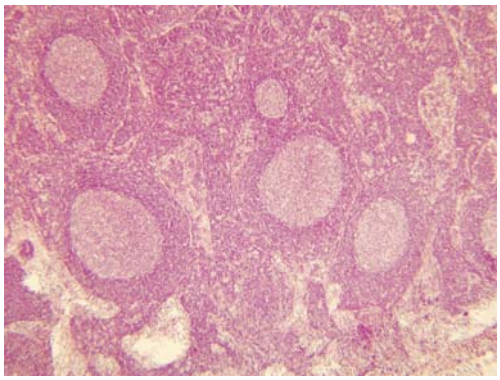

Fig. 4 – Primary follicular structures with interfollicular distension. Blood vessels and mass of plasma cell (HE, ×40).

Interfollicularly, the massive plasmocytosis was present (CD138+/Kappa+/lambda+) with numerous Russell bodies with rare individual CD20+/PAX5+/Bcl 6-/, as well as the copious and noticiable vascular postcapillary proliferation of blood vessels with thickening of hyaline walls (Figures 5 and 6). An enlarged lymph node (the reactive lymphadenopathy) was found.

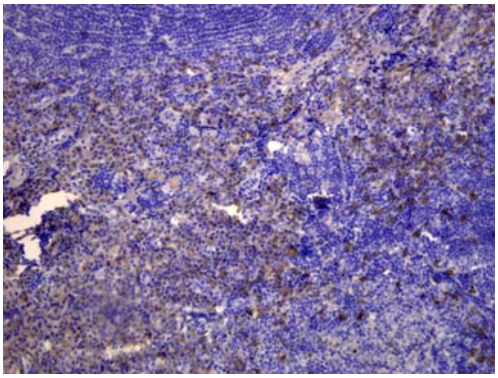

Fig. 5 – Intracytoplasmic kappa light chains in plasma cells (Kappa staining reagent system, ×200).

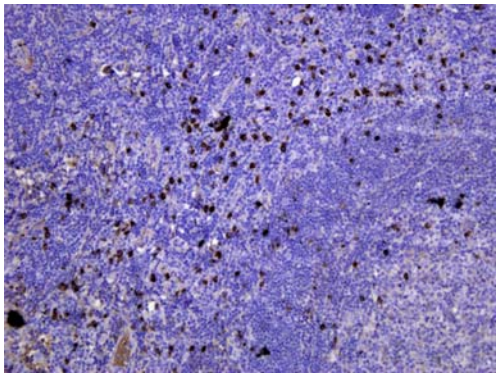

Fig. 6 – Intracytoplasmic lambda light chains in plasma cells (Lambda staining reagent system, ×200).

Discussion

Clinical manifestation and symptoms of CD are various depending on the disease type. They can be absent in some cases while in others they can be various and related to the infection, autoimmune disease or some tumor forms. In the localized, unicentric form, an enlarged lymph node if it is localized in the abdomen, could be asymptomatic and found by chance, as it was in the presented patient. Most often symptoms in unicentric, plasma cell form are anaemia, high value of SE, thrombocytopaenia, hyperglobulinemia, splenomegaly, night perspiration, loss of weight, exhaustion [5, 6]. In the presented patient, as the dominant findings, a 2-year anaemia and increased SE were present, but other laboratory parameters were in the normal limits.

CD occurs in adult persons and children, more often in the unicentric form. The first case with this disease was described by Dr. Benjamin Castleman at patients with the hyperplastic mediastinal lymph node classifying it as the unicentric form [1].

The multicentric form is related to viral infections. Researches show the presence of human herpesvirus 8 (HHV-8) or Kaposi sarcoma herpesvirus (KSHV) in lymph nodes in patients with ulticentric forms, and this form occurs most often in persons with HIV infection [3].

The dignosis of CD can be made by pathohistological findings. The first patient with CD, with the dominance of plasma cells, was operated in 1969, and classification of CD to the hyaline vascular and plasma cell histopathological type was done by Keller et al. [7] in 1972. In some papers classification of pathological changes is performed based on the localization, *ie* spreading of changes, so the unicentric form includes the hyeline vascular and plasma cell variant while at multicentric form the plasma cell type is dominant with the occurrence of plasmablastic characteristics [8]. Most recent recommendations of the American Cancer Society classify CD in 4 forms. Above mentioned researches showed that the unicentric plasma cell form occurred in 10−20% of patients and more often in younger patients (in the third and the fourth decade of life) and hyaline-vascular type, the most frequent, was described with the incidence of 70−90% [1, 8–10]. In the plasma cell form of the disease, pathohistological findings show the hyperplastic germinal center with a vascularized interfollicular part of the node and leaves of the polyclonal plasma cells. This histological findings are not specific only for CD, but it can be found also at hyperplastic reactive lymph nodes, for example at rheumatoid arthritis and viral lymphadenitis [8, 11].

The most frequent localization of the unicentric form is the mediastinum, and in the abdomen and the pelvis it was described as the focal mass differently localized: as retroperitoneal mass, in the mesentery, *porta hepatis* and in the pancreas [5].

CD treatment depends on the form of the disease and histological type. Surgical mode of unicentric form treatment is supposed to be the gold standard and it offers excellent results in over 90% of cases [12–14]. When lymph nodes could not be completely removed or when the surgical intevention is contraindicated, radiotherapy is used [2]. In the presented patient, surgical procedure led to the restoration of health, taking into consideration that additional diagnostic methods like positron emission tomography (PET) scan of the thorax and abdomen, did not suggest any pathological changes. In the multicentric form other modes of treatment are also applied: chemotherapy, application of corticosteroids, antiviral and immunomodulatory therapy [11].

Conclusion

Abdominal localization of the unicentric plasma cell form of Castleman disease has a small incidence, and the surgical method of treatment represents the gold standard as it was confirmed by the presented patient.

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CASE REPORT

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Unexpected bony structure in tonsillar fossa during tonsillectomy

Neočekivana koštana struktura u tonzilarnoj jami tokom tonzilektomije

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Abstract

Introduction. The elongated styloid process is a very rare clinical entity. In most cases it is asymptomatic, but also could cause Eagle's syndrome. We presented a rare case of the anatomic variation of styloid process and its clinical implication. **Case report**. In the left tonsillar fossa an unexpected bony structure was found during the routine tonsillectomy on a 16-year-old female patient. Computed tomography showed the elongated styloid process. No further treatment was necessary because it was asymptomatic in the follow-up period. **Conclusion**. The elongated styloid process is a very rare condition, but physicians should be aware of it and keep it in mind in order to make the diagnosis in patients with suggestive symptoms.

Key words:
temporomandibular joint disorders; ossification, heterotopic; diagnostic techniques and procedures; tonsillectomy.

Apstrakt

Uvod. Produžen stiloidni nastavak je veoma redak klinički entitet. U većini slučajeva je asimptomatski, ali može uzrokovati Eagle-ov sindrom. Prikazali smo anatomske varijacije i kliničku prezentaciju stiloidnog nastavka, kao i indikacije za lečenje bolesnika sa tim sindromom. **Prikaz bolesnika**. Tokom rutinske tonzilektomije kod 16-godišnje bolesnice nađena je neočekivana koštana struktura u levoj tonzilarnoj jami. Načinjena je kompjuterizovana tomografija i nađen je produženi stiloidni nastavak. U periodu praćenja bolesnica nije imala simptome produženog stiloidnog nastavka, pa dalje lečenje nije bilo potrebno. **Zaključak**. Produženi stiloidni nastavak veoma je redak entitet. Radi pravilne dijagnoze i mogućeg daljeg lečenja bolesnika sa tim sindromom, lekari moraju biti upoznati sa njegovom kliničkom prezentacijom i simptomatologijom koju daje.

Ključne reči:
temporomandibularni zglob, poremećaji; osifikacija, heterotopična; dijagnostičke tehnike i procedure; tonzilektomija.

Introduction

An elongated styloid process is an extremely rare clinical entity. It could cause Eagle's syndrome with vague symptomatology [1], facial pain or be silent and incidentally find during tonsillectomy [2], but also could cause a sudden death [3].

Data from literature show that 0.04–0.08% of population suffer from this disease and only 0.16% patients are actually symptomatic [4].

We presented a rare case with unexpected bony structure in the left tonsillar fossa without characteristic symptoms for elongated styloid process, found during the routine tonsillectomy.

Case report

A 16-year-old female patient underwent tonsillectomy under general anesthesia due to chronical tonsillitis. The patient did not suffer any characteristic symptoms for elongated styloid process. After removal of the left tonsil, a straight hard mass about 2 cm (intraoperatively) was seen in lateromedial direction (Figure 1). Normally, the styloid process of normal length are not palpable in the tonsillar fossa, and if it is possible, it is elongated styloid.

After the operation, computed tomography (CT) scan was done showing one-sided elongated styloid process (Figure 2). It was an unexpected finding during tonsillectomy because the patient was symptom-free, so no further treatment was necessary. Unusual, asymptomatic, hard mass in tonsillar fossa only need further imaging and precise information for patient.

The Ethic Committee of the Clinical Hospital Center "Zemun", Faculty of Medicine, University of Belgrade approved this case report.

Disscusion

The stylohyoid components are derived embryologically from the first and second branchial arches. The styloid process

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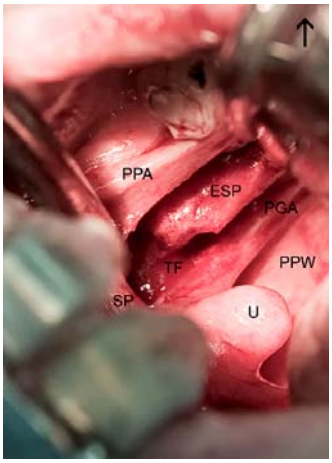

Fig. 1 – After removal of the left tonsil, a hard mass about 2 cm long was pointed into tonsillar fossa intraoperatively. SP – soft palate; U – uvula; PPW – posterior pharyngeal wall; PPA – palatopharyngeal arch; PGA – palatoglossal arch; TF – tonsillar fossa; ESP – elongated styloid process.

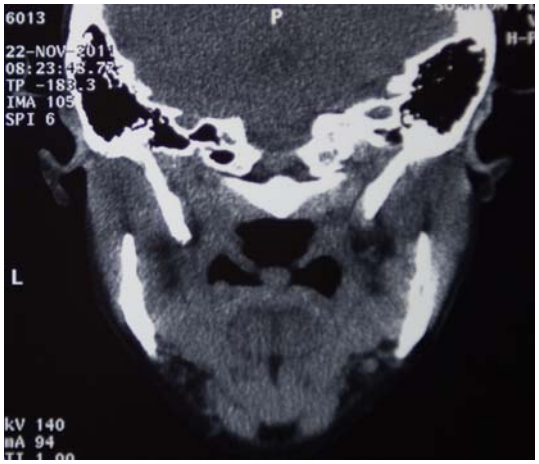

Fig. 2 – Computed tomography (CT) scan (coronal view) shows the left-sided elongated styloid process.

develops from the tympanohyal and stylohyal segments and is usually connected in adolescents [5].

The stylohyoid ligament starts from the tip of the styloid process to the hyoid bone. The stylomandibular ligament extends from the styloid process to the angle of the mandible. There are three muscles: the stylopharyngeus, stylohyoid and styloglossus and innervation comes from IX, VII, XII cranial nerves, respectively. The internal jugular vein and XI, XII, X, IX cranial nerves are located medial to the styloid process. The glossopharyngeal nerve has close relation with the styloid process. It passes from the jugular foramen, medial to the styloid process, where it curves around the posterior border of the stylohyoid muscle. This anatomic relationship is important due to explanation of glossopharyngeal neuralgia in cases with the elongated or fractured styloid process.

The usual length of the styloid process in an adult is approximately 2.5 cm and could not be detected in tonsillar fossa after tonsillectomy. An elongated styloid is defined as greater than 3 cm[3]. The longest symptomatic elongated styloid process was around 6.3 cm and underwent surgery [6]. Nevertheless, the length as a single parameter is not a risk factor but its combination with direction and curvature is important for severity of symptoms [7].

The males had greater styloid process lengths than the females [8].

The elongated styloid process rarely occurs in childhood or adolescence [9]. Nevertheless, in this study the presented patient was 16 years old.

Although the elongated styloid process is usually bilateral [10], in the presented patient was unilateral. Nevertheless, bilateral cases do not always involve bilateral symptoms.

Etiology of the elongation is a poorly understood process and there are three theories for explaining the development of elongated styloid process. The first theory is the hyperplasic reaction of the styloid ligament stimulated by pharyngeal trauma that caused ossification of the ligament. According the second theory there is a metaplastic reaction of styloid ligament, also due to traumatic stimulus, which results in ossification. The third theory is that the styloid process and the styloid ligament are anatomic variations [11].

Eagle's syndrome is characterized most frequently by neck, throat or ear pain, pharyngeal foreign body sensation or dysphagia. The pathophysiological mechanism of symptoms could be several. It could be a traumatic fracture of the styloid process with causing proliferation of granulation tissue pressure on the surrounding structures, or compression of adjacent nerves, the glossopharyngeal, trigeminal or chorda tympani [12]. Degenerative and inflammatory changes could be in the tendonous portion of the stylohyoid insertion. Also, there are the irritation of the pharyngeal mucosa by direct compression or post-tonsillectomy scarring or striking of the carotid vessels, producing irritation of the sympathetic nerves in the arterial sheath [13].

CT with coronal and sagittal views is necessary for the accurate diagnosis of the elongated styloid process and for defining its angulation and anatomic relationship [14]. Three-dimensional CT (3D-CT) has several advantages over conventional coronal and axial CT images, because of its ability to accurately image the anatomy and for defining angulation and direction of the styloid process and its anatomic relationship [15, 16]. CT finding could show several possible variations: elongated, pseudoarticulated or segmented styloid process, and according to the calcification: peripheral, partial, complete or nodular type of calcification [6].

Eagle's syndrome can be treated medically and surgically. Conservative treatment includes transpharyngeal infiltration of steroids or anesthetics into the tonsillar fossa. The surgical approaches for styloidectomy are intraoral or extraoral approach [17]. Asymptomatic cases need only follow-up, good patient information and subsequent health monitoring.

Conclusion

The elongated styloid process is a very rare condition, but physicians should be aware of it and keep it in mind in order to make the diagnosis in patients with suggestive symptoms.

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CASE REPORT

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Oral rehabilitation of a patient with temporomandibular joint ankylosis caused by ankylosing spondylitis: A case report

Oralna rehabilitacija bolesnika sa ankilozom temporomandibularnog zgloba uzrokovanom ankilozirajućim spondilitisom

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Abstract

Introduction. Ankylosing spondylitis (AS)/Morbus Bechterew is a chronic inflammatory rheumatoid disease. The temporomandibular joint (TMJ) dysfunction is involved in 4–35% of AS cases, and is correlated to the severity and extension of AS. Even though AS-caused TMJ ankylosis is exceptional, one should have high index of suspicion of TMJ ankylosis in AS for an early detection, because it is an extremely serious and disabling condition that causes problems with mastication, swallowing, digestion, speech, appearance and poor oral hygiene with heavy caries. **Case report.** A 54-year-old male patient sought medical attention at the Department of Periodontology and Oral Medicine, Clinic for Dentistry at the Military Medical Academy, Belgrade, Serbia, with the chief complaint of pain in the area of the upper left canine in the presence of limited mouth opening. The treatment plan consisted of upper left canine management and rehabilitation of the remaining teeth in the frontal and the premolar region in both, the upper and lower jaw. Even though molar region needed to be treated, unfortunately it was not in the treatment plan because ankylosis of TMJ made the treatment impossible. **Conclusion.** The patients with AS-caused TMJ ankylosis are considered a diagnostic challenge to routine dentistry. Accent should be given to early diagnosis and multidisciplinary approach in the treatment of the AS patients towards the favorable disease course and outcome.

Key words:
spondilitis, ankylosing; temporomandibular joint; ankylosis; diagnosis; dental care for chronically ill; treatment outcome.

Apstrakt

Uvod. Ankilozirajući spondilitis (AS)/Behterova bolest je hronično zapaljensko reumatsko oboljenje. Kod 4–35% bolesnika obolelih od AS zahvaćen je i temporomandibularni zglob (TMZ), a njegova zahvaćenost zavisi od težine samog oboljenja i dužine trajanja AS. Iako je AS-uzrokovana TMZ ankiloza veoma retka, mora se obratiti posebna pažnja na njeno rano prepoznavanje kao veoma teškog oboljenja koje uzrokuje velike probleme pri mastikaciji, gutanju, govoru, izgledu i lošoj oralnoj higijeni s pojavom karijesa. **Prikaz bolesnika.** Muškarac, star 54 godine, potražio je medicinsku pomoć u Odeljenju za bolesti usta i zuba na Stomatološkoj klinici Vojnomedicinske Akademije u Beogradu, Srbija, zbog bola u gornjem levom očnjaku uz smanjenu mogućnost otvaranja usta. Terapijski plan se sastojao od konzervativno-endodntskog zbrinjavanja gornjeg levog očnjaka, i ostalih zuba frontalnog, kao i premolarnog područja gornje i donje vilice. Iako je i molarno područje bilo za zbrinjavanje, to je usled napredovanja oboljenja (ankiloze TMZ) bilo nemoguće sprovesti. **Zaključak.** Stomatološko zbrinjavanje bolesnika sa AS-uzrokovanom ankilozom TMZ smatra se pravim izazovom za rad u svakodnevnoj stomatološkoj praksi. Stoga, trebalo bi naglasiti izuzetnu važnost postavljanja rane dijagnoze oboljenja, kao i multidisciplinarni pristup u lečenju AS bolesnika u cilju unapređenja toka i ishoda samog oboljenja.

Ključne reči:
spondilitis, ankilozirajući; temporomandibularni zglob; ankiloza; dijagnoza; zubi, nega i lečenje hronično obolelih; lečenje, ishod.

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Introduction

Ankylosing spondylitis (AS)/Morbus Bechterew is a chronic inflammatory rheumatoid disease [1]. It occurs in 0.1–0.2% of adult population, mostly in the second or third decade of life, and 3–5 times more often in males [1–4]. AS causes progressive synovial changes that eventually involve all of the axial joints (mainly joints in the spine/"bamboo spine" and the sacroiliac joint in the pelvis), but peripheral joint involvement may also be an important feature [5–7]. The disease course, although highly variable, will progress to severe disability of the temporomandibular joint (TMJ) in almost one third of AS patients [1–4]. TMJ disability is correlated to the severity and extension of AS [1].

Involvement of the TMJ appears to present with increasing pain on eating, often associated with progressive limitation of mouth opening, stiffness and gross restriction of jaw movement, accompanied by radiographic evidence of joint degeneration (loss of joint space, osteophytes, surface erosion and ankylosis). The available literature suggests that AS-caused ankylosis of the TMJ is exceptional [1–3]. Even though exceptional, one should think about its possibility as it is a serious and disabling condition that causes problems with mastication, swallowing, digestion, speech, appearance and poor oral hygiene with resultant rampant caries [1–3]. For these patients access to routine dentistry is limited and they are considered a diagnostic challenge for the dentist [4]. The aim of this study was to present caries management in a patient with AS-caused TMJ ankylosis and to draw attention to early detection and multidisciplinary approach in the treatment of AS patients towards to the favorable disease course and outcome.

Case report

A 54-year-old male patient sought medical attention at the Department of Periodontology and Oral Medicine, Clinic for Dentistry, Military Medical Academy, Belgrade, Serbia, due to pain in the area of the upper left canine in the presence of limited mouth opening.

Clinical dental examination revealed painful and limited mouth opening. His feeding was characterized by the inability to masticate food, limiting intake of liquids or semisolids. The TMJ range of motion was limited and associated with preauricular pain, while maximum unassisted interincisal mouth opening was 15 mm (Figure 1). Assisted (passive) mouth opening did not increase the interincisal distance. No clicking sounds on opening, closing and lateral movements of the TMJ were noticed. The patient had no history of trauma or infection of the TMJ. Due to the degenerative changes in TMJ, the patient had occlusal changes (protruded mandible) and anterior open bite.

The detailed medical history of the patient revealed that the patient due to AS was for 25 years under the appropriate treatment in the Institute of Rheumatology of Serbia (IRS). The IRS gave a detailed description of the patient's condition along with the laboratory findings (presence of HLA-B27 antigen) and conventional radiographic findings of spine and sacroiliac joint in the pelvis (Figures 2 and 3).

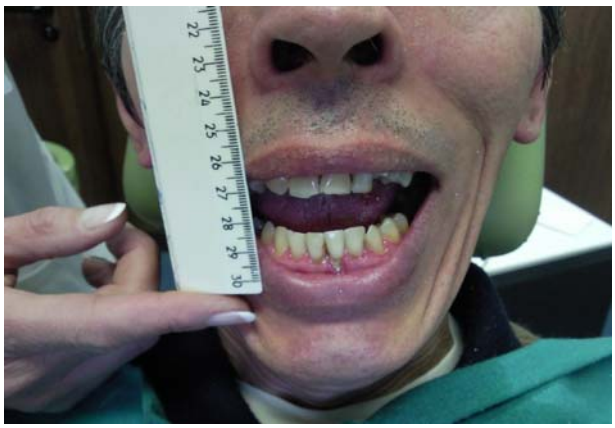

Fig. 1 – Maximum unassisted interincisal mouth opening (15 mm) on the first visit to the clinic.

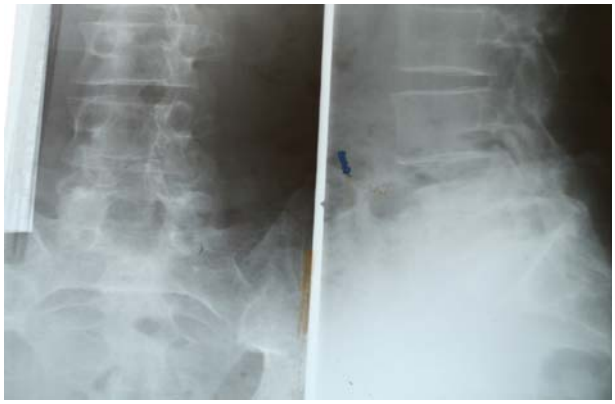

Fig. 2 – Lumbar spine radiograph shows syndesmophytes bridging the vertebral bodies with the classic "bamboo spine" appearance.

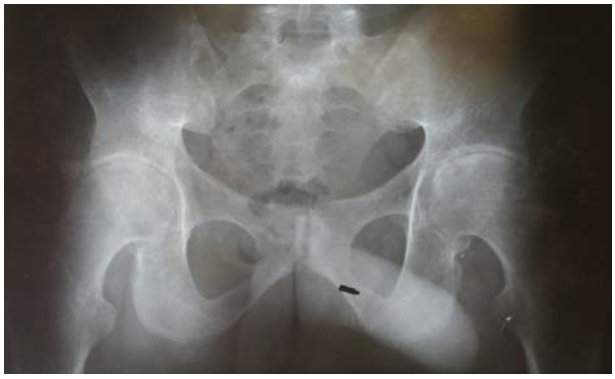

Fig. 3 – Conventional radiograph of the pelvis shows bilateral sacroiliitis.

After clinical (anamnesis and physical examination) and imaging evaluations, the diagnosis was bilateral TMJ ankylosis secondary to AS.

Intraorally, teeth were severely damaged. Due to poor oral hygiene habits almost all present teeth, apart from the painful left canine, were affected by extensive multiple caries and periodontal disease (Figure 4). The treatment plan in accordance with the possibilities of opening the mouth was made. The plan consisted of the upper left canine management and rehabilitation of the remaining teeth in the frontal and the premolar region in both, the upper and lower jaw. Even though molar region needed to be treated, it was

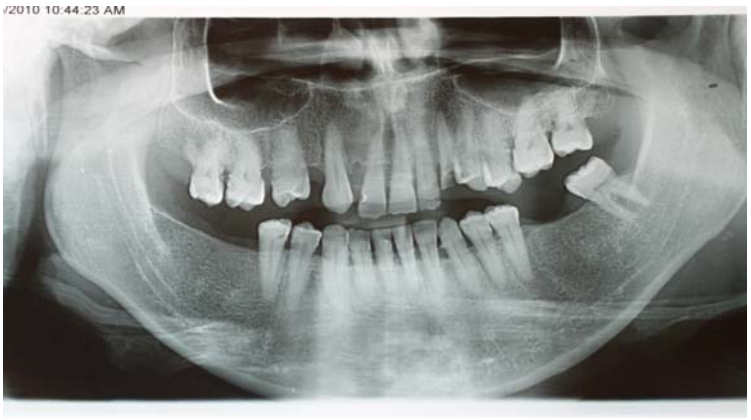

Fig. 4 – Digital panoramic radiograph of the patient before the upper left canine treatment.

not in the treatment plan because TMJ ankylosis made the treatment impossible, even for tooth extraction.

The treatment of upper left canine consisted of necrotic pulp removal by chemomechanical instrumentation of the root canal. Mechanical treatment was carried out by a hand NiTi file with the passive step-back technique. Root canal drying was done with paper points and it was temporary filled, with aqueous suspension $Ca(OH)_2$ with pH value 12 (Calcipro, VOCO Germany); after that, the tooth was closed with temporary cement (Provis, Favodent, Austria). Control exam was scheduled in two weeks. After two weeks, since patient had no clinical and subjective symptoms, the definitive root canal filling was done with the paste based on ZOE (Endomethasone N, Septodont, France) and gutta-percha points (Beutelrock, VdW dental, Germany) using cold lateral compaction of gutta-percha (Figure 5).

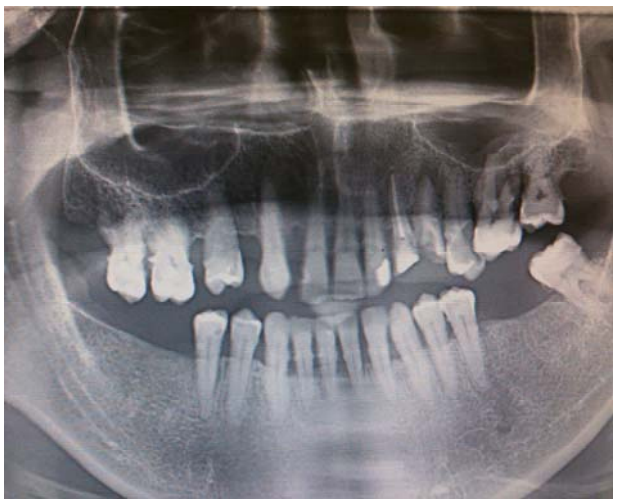

Fig. 5 – Digital panoramic radiograph of the patient after upper left canine treatment.

For all teeth in the frontal region of the upper and lower jaw, calculus deposits were removed by an ultrasonic scaler.

Discussion

AS is a chronic idiopathic rheumatic disease of multifactorial etiology, associated with the combination of infection (such as *Klebsiella*), autoimmunity and strong genetic factors [1–3]. AS is diagnosed through a combination of clinical history, examination, radiological imaging and determination of HLA genotype (the antigen HLA-B27 in the blood is present in more than 90% of patients with AS). To date, the diagnosis of AS is usually made with an average 5–10 years of delay [1–5].

The available literature suggests that TMJ involvement is found in 4–35% of AS patients, usually at a relatively late stage, after 10–30 years of presence [5, 6]. Therefore, evaluating TMJ function is strongly recommended in patients who have the long history of AS.

Having all this in mind, it is obvious that the accent should be put on the early diagnosis of AS. For the early AS diagnosis we should be informed about age of patients, symptoms and signs of initial or early manifestation, the significance of laboratory findings and radiological changes, especially on sacroiliac joints. When the early diagnosis of AS is set, the clinician should be suspicious of possible TMJ involvement and make a routine clinical examination in order to detect the condition and possibly present rare complications of TMJ. If involved, clinical findings in the TMJ are pain, swelling, movement impairment, crepitation and malocclusion of the teeth (in advanced stages) [7].

Conclusion

The early diagnosis of AS will give a chance to the reumatologist to make a multidisciplinary approach by team work (such as maxillofacial surgeon, dentist, psychiatrist, physiotherapist, speach therapist...) towards the most favorable disease course and outcome. The patient should be adviced to accept recommendations concerning his behaviour regarding, among others, dental health (periodical dental health examinations) before the TMJ ankylosis occur. The patient should know that all the teeth should be rehabilitated, not extracted. Even if TMJ ankylosis occurs in later stages, the patient can have a surgical treatment of ankylosis, and in that case all his teeth will have an important role in the future coordination of mandibular movement after the operation.

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C A S E R E P O R T

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Left atrial appendage closure with Watchman device in prevention of thromboembolic complications in patients with atrial fibrillation: First experience in Serbia

Zatvaranje aurikule leve pretkomore Watchman uređajem u prevenciji tromboembolijskih komplikacija kod bolesnika sa atrijalnom fibrilacijom: Prva iskustva u Srbiji

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Abstract

Introduction. Atrial fibrillation (AF) is the major cause of stroke, particularly in older patients over 75 years of age. European Society of Cardiology guidelines recommend chronic anticoagulation therapy in patients with atrial fibrillation if CHA_2DS_2-VASc score is ≥ 1 [CHA_2DS_2-VASc score for estimating the risk of stroke in patients with non-rheumatic AF consisting of the first letters of patients condition: C – congestive heart failure; H – hypertension; A_2 – age ≥ 75 years; D – diabetes mellitus; S_2 – prior stroke, transitory ischaemic attack (TIA) or thrombolism; V – vascular disease; A – age 65–74 years; Sc – sex category]. However, a significant number of patients have a high bleeding risk, or are contraindicated for chronic oral anticoagulation, and present a group of patients in whom alternative treatment options for thromboembolic prevention are required. Transcatheter percutaneous left atrial appendage closure (LAAC) devices have been recommended in patients with contraindications for chronic anticoagulant therapy. **Case report.** We present our first three patients with nonvalvular AF and contraindications for chronic anticoagulant therapy who were successfully treated with implantation of LAAC Watchman device in Catheterization Laboratory of the Clinic for Cardiology, Clinical Center of Serbia in Belgrade **Conclusion.** Our initial results with Watchman LAAC device are promising and encouraging, providing real alternative in patients with non-valvular AF and contraindication for chronic anticoagulant therapy and high bleeding risk.

Key words:
atrial fibrillation; cerebrovascular disorders; risk assessment; therapeutic occlusion; heart atria.

Apstrakt

Uvod. Atrijalna fibrilacija (AF) je glavni uzrok moždanog udara, posebno kod starijih bolesnika preko 75 godina. Preporuke Evropskog udruženja kardiologa preporučuju hroničnu antikoagulantnu terapiju kod bolesnika sa atrijalnom fibrilacijom i CHA_2DS_2-VASc skorom ≥ 1 [CHA_2DS_2-VASc skor za procenu rizika od nastanka moždanog udara kod bolesnika sa ne-reumatskom AF koji se sastoji od početnih slova bolesti: C – kongestivna srčana slabost; H – hipertenzija; A_2 – starost preko 75 godina; D – dijabetes melitus; raniji moždani udari ili tranzitorni ishemijski atak (TIA) ili tromboembolizam; V – vaskularne bolesti; A – starost 65–74 godine; Sc – pol]. Međutim, značajan broj bolesnika ima visok rizik od krvarenja ili kontraindikacije za hroničnu antikoagulantnu terapiju, i predstavljaju bolesnike kod kojih su neophodni alternativni načini lečenja u prevenciji tromboembolijskih komplikacija. Transkatetersko perkutano zatvaranje aurikule leve pret-

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komore (LAAC) putem posebnih zatvarača preporučuju se kod bolesnika sa kontraindikacijama za hroničnu antikoagulantnu terapiju. **Prikaz slučaja.** Predstavljamo naša prva 3 bolesnika sa ne-valvularnom AF i kontraindikacijama za hroničnu antikoagulantnu terapiju kod kojih su uspešno ugrađeni Watchman LAAC zatvarači u Sali za kateterizaciju Klinike za kardiologiju Kliničkog centra Srbije u Beogradu. **Zaključak.** Naši inicijalni rezultati sa Watchman LAAC zatvaračima su obećavajući i ohrabrujući, a predstavljaju pravu alternativu za bolesnike sa ne-valvularnom AF i kontraindikacijom za primenu dugotrajne antikoagulantne terapije, odnosno za one kod kojih postoji visok rizik od krvarenja.

Ključne reči:
fibrilacija pretkomora; cerebrovaskularni poremećaji; rizik, procena; okluzija, terapijska; srce, pretkomora.

Introduction

Left atrial appendage closure (LAAC) by transcatheter technique has been developed to prevent thromboembolic complications in patients with nonvalvular atrial fibrillation (AF) who cannot tolerate chronic oral anticoagulant therapy [1, 2]. In fact, it has been demonstrated that LAAC device can be used as an alternative to chronic anticoagulant therapy for stroke prevention in patients with nonvalvular AF, but the primary indications for LAAC include the patients with contraindications to chronic anticoagulant therapy [3].

In patients with nonvalvular AF, left atrial appendage is in vast majority of cases the origin of thrombi and thromboembolic complications with stroke being most devastating and life-threatening [4]. Oral anticoagulant therapy with vitamin K antagonists, have been used for years to prevent thromboembolic complications but still a number of patients are undertreated with large periods of time out of therapeutic range [5]. Newer oral anticoagulant drugs have demonstrated superiority in relation to vitamin K antagonists in regard to efficacy and less intracranial bleeding, but still a number of patients cannot tolerate these agents due to high bleeding risk or adverse effects [6].

Several transcatheter LAAC devices have been developed, but only Watchman (Boston Scientific, USA) has demonstrated long-term superiority over warfarin in two large randomized clinical trials, PROTECT AF and PREVAIL [7–10]. Thus, here we present our first three patients with nonvalvular AF and contraindications for chronic anticoagulant therapy who were treated with implantation of Watchman device in Catheterization Laboratory of the Clinic for Cardiology, Clinical Center of Serbia, Belgrade.

Case report

Study population

Implantation of Watchman LAAC device was performed between March 2014 and April 2015 in three patients with nonvalvular AF and contraindications for chronic anticoagulant therapy or high bleeding risk [11]. The patients were considered for LAAC if noninvasive and invasive cardiologists concluded that they were not candidates for chronic anticoagulant therapy. All patients were informed about the risks and benefits of the procedure and provided informed consent for LAAC. All procedures were performed with the guidance of the experienced proctors for LAAC (MG and AVP).

Pre-procedure screening

Pre-procedure screening included detailed clinical examination, 2D echocardiographic and transesophageal echocardiographic (TEE) examination. Baseline TEE was required to exclude existing thrombus, to evaluate feasibility of the intervention and the morphology of the appendage ("WindSock type", "Chicken Wing type", or "Broccoli type") (Figure 1). Accurate TEE measurements in several planes (at 0°, 45°, 90°, 135°) (Figure 2) were important to determine dimensions of left atrial appendage (LAA) ostium and depth of the appendage [12]. The sizing of the Watchman device is based on largest ostium diameter which should be in the range of available device diameter, with certain (up to 20–25%) recommended oversizing. The maximum LAA ostium size should be > 17 mm or < 31 mm to accommodate available Watchman device sizes.

Watchman device

Watchman LAAC device (Figure 3) consists of self-expanding nitinol frame covered with permeable (160 microns) polyethylene terephthalate (PET) membrane, with 10 anchors on the nitinol frame designed to fix and stabilize appendage tissue with device nitinol frame [8, 9]. They are manufactured in 5 sizes (21, 24, 27, 30 and 33 mm) that are delivered through 14F sheath inserted in the femoral vein. In most of the cases, double-curve sheath is used for implantation of the device. The Watchman device has CE and FDA marks.

Implantation procedure

All three patients were premedicated with aspirin 100 mg, clopidogrel 75 mg, and the left atrial appendage was one day before procedure checked for the presence of left atrial thrombus by TEE. In case of the presence of left atrial thrombus, the patient received anticoagulation for at least 15 days, and then again the left atrium was re-evaluated by TEE.

The procedure was performed in catheterization laboratory under general anesthesia and TEE monitoring, with pure percutaneous approach from right femoral vein. After insertion of 8F sheet into right femoral vein, transseptal puncture with Brocken-brough needle and insertion of transseptal sheath were performed. Transseptal puncture was performed with TEE guidance in the middle lower part of interatrial septum, using TEE bicaval view and aortic short axis view. At this time point, unfractionated heparin dose of

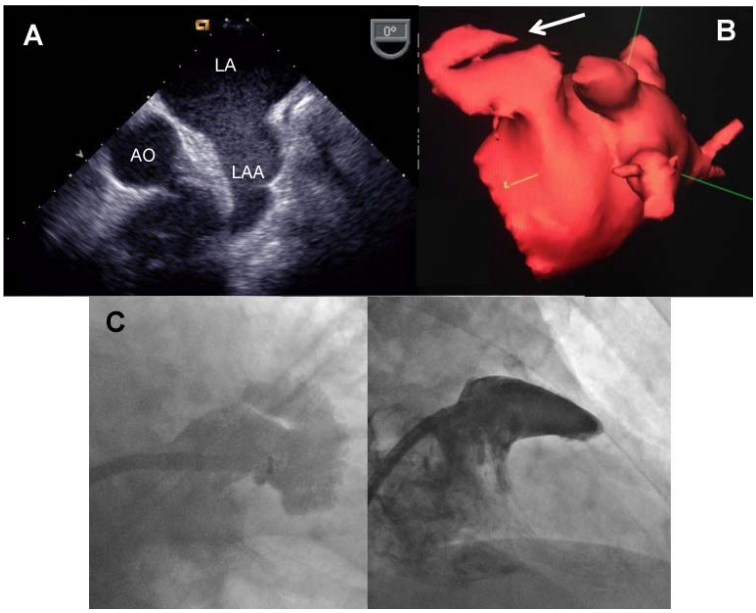

Fig. 1 – The "Chicken Wing" left atrial appendage morphology. Imaging modalities such as (A) transesophageal echocardiography, (B) computed tomography or (C) contrast angiography can be used to determine the shape of the left atrial appendage (arrow) to help planning device placement.
AO - aorta; LA - left atrium; LAA - left atrial appendage.

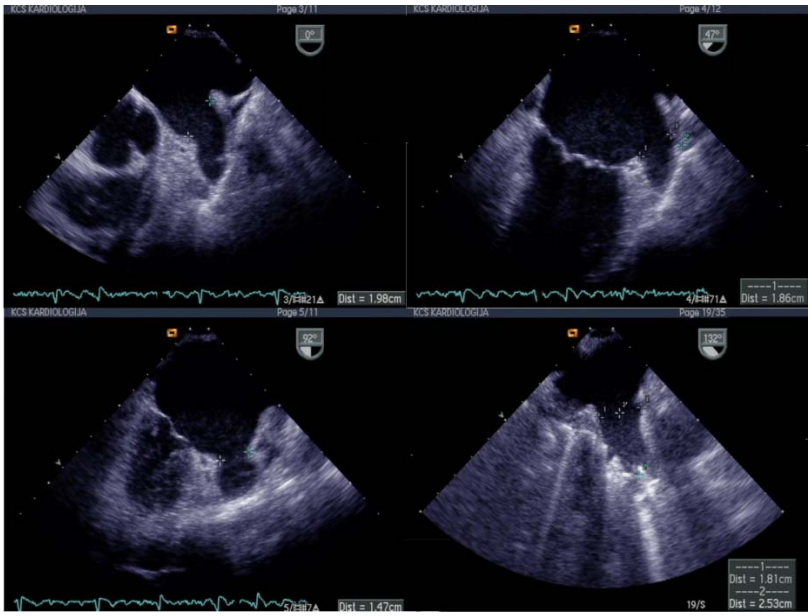

Fig. 2 – Transesophageal echocardiography measurements in several planes (at 0°, 47°, 92°, 132°) are important to determine maximal ostial diameter and depth of the left atrial appendage.

Fig. 3 – Watchman: Left atrial appendage closure device is a nitinol cage with a polytetrafluoroethylene membrane on the surface, and fixation anchors around the perimeter (Courtesy of Boston Scientific).

100 units kg body weight was administered to reach activated clotting time of at least 250 seconds which was repeated every 30 min.

After transseptal puncture, super stiff J-tip guide wire 0,035" was positioned into the upper left pulmonary vein, and the device sheath was introduced into the left atrium. Then, pigtail catheter was introduced and positioned in the LAA for angiography performed in several views (right anterior oblique with caudal and cranial angulations) for LAA shape visualization and measurement. At the same time, once again TEE measurements of LAA orifice and depth were rechecked, and optimal device size was selected.

The 14F double curved sheath was advanced over pigtail catheter into LAA dominant lobe and positioned at the LAA orifice. Then, pigtail catheter was removed and the device preloaded into 12F delivery system was advanced and aligned with 14F sheath positioned at the orifice. Once the sheath was slightly retracted from the orifice, with the delivery system stable positioned at the orifice, the whole delivery system and 14F sheath was withdrawn to expose Watchman device to adapt to LAA (Figure 4). The device was ready for the release when following criteria were met: position (device distal or at ostium with less the 40–50% of device depth protrusion of the shoulders), anchor stability (return to original position when retracting); size (device shoulder compressed up to 20% of original size by TEE); seal (residual flow less then 5 mm by TEE). When all these criteria were met, the device could be released by counterclockwise rotation. The position was finally checked by angiography (Figure 5) and TEE images (Figure 6) for residual seal and position.

One day after the procedure, TEE was repeated for the position of the device and the presence of pericardial effusion, and if absent the patients were discharged on oral anticoagulant therapy and aspirin for the next 45 days. If on repeated TEE after 45 days there was successful sealing around the device (complete or less then 5 mm residual flow) the patient was given clopidogrel for the next 6 months and aspirin indefinitely.

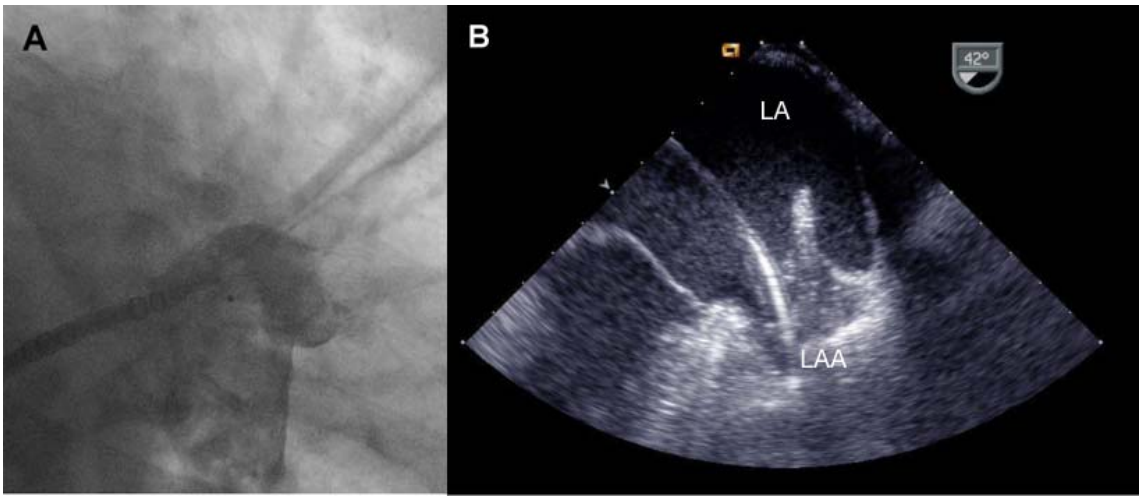

Fig. 4 – Angiography (A) and transesophageal echocardiography (B) images showing the final position of the Watchman device before device release.
LA - left atrium; LAA - left atrial appendage.

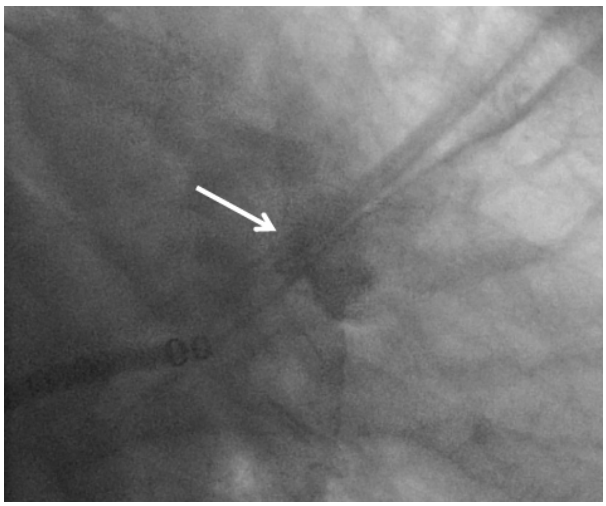

Fig. 5 – Angiography showing the final position of the Watchman device (arrow) after device release without residual peridevice shunt.

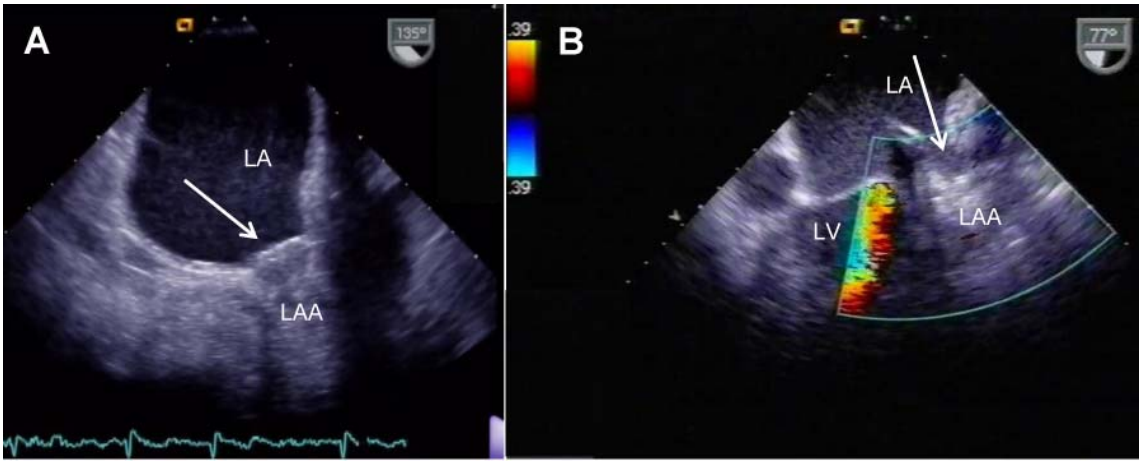

Fig. 6 – A) Transesophageal echocardiography showing the final position of the Watchman device (arrow); B) – Color Doppler echocardiography image showing the absence of residual shunt within the left atrial appendage.
LA – left atrium; LAA - left atrial appendage; LV – left ventricule.

Case 1

A 59 male patient was admitted to our hospital with a history of frequent episodes of paroxysmal AF for the implantation of LAA occluder. He had a moderately diminished renal function (stage 3A), well controlled arterial hypertension and prior history of two transient ischemic attacks (in January 2002 and in February 2014) even though he was on warfarin and later on dabigatran anticoagulant therapy. The patient also had at the end of 2013 and at the beginning of 2014 duodenal ulcer perforation which was conservatively treated. Thus, due to prior stroke despite anticoagulant therapy and high bleeding risk HAS-BLED score 4) [H – hypertension; A – abnormal real and liver function; S – stroke; B – bleeding; L – labile INRs (International normalized ratio) D – drugs or alcohol], this patient was referred to implantation LAAC in order to prevent further thromboembolic complication of AF. He denied chest discomfort during physical activity but he had palpitations and dyspnea on effort. On transthoracic echocardiography (TTE), left ventricular (LV) dimensions were normal, end-diastolic dimension (EDD) was 50 mm, and end-systolic dimension (ESD) 38 mm with preserved ejection fraction (EF), 67%. Left atrium (LA) was enlarged, 52 mm (volume 85 mL). Prior to the intervention on TEE, the presence of thrombus in LAA was excluded and maximum measured ostial dimension of LAA was 21 mm. Watchman LAA occluder size 24 mm was implanted in March 2014. Even though at the end of the procedure we found on TEE the residual jet leak measured about 5.3 mm (Figure 7A), angiographically we found only mild leak – dye filling of one-third of the LAA (Figure 7B). We decided to stop the procedure since the angiographic result was acceptable. As a result of the residual leakage due to probably smaller device size, the patient was left on anticoagulant therapy including dabigatran.

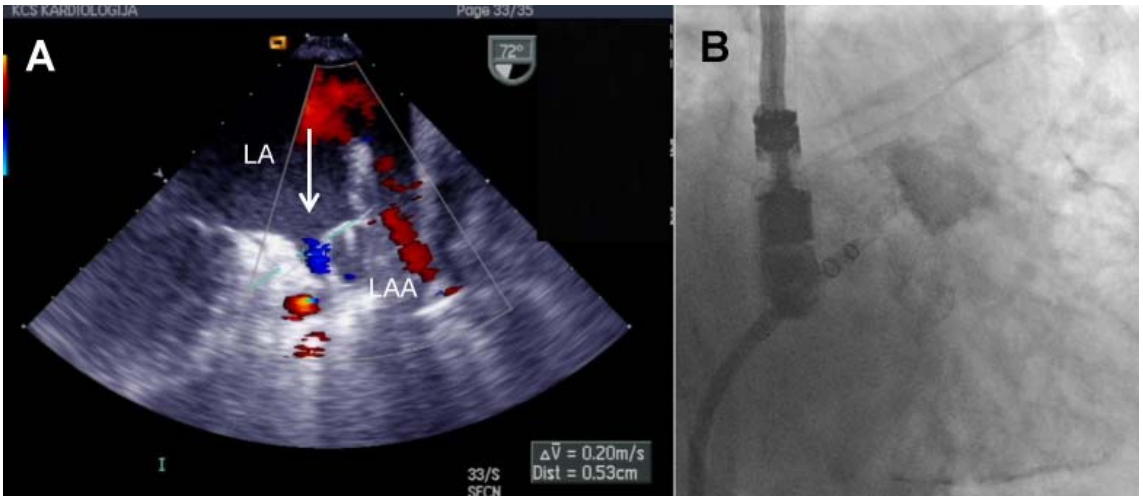

Fig. 7 – A) Color Doppler echocardiography image showing residual peridevice shunt within the left atrial appendage (arrow); B) Angiography showing mild leak – dye filling one-third of the left atrial appendage.
LA – left atrium; LAA – left atrial appendage.

Case 2

A 53 years old male patient with a prior history of permanent AF, kidney transplantations (1989 and 2003) due to terminal renal insufficiency and with chronic hepatitis B was referred to our hospital. He knew for the AF since 2013, and was unsuccessfully medically converted to the sinus rhythm both with amiodarone due to the rise of the liver enzymes and with propafenone therapy. Thus 2013, he was on the heart rate control therapy with beta blockers and on warfarin therapy. Also, because of chronic hepatitis B and moderately elevated liver enzymes, he was on antiviral therapy. Due to kidney transplant he was on immunosuppressive therapy. He denied chest pain at rest and during physical activity, palpitations or dyspnea on effort. He had also well controlled arterial hypertension. Because of the need of chronic anticoagulant therapy and high risk of bleeding due to his comorbidities (HAS-BLED score 3) he was referred to the percutaneous device closure of the LAA. Prior to the intervention, TTE and TEE were done. On TTE, LV dimensions were normal (EDD 47 mm, ESD 37 mm with preserved EF, 55%). LA was enlarged, 50 mm (volume 80 mL), with spontaneous echo contrast, but without thrombotic masses in LA. On TEE, prior to the LAAC, the presence of thrombus in LAA was excluded and the maximal dimension of LAA ostium was 23 mm. Initially, Watchman device 24 mm was selected and positioned, but due to high peridevice leak it was not released, but retracted and replaced for bigger device size 27 mm which was successfully implanted with good device deployment, very mild peridevice leak of up to 2 mm and angiographically only the trace of the dye in the LAA. After 45 days, TEE showed residual peridevice leak of up to 4mm, without thrombotic formations but because of spontaneous echo contrast in LA we decided to continue with anticoagulant therapy.

Case 3

A 53 years old male patient with a permanent AF was admitted to our hospital. He had a prior history of paroxysmal AF from 2006, with several unsuccessful cardioversions to the sinus rhythm, and since 2006 he was on warfarin. He had also well controlled hypertension and smoking habit. In July 2013, he suffered hemorrhagic stroke. He denied chest discomfort during physical activity but he had palpitations and dyspnea on effort. The decision to implant Watchman device was based on previous hemorrhagic stroke and consequently high bleeding risk (HAS-BLED score was 3) on anticoagulant therapy. Prior to the implantation of device TTE showed enlarged LV: EDD 58 mm and ESD 44 mm with slightly reduced EF 45%, without wall motion abnormalities. LA was enlarged 55 mm (volume 103 mL), with spontaneous echo contrast, but without thrombotic masses. On TEE precise measurements (maximal ostial LAA dimension of 26 mm), anatomy (lobularity and shape) and potential presence of thrombus in LAA were assessed. Next day, the implantation of Watchman device, size 30 mm, was successfully done with TEE periprocedure guidance. At the end of the procedure TEE showed good device deployment, stability, no interference with surrounding structures and peridevice leak. Also after 45 days, TEE showed excellent device sealing of the LAA, without any peridevice leakage. Therefore, only antiplatelet therapy including clopidogrel for the next 6 months and aspirin indefinitely was continued.

Disscusion

Our initial results with Watchman LAAC device in patients with non-valvular AF are promising and confirm previous experience and data [8–11]. The technique appeared to be effective and safe in preventing thromboembolic complications in high risk patients.

AF is not only the major cause of stroke, particularly in older patients over 75 years, but also those strokes generating from AF are clinically more severe and disabling [13]. Thus, prevention of strokes in patients with AF is cornerstone of treatment and the European Society of Cardiology (ESC) guidelines recommend chronic anticoagulation if CHA_2DS_2-VASc [congestive heart failure, hypertension, age $\geq$ 75 years, age 65–74 years, diabetes mellitus, stroke/transit ischemic attack/thromboembolism, vascular disease, sex (female)] are ≥ 1 [3]. However, a significant number of patients have a high bleeding risk, or are contraindicated for chronic oral anticoagulation, and represent a group of patients who required the alternative treatment options. In addition, although the rate of intracranial bleeding is less with novel anticoagulant (NOAC) drugs, the overall risk of bleeding is not significantly lower with rivaroxaban and dabigatran in comparison to warfarin [14, 15]. Other concerns and contraindications for oral anticoagulant therapy include renal and liver dysfunction, noncompliance and discontinuation (even more with NOAC), low adequate therapeutic range with warfarin, as well as interaction with food and drugs [1].

LAAC device technology has evolved significantly over last 15 years, with several devices being under clinical investigation [7–11]. Out of few of them, Watchman device has demonstrated most relevant clinical results confirmed in 2 large randomized trials. First, the PROTECT AF study [8, 9] included 707 patients with $CHA_2DS_2 \geq 1$, randomized to device therapy and warfarin. Watchman was successfully implanted in 91% of the patients. The primary outcome was similar for Watchman and warfarin, with Watchman having more procedure adverse events and bleeding. However, after 45 months the primary efficacy endpoint was lower with the Watchman, as well as hemorrhagic stroke, cardiovascular death and overall mortality [8, 9]. In the second study, PREVAIL [10] 407 patients were randomized to Watchman and warfarin, and successful implantation of the device increased to 95%. In addition, procedure time was significantly reduced, and there was also a decline in procedure-related adverse events. Even though recent meta analysis of the 2 randomized clinical trials and 2 nonrandomized registries demonstrated all-cause stroke rates similar between the device and warfarin group, pathophysiology of stroke was significantly different - more device patients experienced ischemic strokes, while more warfarin patients experienced hemorrhagic strokes [16]. Higher ischemic strokes rates might be explained

either by development of thrombus on the device or to the failure to completely obliterate LAA flow and as a result to have residual leak that might have embolic potential [8, 9, 15]. Also, according to the same meta analysis, patients randomized to the LAAC had significant improvement in survival (freedom from cerebrovascular death) and significantly less bleeding complications compared to the patients on warfarin therapy when periprocedural bleeding was excluded [16].

Implantation of the Watchman device carries substantial upfront procedural risk mostly observed at the beginning of the learning curve and became less frequent with more experience. Pericardial effusion either as cardiac tamponade or as an asymptomatic effusion is one of the most serious complications in LAA-occlusion procedures [8]. Transseptal puncture, manipulation of the guiding catheters, stiff wires and even aggressive movement of device itself might result in LAA injury causing pericardial effusion [17]. In the PROTECT AF trial 5% of patients with pericardial effusion required drainage or surgery [8, 17]. In the Continued Access Protocol (CAP) registry, where experienced operators implanted Watchman devices, the rate of pericardial effusions decreased to 2.2% [17]. During the LAA occlusion procedure ischemic stroke due to air or thromboemboli occured in 0.9% in the PROTECT AF trial while none in the CAP registry [17]. Percutaneous closure of the LAA may be also complicated by immediate or late device embolization which occurred in 3 (0.6%) patients in the PROTECT AF trial and none in the CAP registry, thus proper selection of patients with favorable LAA morphology and appropriate device sizing are crucial to prevent this very serious complication [17].

In regard to indication for implantation of LAAC devices, ESC has recently issued guidelines stating that LAAC device may be considered in patients with high bleeding risk and contraindications for long-term oral anticoagulant therapy, but also in patients with previous stenting and prolonged need for triple antithrombotic and anticoagulant therapy, previous stroke on warfarin, labile and poorly regulated INR and severe renal and hepatic diseases that preclude chronic anticoagulant therapy [3, 18]. Practically, in those, and most often older patients with co-morbidities a number of clinical situations can be anticipated where long-term oral anticoagulant therapy should be avoided.

Conclusion

Our initial results with Watchman LAAC device are promising and encouraging, providing real alternative in patients with non-valvular atrial fibrillation and contraindication for chronic anticoagulant therapy and high bleeding risk. This initial results in our Center need to be extended with consistent application and performance as this and other highly sophisticated procedures for percutaneous treatment of "structural and valvular" heart diseases, but require considerable experience and a learning curve.

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Zsuzsanna Kossuth – the first volunteer nurse in Hungary and her activities in the War of Independence (1848–1849)

Žužana Košut – prva bolničarka Mađarske i njen rad za vreme Rata nezavisnosti (1848–1849)

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Key words:
history of medicine; military medicine; hospitals, military; nursing; volunteers.

Ključne reči:
istorija medicine; medicina, vojna; bolnice, vojne; nega bolesnika; dobrovoljci.

Introduction

The creator of nursing is considered to be a British nurse Florence Nightingale (1820–1910), who worked as a volunteer nurse in the Crimean War (1853–1856), she rescued and nursed the wounded, and later founded a school for nurses. In memory of her, May the 12th (her birthday) is celebrated as International Nurses Day. However, even before her, it was Zsuzsanna Kossuth (1817–1854) who devoted herself to nursing in more difficult conditions. Zsuzsanna greatly contributed to the development of health care and the recognition of women's work, but her name has almost been forgotten.

The participation of women in caregiving during the War of Independence

From the aspect of health care and patients care two important facts should be pointed out, at which the Hungarian health care during the War of Independence (1848–49) was far ahead of its time. The first is the activation of social self-preservation, the other is the use and evaluation of women's work. Both are related to the organization of field work, military hospitals, which mainly took place in Debrecen. Due to the chronic lack of bandages and hospital equipment, in 1849 the proposal to seek the help from the Association of Women volunteers was accapted. The wife of Lajos Kossuth*, who was the president of the Association, gave a call to women and girls to gather bedding, linen, pillows to make them into bandages. Already, more women were included in work at hospitals in order to maintain purity, keeping the hospital kitchen clean, but also to participate in the care of the wounded. Officially, participation of women in caregiving became institutionalized after April 16, 1849, when the youngest sister of Lajos Kossuth, Zsuzsanna, was declared as the first national head nurse [1] (Figure 1).

Fig. 1 – Zsuzsanna Kossuth (1817–1854).

*Lajos Kossuth (1802–1894), lawyer, politician, prime minister of the provisional government of Hungary. Known and recognized as a freedom fighter, one of the most prominent leaders of the 1848 Revolution.

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Zsuzsanna Kossuth – family life and the first volunteer nurse engagement

The first volunteer nurse of Hungary, Zsuzsanna Kossuth was born on February 19, 1817 in Sátoraljaújhely, as the fifth child to the family of Laslo Kossuth and Karoline Wéber. The only son in the family, Lajos Kossuth, whose favourite sister was Zsuzsanna, were almost inseparable. As teenagers, in 1833 the family relocated to Pest, where they were supported by Lajos Kossuth. Through the delivery of government notifications Zsuzsanna was introduced to politics. Together with his brother in 1831 during a cholera epidemic, she organized care of the infected put in quarantine [2] (Figure 2).

Fig. 2 – Quarantine cholera.

In 1841 Zsuzsanna married the best friend of her brother, Rudolf Meszlényi, and during the same year Lajos Kossuth married his sister Tereza Meszlényi. Rudolf Meszlényi was a well-known lawyer and politician on the side of Kossuth. He accepted his wife's involvement in politics, for which the newly established National Protective Association gave an opportunity. They lived in a happy, balanced marriage, they had three children, two girls and one boy. However, the birth of the third child was preceded by a terrible tragedy. In January 1847 Zsuzsanna husband suddenly died [1]. Zsuzsanna's deep sorrow was interrupted by the historical events and a lifelong desire to help his brother. She chooses a self-sacrificing profession, and takes care of the wounded during the War of Independence (1848–1849). She noted not enough nurses in the country, health care lacking, the equipment in the hospital very scarce and their number insufficient [3]. Zsuzsanna, her family and her mother run away from Budapest in January 1849. They found refuge in Debrecen, with Mihály Tóth[#], a Calvinist priest. At the suggestion of Zsuzsanna, the Association of Women Volunteers was founded to assist patients in military hospitals. For the president of the Association of Women the wife of Lajos Kossuth was elected. Near their house a warehouse was opened where they gathered equipment and underwear for hospitals [4].

Appointment for the head nurse of all the military hospitals in Hungary

Lajos Kossuth, after being appointed as the Governor, on April 16, 1849, appoints Zsuzsanna as the national head nurse of all the military hospitals in Hungary and at the same time lays down the responsibilities and obligation of commanders to hospital nurses, which through a letter is delivered to the General Görgei and Kossuth's sister "...Zsuzsanna, now I declare you as the head nurse of all the military hospitals, giving you the power and support to take two nuns with our funding, that will help you to do everything at the hospitals (except for medical care), what you think is useful in the purpose of taking care of the wounded. In order for your efforts and investments to be as successful as possible, I'm inviting and ordering all government bodies, and leading doctors that they have a civic duty to facilitate and assist the implementation of all your actions..." The letter of Lajos on the declaration of the head nurse, that appeared in the bulletin, caused a negative reaction of the military commanders and doctors, because they did not understand its true meaning [2].

Zsuzsanna after her appointment, by the President of the Women's Association, launched a campaign to prepare bandages. At that time there were not enough bandages for the wounded, Zsuzsanna through the newspapers called for help from all women and girls, asking them to tear clean cloths, and prepare dressings from them. Zsuzsanna was given the responsibility to organize the entire military medical system in Hungary. The National Sister Office was put under the supervision and management of the head nurse, which was located in Debrecen, in the building of Trade. The function of the institution was to keep records of nurses, coordinate issues of national importance. There was also the central storage of all hospitals in order to collect, store and distribute the necessary equipment for hospital care, which they also organized. In stock they kept supplies needed for the hospitals (bed, linen, blankets, underwear, dressings), while the

[#]Mihály Tóth, known revolutionary, friend of Petőfi, after the loss of the War of independence he was sentenced to more years in prison, because helped the Kossuth family.

medical equipment and medicines were kept separately. Zsuzsanna after declaring, in April 1849 invited all the directors of the military hospitals: "...please let me promptly notify you of any shortcomings that have emerged in the hospitals, which are essential in patient-care, diet and sanitation services, as to get them eliminated as soon as possible ..." Based on the responses, she organized work, *eg* in April 1849 the delivery of 125 medical sets to the hospitals was organized [4].

Invitation to all girls and women to voluntarily join nursing and helping

Also, in May 1849, using newspapers she invited all girls and women to voluntarily join the profession: "... do not wait until the official letters or the publication of regulations, but look for the nearest hospital and help. If you notice that in the hospitals scarce material is in disrepair immediately consult the nearest civilian authority or municipal authorities and report on the need for the urgent procurement procedure for necessary hospital supplies, if the situation demands, even through requisition " "...if by authorities, hospital director, or staff notice inappropriate attitude towards your volunteer work, please contact me immediately, to replace them as soon as possible with worthier workers. Grouped by cities, neighborhoods and split each working day, but every minute, even in your dreams take care of those who fight for our freedom..." [5] (Figure 3).

Fig. 3 – Helping to wounded.

Her work was a great help in the health sector. Its success was largely helped by the support and trust of Ferenc Flór, president of Medical Center of the Ministry of Defence. Zsuzsanna received authorization to be superior to women volunteers who were answering to provide care.

In April 1849, György Klapka, Deputy Minister of Defense, by decree provides strengthening of the labor force of military hospitals, "...hospital commanders are obliged to accept the voluntary work of women and girls, and on their training will be taken care by the doctors..." In May 1849 the Regulation of Ferenc Flór appeared in a bulletin, "...until the newly developed regulations for care are not officially announced, it should be known that all military commanders, and doctors are obliged to act on the orders of the head nurse, provide support, and comply with its orders related to hospital care..." [6].

However, it must be noted that in April–May 1849 the inclusion of women in clinical work was still only at the developmental level, in life completely.

On May 14, 1849 Ferenc Flór gives the following statement: "...the proposal of the national head sister, hospital care will be performed by women, however due to the large number of hospitals we believe that we will not have them in sufficient numbers, and we propose to all directors and commanders of hospitals that due to the said experiment do not fire those who already work there and have enough skills, but to liberate them from field service and let them be exclusively in hospitals, since gentle female hands are not always enough for performing tasks that require greater physical strength...".

...And to men, too

In May 1849 within the Association of Women was established the Department for the Care of Patients, whose members were actually involved in hospital work. Public information increasingly showed that these women enthusiasts, dedicated to nursing and helping, achieved great success and great reputation throughout the country. Zsuzsanna favored inclusion of women in clinical work, however, accepted the need for male labor, so for example in June 1849, when General Joseph Schweidl, chief commander of Pest, requested medical personnel to a military hospital, Zsuzsanna taking into account the current situation still suggested male workers [3].

The health service developed a precise itinerary for Zsuzsanna, which included monitoring of faraway hospitals, especially those with no official figures, or known to be in difficult situation and to be under difficult circumstances. As a co-leader, Ignác Barna was appointed, as the head physician, although Lajos wanted nuns as escorts because they had experience in patient care, however, in the organization of the hospital they would not be able to make appropriate recommendations for improvement.

Zsuzsanna's effort to open and organize new military hospitals

The head nurse of Hungary collected a lot of personal experience, tirelessly traveling around the country. As a result of its work during the summer 1849, 172 millitary hospitals started to work. She personally nursed the wounded, and offered them spiritual support. Careful attention was reflected even in housing. She drew attention to the patients from the same village to be located in the same room. Not only did they nurse the Hungarian wounded soldiers, but also the Austrian troops, not taking into consideration the current war conflicts. The wounded remembered that "they were nursed with maternal tenderness by Zsuzsanna" [2] (Figure 4).

Travelling across the country not only to check the functining of hospitals, but also to provide a proposal for ope-

Fig. 4 – Nurses in military hospitals.

ning new hospitals where necessary, correctly indicated by schools and other buildings that could, if necessary, be used for care of the wounded, in addition, to deal with the issues related to trapped patients, sticking to the profession that she voluntarily chose to the end. Before the end of the war, she visited many hospitals, toured the rooms of seriously wounded, and in addition there was a cholera outbreak which reduced to half the number of wounded who survived. The situation was hopeless, all the efforts of doctors and nurses seemed insufficient because of the insufficient amount of bandages, medicines, and even food [2].

After the conquest of Pest, Zsuzsanna is back, and invests huge amount of work and organization for the establishment and equipment of the central military hospital in Budapest, which often had the problem of acquiring the provisions from the military and civilian authorities. In the military hospital there was already a great number of women working, approximately in the same numbers as paid defenders and civilian medical workers. Training of voluntarily reported female doctors was performed at hospitals, there was no centralized training.

In June 1849 due to the large number of Austrian and Russian troops, the government counted on the submission of Pest and Buda, and the defenders withdrew to the southern parts of the state. The main task of Zsuzsanna was to transfer the wounded to the southern part of the state for opening of the new hospital. Zsuzsanna did all the necessary steps to transport the wounded, however, she recognized that the seriously injured would not have survived such a trip. Recalling all the women: "...to accept the wounded in their home, that they cherish, preserve and treat them, to keep them alive so that they can experience and rejoice in the freedom for which they fought for..."

To realize all of this, she chooses three voluntary women: Júlia Szekáts, Amália Balogh, Karoline Foszter – and sends a letter to all the directors of the hospitals that on the request of the said women they are obliged to give up all military casualties for appropriate care [3].

She managed to organize for the soldiers who could not be accepted in civilian homes, to be located in the hospital in Buda. Poor families that embraced the wounded soldiers were given "tax for the care of" a private fund of the Kossuth family (the total amount of funds were the salaries for two hundred soldier). In Szeged voluntary women gathered in large numbers, but the problem appeared to be the contradictions between the doctors and nurses of the hospital. Also, she had a huge help with relocation, equipping and relocating the hospital from Pest [5].

The War lost and Zsuzsanna unjustly accused

After losing the War for Independence, the family Kossuth was forced to emigrate, they were on the run (four women and nine children). On the way the youngest son of Zsuzsanna died, being only 18 months old. In the north, they were first captured by the Russians, and put under house arrest. With another 12 refugees they lived together in one room, under inhumane conditions: slept on the ground sprinkled with straws, between damp walls, so Zsuzsanna got sick with tuberculosis. A few weeks later, at the request of Julius von Hajnau, the Russians handed Zsuzsanna to her mother over to the Austrians, but after a year and a half they were forced to return to Budapest, which was soon followed by the trial to the head nurse. Zsuzsanna was accused for the greatest sin, for high treason.

At the hearing, very impressively, she logically proved that during the war she did only what was right, and her duty and mission were "nursing the sick and where she could she eased the suffering of victims …" The investigating judge was confused. Then he looked at the paper in front of him and underlined in red color by a pen: "greatest penalty should be applied". However, the Austrian military officers who were treated in the Hungarian hospitals came to her defense, claiming that they got the same care and concern as the Hungarian wounded: "This woman, we owe our lives to her, she did not see the enemy in us, but the people who suffer, nurtured us and helped us..." [1].

Released, continues to develop patriotism

Thus, the first head nurse was cleared of all chargers. Although advised to leave the country, she still stayed, believing that the Hungarian people were waiting back of her

brother, and being his sister she could not escape. During the exile of Lajos Kossuth, Zsuzsanna assumed the role of mediator and messenger between her brother and his friends, using letters to inform him about the political developments. To support the family, Zsuzsanna was forced to take disciples that she took care of, but she also helped the prisoners.

Later she opens an educational home to teach history and Hungarian language, and intensively develop patriotism in her students. In 1851, Austrian authorities prohibited this institution. Despite all the measures of the government, in December 1851 Zsuzsanna was still involved in the planning of conspiracy, hoping to bring back his brother, Lajos from exile and to organize an internal uprising against the Austrians [4].

Prison, exile, illness and early death of Zsuzsanna Kossuth

However, the conspiracy was revealed, Zsuzsanna arrested and threatened with jail again, and also ill with tuberculosis. In a very difficult position she was moved to Vienna to the hospital for prisoners, where with the help of the American Embassy she managed to free herself. The charges for conspiracy luckily could not be proven, and provided that all members of the family Kossuth leave territory of the Habsburg Empire and that she would never return to Hungary, she was released. First she emigrated to Brussels, very sick, but she learned sewing and opened a sewing's shop, which was maintained by the family. She worked tirelessly to leave some heritage for her children, so she raised her daughters to work and be independent. However, due to the frequent harassment of the Austrian Embassy, she was forced to leave Brussels, and in 1853 received permission to emigrate to America, to New York. Difficulties, torture and constant struggle exhausted her and on June 29, 1854 died being 37 years old. Zsuzsanna Kossuth was buried in New York. We hope that the time will come when her remains reach Hungary, the country for which she sacrificed her life and where she always wanted to return.

"... It was an immense consolation that I could defy the storm, share the misery, to fight and be tortured in this country, which is bleeding. I was happy when I wiped my tears, when I eased the burden on the poor of heart. My torments I hardly felt.... They drove me from my home country, which is suffering, I did not have the opportunity to fall on my knees and bow to her, to say goodbye. And it was not my fault..." [7].

The sculpture of Zsuzanna Kossuth in Budapest is shown in Figure 5.

Fig. 5 – The sculpture of Zsuzsanna Kossuth.

Conslusion

Ideas, moral values, hard work of Zsuzsanna are todays foundation for nursing. To pay tribute to her, secondary medical schools in Szeged and Eger bear her name. In recognition, for respect and as gratitude to Zsuzsanna Kossuth and those who choose the medical profession, the Hungarian parliament declared the 19th of February the day of health workers in Hungary.

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E R R A T U M

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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.

Ilustracije

Slikama se zovu svi oblici grafičkih priloga i predaju se kao dopunske datoteke u sistemu **aseestant**. Slova, brojevi i simboli treba da su jasni i ujednačeni, a dovoljne veličine da prilikom umanjivanja budu čitljivi. Slike treba da budu jasne i obeležene brojevima, onim redom kojim se navode u tekstu (**Sl. 1; Sl. 2** itd.). Ukoliko je slika već negde objavljena, obavezno citirati izvor.

Legende za ilustracije pisati na posebnom listu, koristeći arapske brojeve. Ukoliko se koriste simboli, strelice, brojevi ili slova za objašnjavanje pojedinog dela ilustracije, svaki pojedinačno treba objasniti u legendi. Za fotomikrografije navesti metod bojenja i podatak o uvećanju.

Skraćenice i simboli

Koristiti samo standardne skraćenice, izuzev u naslovu i apstraktu. Pun naziv sa skraćenicom u zagradi treba dati kod prvog pominjanja u tekstu.

Detaljno uputstvo može se dobiti u redakciji ili na sajtu: www.vma.mod.gov.rs/vsp

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Časopis „Vojnosanitetski pregled“ izlazi godišnje u 12 brojeva.

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Poreski identifikacioni broj (PIB) za ustanove	
Mesto	
Ulica i broj	
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Pretplata na časopis „Vojnosanitetski pregled“ (zaokružiti):

1. Lično. Dokaz o pretplati dostavljam uz ovu prijavu.
2. Za pripadnike MO i Vojske Srbije: Dajem saglasnost da se prilikom isplate plata u Računovodstvenom centru MO iz mojih prinadležnosti obustavlja iznos mesečne rate (pretplate).
3. Virmanom po prijemu profakture.

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