Srp Arh Celok Lek ISSN 0370-8179 COBISS.SR-ID 3378434 UDC 61(497.1)=861

СРПСКИ АРХИВ

ЧАСОПИС СРПСКОГ ЛЕКАРСКОГ ДРУШТВА ОСНОВАН 1872. ГОДИНЕ

ЗА ЦЕЛОКУПНО ЛЕКАРСТВО

ГОДИШТЕ 144. | VOLUME 144
МАЈ–ЈУН 2016. | MAY–JUNE 2016
СВЕСКА 5-6 | NUMBER 5-6

SERBIAN ARCHIVES OF MEDICINE

OFFICIAL JOURNAL OF THE SERBIAN MEDICAL SOCIETY FOUNDED IN 1872

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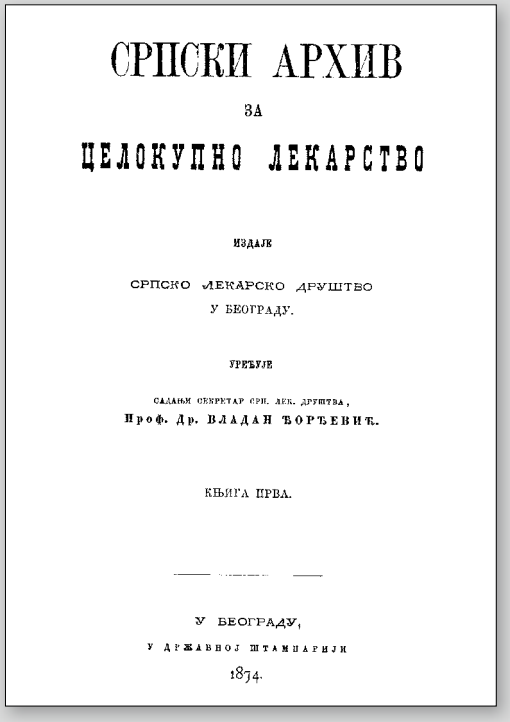

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У БЕОГРАДУ,

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1874.

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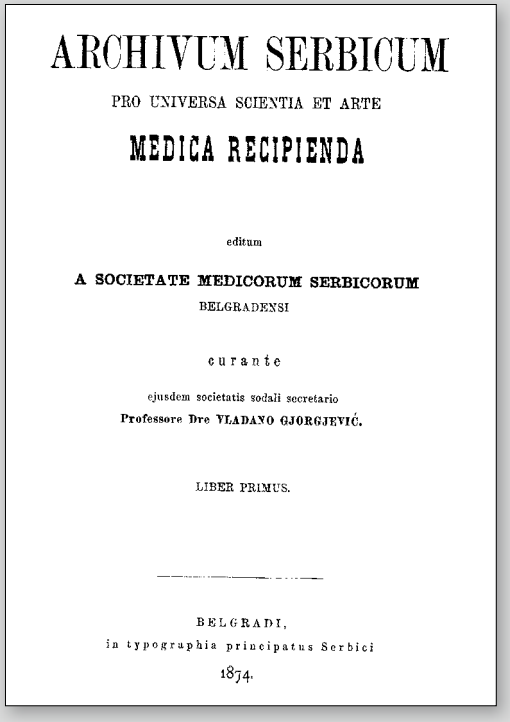

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Srp Arh Celok Lek
ISSN 0370-8179
UDC 61(497.1)=861
COBISS. SR-ID 3378434

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**ISSN 0370-8179; ISSN Suppl 0354-2793;
ISSN Online 2406-0895**

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СРПСКИ АРХИВ
ЗА ЦЕЛОКУПНО ЛЕКАРСТВО

ГОДИШТЕ 144. МАЈ–ЈУН 2016. СВЕСКА 5-6

Часопис „Српски архив за целокупно лекарство" је индексиран у базама: Science Citation Index Expanded, Journal Citation Reports/Science Edition, Index Medicus (Medline, PubMed), Web of Science, Scopus, EBSCO, Directory of Open Access Journals, DOI Serbia.

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Тираж: 700 примерака

SERBIAN ARCHIVES OF MEDICINE

VOLUME 144 MAY–JUNE 2016 NUMBER 5-6

Srp Arh Celok Lek
ISSN 0370-8179
UDC 61(497.1)=861
COBISS. SR-ID 3378434

Serbian Archives of Medicine is an official publication of the Serbian Medical Society, and is published six times per year.

The journal "Srpski arhiv za celokupno lekarstvo" (Serbian Archives of Medicine) is indexed in: Science Citation Index Expanded, Journal Citation Reports/Science Edition, Index Medicus (Medline, PubMed), Web of Science, Scopus, EBSCO, Directory of Open Access Journals, DOI Serbia.

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Printed by: JP "Službeni glasnik", Beograd

Circulation: 700 copies

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Serbian Medical Society
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Calendar year subscriptions prices are: 3,000 dinars for individuals, 6,000 dinars for institutions, and 100 Euros for readers outside Serbia. The price of a current issue is 600 dinars, and of a back issue 300 dinars.

The publishing of the Serbian Archives of Medicine during 2016 is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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ISSN 0370-8179; ISSN Suppl 0354-2793; ISSN Online 2406-0895

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ПРИКАЗИ БОЛЕСНИКА • CASE REPORTS

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):262-265
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1606262D
UDC: 616.832-004

The effect of fatigability on Expanded Disability Status Scale components in multiple sclerosis

Irena Dujmović[1,2], Simon Demetz[3], Alban Millonig[3], Florian Deisenhammer[3]

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SUMMARY

Introduction The Expanded Disability Status Scale (EDSS) is the most widely used disability measure in multiple sclerosis (MS). The effect of fatigability on EDSS components has been underreported to date.

Objective We investigated daytime variability in EDSS score and EDSS components – functional scores (FS) and walking distance (WD) up to 500 m, in MS patients who underwent a standardized fatiguing exercise.

Methods Twenty-four patients with relapsing-remitting MS (n = 7), secondary-progressive MS (n = 8) and primary-progressive MS (n = 9) were included. Exclusion criteria were as follows: current MS relapse, infection/fever/flu-like symptoms, conditions prohibiting safe exercise testing, current medication affecting fatigue. One trained examiner performed baseline (BL) and follow-up (FU) assessments (FU1 after a standardized fatiguing exercise, FU2 after rest) over a single day. EDSS score change of ≥1 point if BL EDSS score was <5.5 or of ≥0.5 point if BL EDSS score was ≥5.5 were considered clinically meaningful.

Results In progressive MS subtypes, WD decreased at FU1, but recovered at FU2, more so in secondary-progressive MS subgroup with the highest BL EDSS score. Although BL EDSS scores (median, 5.0; range 4.0–6.5) and FS remained relatively stable over repeated assessments in the total group, a clinically meaningful transitory post-exercise EDSS score increase was observed in three patients with progressive MS.

Conclusion WD seems to be more influenced by fatigability than the total EDSS score, more so in patients with progressive MS and higher disability. WD should be assessed after rest and this strategy should be implemented into protocols of clinical trials recruiting patients with progressive MS phenotypes.

Keywords: multiple sclerosis; disability evaluation; Expanded Disability Status Scale; walking distance; fatigability

INTRODUCTION

An increasing number of clinical trials are testing new therapeutic compounds in multiple sclerosis (MS) [1]. However, outcome assessment in MS remains challenging due to diversity and fluctuating nature of MS symptoms [2], whose transitory worsening could be underlain by several factors including fatigability [1]. Many trial designs have no standardized interval between previous physical activity and clinical examinations. Expanded Disability Status Scale (EDSS) is the most widely used clinical scale in MS, but has been debated because of low sensitivity [1, 3]. Daytime variability in EDSS components has been rarely investigated, but the influence of physical exercise on these outcomes has not been addressed at all except for sensory symptoms [4, 5].

OBJECTIVE

The aim of the study was to investigate daytime variability in EDSS score and EDSS components – functional scores (FS) and walking distance (WD) up to 500 m in MS patients exposed to a standardized fatiguing exercise.

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METHODS

Patients

MS patients who had scheduled visits at the MS Clinic, Department of Neurology, Innsbruck Medical University Hospital, were screened for participation in the study. Inclusion criteria were as follows: MS according to the McDonald criteria [6], EDSS score 3.5–6.5, and no current medication affecting fatigue. Exclusion criteria were the following: current MS relapse, interferon-beta-related flu-like symptoms, current infection or fever or other condition prohibiting safe exercise testing.

To detect a difference of 0.5 EDSS points with a standard deviation of 0.3 and a power of 80% at an alpha level of 0.025 (multiple testing), a sample size of 24 patients was calculated and patients were recruited until this sample size was reached. Twenty-four MS patients with relapsing-remitting (RR) MS, secondary-progressive (SP) MS, and primary-progressive (PP) MS were included (female/male ratio, 1.19:1) (Table 1).

All study participants gave written consent prior to entering the study, which was approved by the Innsbruck Medical University Ethics Committee.

Study procedures

All included patients were admitted for study purposes to the hospital at 08:00 a.m. and were examined at 09:00 a.m. (baseline, BL), at 11:00 a.m. without delay following fatiguing exercise (follow-up, FU1), and at 05:00 p.m. (FU2) following rest in a hospital area.

All patients underwent standardized physical exercise before FU1 on a standard bicycle ergometer at a heart rate of 100–120 beats/minute for 30 minutes, but were allowed to stop the exercise if they subjectively reached maximal exertion. Time to maximal perceived exertion in seconds (s) and maximal exercise mechanical power output in Watts (W) were recorded (Table 1). All study procedures were performed in a hospital area with stable ambient temperatures (average, 21°C).

To avoid inter-rater variability [4], neurological disability level was assessed in all patients by one trained examiner (SD), according to EDSS grading using Kurtzke FS, along with WD assessment up to 500 m [7, 8]. EDSS score change of ≥1 point in patients with BL EDSS score <5.5 or of ≥0.5 point change if BL EDSS score was ≥5.5 were considered clinically meaningful [9].

Statistical analysis

The Kolmogorov–Smirnov test was used to determine normality of data distribution. The significance of changes over time was tested by repeated measures analysis of variance (ANOVA) with Tukey's posttest or Friedman's test with Dunn's posttest while the differences between subgroups were assessed by one-way ANOVA (with Tukey's posttest) or Kruskal–Wallis test (with Dunn's posttest). Spearman's or Pearson's model were used for correlations. P-values <0.05 were considered significant. We used GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) for statistical analyses and Power and Sample Size Calculation (PS, downloaded from: http://ps-power-and-sample-size-calculation.software.informer.com) software for sample size calculation.

RESULTS

Tables 2 and 3 summarize all the results. In the total group, EDSS score and single FS did not change significantly over repeated assessments (Table 2), although clinically meaningful post-exercise EDSS deterioration was observed in three patients (two SPMS and one PPMS) (12.5% of all the patients). WD decreased following fatiguing exercise and improved after rest in SPMS and PPMS, but not in RRMS patients (Table 3). A post-exercise decrease in WD was statistically significant in SPMS patients, a subgroup with the highest BL EDSS score (Table 3).

The total EDSS score was significantly driven by pyramidal and cerebellar FS as well as by WD (Table 2).

Performance on any scale did correlate, not significantly though, with age and disease duration. BL-FU1 changes

Table 1. The characteristics of multiple sclerosis (MS) patients with exercise parameters

MS subgroup	Number of patients (%)	Age[a] (years)	Disease duration[b] (years)	Maximal exercise power output[b] (W)[†]	Time to maximal perceived exertion[a] (s)[†]
RRMS	7 (29.2)	38.3 ± 7.2* (31.6–45.0)	9 (3–22)	60 (30–80)	615.9 ± 142.0 (484.5–747.2)
SPMS	8 (33.3)	51.9 ± 8.8 (44.5–59.2)	18 (9–27)**	40 (20–70)	453.8 ± 226.9 (215.7–692.0)
PPMS	9 (37.5)	54.3 ± 11.7 (45.4–63.3)	7 (2–17)	50 (25–75)	550.0 ± 178.8 (412.5–687.5)
Total MS	24 (100)	48.8 ± 11.6 (43.9–53.7)	12 (2–27)	50 (20–80)	544.7 ± 185.1 (462.7–626.8)

RRMS – relapsing-remitting MS; SPMS – secondary-progressive MS; PPMS – primary-progressive MS; [a] mean with standard deviation (95% confidence interval of the mean); [b] median with range; [†] no statistically significant difference between subgroups; * significantly lower than in SPMS or PPMS (one-way ANOVA p = 0.0083, Tukey's posttest); ** significantly longer than in RRMS or PPMS (one-way ANOVA p = 0.0140, Tukey's posttest)

Table 2. Expanded Disability Status Scale (EDSS) components over three repeated assessments and their correlation with the overall EDSS score at baseline; values are given in median (ranges) unless otherwise stated

EDSS component	BL	FU1	FU2	P[a]	r
FS: Visual	1 (0 –2)	1 (0–3)	1.5 (0–3)	0.03[b]	-0.28
FS: Brainstem	2 (1–3)	2 (1–3)	2 (1–3)	n.s.	0.18
FS: Pyramidal	3 (3–4)	3 (3–4)	3 (2–4)	n.s.	0.52*
FS: Cerebellar	3 (2–4)	3 (2–4)	3 (2–4)	n.s.	0.66*
FS: Sensory	3 (1–4)	3 (2–4)	3 (1–4)	n.s.	-0.33
FS: Bowel/bladder	1 (0–4)	1 (0–4)	1 (0–4)	n.s.	0.05
FS: Cerebral	0 (0–2)	0 (0–2)	0 (0–2)	n.s.	0.02
WD[c] [m]	305.0 (175.0–500.0)	235.0** (128.8–487.5)	372.5 (178.8–500.0)	0.007	-0.82*
EDSS score	5.0 (4.0–6.5)	5.2 (4.0–6.5)	4.5 (4.0–6.5)	n.s.	---

FS – functional score; WD – walking distance up to 500 m; BL – baseline; FU – follow-up assessment; r – Spearman's correlation coefficient (with EDSS score at BL); n.s. – not significant; [a] p-values obtained by Friedman test between BL, FU1 and FU2 assessment, except repeated-measures ANOVA for WD; [b] Dunn's post-hoc multiple comparison test revealed no statistically significant difference; [c] median (interquartile range); * statistically significant correlation with EDSS score at BL; ** statistically significant difference from BL

Table 3. Expanded disability status scale (EDSS) scores and walking distance up to 500 m (WD) over three repeated assessments in multiple sclerosis (MS) phenotype strata

Disability measure	RRMS			SPMS			PPMS		
	BL	FU1	FU2	BL	FU1	FU2	BL	FU1	FU2
EDSS[a]	4.0[†] (4.0–5.5)	4.0 (4.0–5.5)	4.0 (4.0–5.5)	6.0 (4.0–6.5)	6.0 (5.0–6.5)	6.0 (4.0–6.5)	5.0 (4.0–6.0)	5.0 (4.0–6.0)	4.5 (4.0–6.0)
WD[b] (m)	500.0[††] (420.0–500.0)	500.0 (450.0–500.0)	500.0 (365.0–500.0)	170.0 (37.5–307.5)	132.5* (21.7–181.3)	242.5 (42.5–265.0)	300.0 (215.0–500.0)	250.0 (160.0–302.5)	410.0 (255.0–500.0)

RRMS – relapsing-remitting MS; SPMS – secondary-progressive MS; PPMS – primary-progressive MS; BL – baseline; FU – follow-up assessment; [a] median (range); [b] median (interquartile range); [†] significantly lower than in SPMS (Kruskal–Wallis test p = 0.0146, Dunn's posttest); [††] significantly longer than in SPMS (one-way ANOVA p = 0.0184, Tukey's posttest); * change from BL was statistically significant (repeated-measures ANOVA p = 0.0155, Tukey's posttest)

in studied scores did not correlate significantly with maximal exercise power output or time to maximal perceived exertion (data not shown).

DISCUSSION

Decades ago, Kurtzke [10] indicated that one good reason for quantitative schemes in MS was to document changes. In contrast to the total EDSS score, WD in our study showed a significant daily variability induced by fatigability, more so in patients with progressive MS phenotypes and higher disability. Considering also day-to-day variability of maximum walking distance [11], our results further necessitate WD assessment in non-fatigued MS patients.

Similarly to previous reports [3], BL EDSS score was predominantly determined by FS mainly affecting walking performance (pyramidal, cerebellar) and WD, but significant daily WD changes were not followed by significant changes in the EDSS score. Although being stable at the total group level, a clinically meaningful transitory post-exercise EDSS score increase occurred in 12.5% of our patients who had progressive MS phenotypes. Such short-term variations should be considered when evaluating disease progression and designing trial outcomes since measurement of changes smaller than the variation intrinsic to the tool might be inappropriate [12]. Additionally, EDSS variability before randomization might limit treatment discovery in MS, as recently shown in primary progressive MS by Zhang et al. [13].

Since a two-year EDSS change might predict later EDSS progression [14], more sensitive outcome variables that strongly correlate with the EDSS such as WD – being metric rather than ordinal measures – might be able to predict long-term disability in a shorter time. This would be advantageous for designing clinical trials but also for assessing individual treatment response in clinical routine. Higher sensitivity could offer improved precision to detect even subtle disability changes and would increase study power / decrease sample sizes in clinical trials [15]. More sensitive disability measures might also provide more clinically relevant information than relapse rates, which are poorly predictive of the long term outcome [14, 16]. However, the validity of WD as a separate assessment tool still has to be investigated in longitudinal studies on a larger sample size.

The phenomenon of fatigability, which contributes to the more general complaint of fatigue in MS [17], has been assumed to be partly due to impaired membrane excitability after a fatiguing exercise [18]. In this context, our results could represent a clinical correlate of neurophysiological findings showing intracortical excitability to be altered at higher EDSS scores and different across disease subtypes as we found motor fatigability to be more pronounced in patients with progressive MS and higher disability [19]. This is in line with results showing the complex paradigm of fatigue to be more frequent in MS patients with progressive MS subtypes and higher disability [20, 21, 22].

Our study was powered to detect minor EDSS changes, which probably has not introduced a bias regarding the sensitivity of WD to detect changes, but correlations done in our study might have been underestimated.

CONCLUSION

The EDSS remains a robust clinical score designed to cover the life span of MS patients and therefore insensitive to short-term changes even after fatiguing exercise. In contrast to the total EDSS score, WD seems to be more sensitive to detect daily functional fluctuations in MS and is influenced by fatigability, more so in patients with progressive MS course and higher disability. WD should be assessed after rest and this strategy should be implemented into protocols of MS clinical trials recruiting patients with progressive MS phenotypes.

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Утицај заморљивости на компоненте проширене скале неуролошке онеспособљености у мултиплој склерози

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КРАТАК САДРЖАЈ

Увод Процена онеспособљености у мултиплој склерози (МС) најчешће се врши проширеном скалом неуролошке онеспособљености (*EDSS*). Утицај заморљивости на све компоненте *EDSS* скале није до сада анализиран.

Циљ рада Циљ рада је био да анализира утицај заморљивости на укупни *EDSS* скор и компоненте *EDSS* скале – функционалне скорове (ФС) и дистанцу хода (ДХ) до 500 *m* код болесника са МС који су подвргнути стандардизованом физичком оптерећењу.

Методе рада У студију су укључена 24 болесника са релапсно-ремитентном МС ($n = 7$), секундарно-прогресивном МС ($n = 8$) и примарно-прогресивном МС ($n = 9$). Искључујући критеријуми су били: актуелни релапс МС, постојање инфекције/фебрилности/симптома налик грипу, употреба лекова са потенцијалним утицајем на замор, контраиндикације за физичко оптерећење. Компоненте *EDSS* скале су процењиване од стране једног процењивача у току истог дана: пре оптерећења, по излагању стандардизованом оптерећењу и након одмора. Клинички значајним је сматрано увећање *EDSS* скора за ≥1 уколико је почетни *EDSS* скор <5,5 или за ≥0,5 уколико је почетни *EDSS* скор ≥5,5.

Резултати ДХ је у подгрупи са прогресивном МС била умањена након замарања, израженије у подгрупи секундарно-прогресивне МС, у којој је почетни *EDSS* скор био највећи. У укупној групи болесника почетни *EDSS* скор (медијана, 5,0; распон 4,0–6,5) и ФС нису се значајно мењали током понављаних процена, али је клинички значајан пролазни пораст *EDSS* скора након оптерећења забележен код три болесника са прогресивним формама МС.

Закључак Утицај заморљивости је израженији на ДХ него на укупан *EDSS* скор и присутан је код болесника са прогресивном МС и већом онеспособљеношћу. Процењивање ДХ након одмора би требало увести у протоколе клиничких истраживања у прогресивним фенотиповима МС.

Кључне речи: мултипла склероза; процена онеспособљености; проширена скала неуролошке онеспособљености; дистанца хода; заморљивост

Примљен • Received: 07/05/2015 **Прихваћен • Accepted:** 09/07/2015

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):266-272
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1606266M
UDC: 616.314.17-002:577.112.85; 616.379-008.64-06

Relationship between serum tumor necrosis factor receptor-2 concentration and periodontal destruction in patients with type 2 diabetes: Cross-sectional study

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SUMMARY

Introduction The role of tumor necrosis factor-α (TNFα) is well documented in pathogenesis of chronic periodontitis (CP) and type 2 diabetes (T2D). Considering short half-life of TNFα, tumor necrosis factor receptor-2 ($TNFR_2$) is used as prosperous surrogate marker of TNFα activity.

Objective The aim was to detect $TNFR_2$ serum concentration and correlate it with periodontal destruction in patients with diagnosed T2D and nondiabetics.

Methods The study included 85 patients divided into three groups: T2D + CP (group T2D, n = 34); nondiabetics + CP (Group PD, n = 27); and healthy controls (group HC, n = 24). T2D was diagnosed according to WHO criteria (2013) and periodontitis was diagnosed using International Workshop for a Classification of Periodontal Diseases and Conditions criteria (1999). $TNFR_2$ level was measured by enzyme-linked immunosorbent assay (ELISA).

Results There was no difference in $TNFR_2$ level among the groups (Kruskal–Wallis, p = 0.482). Significant correlation (Pearson's correlation coefficient) was observed between clinical attachment loss (CAL) and $TNFR_2$ concentration in PD group (r_p = -0.460, p = 0.016). In T2D group, correlations were observed between $TNFR_2$ concentration and CAL (r_p = 0.363, p = 0.005) and periodontal inflamed surface area (PISA) (r_p = 0.345, p = 0.046) and periodontal epithelial surface area (PESA) (r_p = 0.578, p = 0.000).

Conclusion Higher concentration of $TNFR_2$ was associated with higher CAL, PESA, and PISA scores in T2D group. Contrary to that, nondiabetics with higher values of CAL exhibited lower concentration of $TNFR_2$, presenting potential protective effect on periodontal destruction. These results imply that diabetes may alter $TNFR_2$ secretion originated from periodontium.

Keywords: chronic periodontitis; $TNFR_2$; TNF-alpha; type 2 diabetes

INTRODUCTION

Plaque-associated periodontal diseases are chronic infectious diseases caused by mixed microbial flora that lead to inflammation and destruction of tooth attachment and ultimately tooth loss [1]. Although bacteria of dental biofilm are considered to be the main etiological factor in periodontitis, it is a well-known fact that a local immune and inflammatory response to bacteria leads to destruction of periodontium [2].

Key mediator of periodontal inflammation is tumor necrosis factor-alpha (TNFα). TNFα is potent proinflammatory cytokine produced by many cells in response to inflammation, infection and injury. TNFα plays a vital role in host defense processes and immune surveillance. However, increased levels of TNFα exhibit harmful effects [3]. TNFα binds two specific TNFRs – $TNFR_1$ and $TNFR_2$. $TNFR_1$ is expressed in most cell types, whereas immune cells constitutively express low levels of $TNFR_2$ [4]. Both receptors exist in two forms – transmembrane and soluble (s). The soluble forms compete with TNFα for TNF binding sites on transmembrane receptors [3]. Therefore, receptors participate in signal transduction, and regulate TNFα bioavailability [5].

Beneficial and pathological signals diverge at receptor levels. Transmembrane TNFα and $TNFR_2$ are responsible mostly for prosurvival signals, while sTNFα and $TNFR_1$ mediate detrimental effect [6]. Since $TNFR_2$ half-life is longer (4 hours) than TNFα half-life (~ 6 min), it exhibits superior sensitivity and reliability when measuring in frozen plasma. Therefore, $TNFR_2$ is used as an effective surrogate marker of TNFα activity.

Type 2 diabetes (T2D) is a metabolic disorder characterized by hyperglycemia, which results from progressive insulin secretory defect on the background of insulin resistance [7]. For 30 years now, periodontitis has been acknowl-

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edged as the sixth chronic complication of diabetes [8]. There is increasing evidence that the relationship between T2D and periodontitis is bidirectional [9]. Roles of TNFα in pancreatic β-cell destruction and insulin resistance have been established [10].

OBJECTIVE

This study was aimed at detecting $TNFR_2$ serum concentration and correlating it with periodontal destruction in type 2 diabetics and nondiabetics.

METHODS

Study population

The study was conducted as a cross-sectional study, approved by the Ethical Committee of the Faculty of Dental Medicine, University of Belgrade (decision No. 36/9 of 20th of February 2013) between March 2013 and January 2014. A total of 85 subjects (41 men and 44 women, 28–68 years of age) were divided into three groups: healthy control (HC) group consisted of 24 healthy volunteers without clinical signs of periodontitis and free of systematic diseases; periodontitis group (PD) consisted of 27 patients with diagnosed chronic periodontitis (CP) and free of systematic diseases (periodontitis patients were referred to Department of Periodontology and Oral Medicine, Faculty of Dental Medicine, University of Belgrade); 34 patients, hospitalized at the Clinic for Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia, with diagnosed T2D and CP comprised the T2D group. All the subjects who participated in the study signed an informed consent.

Inclusion and exclusion criteria

The subjects included in study had more than 14 teeth and body mass index (BMI) of 18–30 kg/m^2. The exclusion criteria were as follows: pregnancy/lactation, presence of a systemic disease except T2D and its chronic complications, injury in the previous six months, systemic antibiotic/immunomodulatory therapy in the previous three months, therapy of periodontitis during 18 months prior to the study, everyday usage of oral antiseptics.

Anamnesis of the patients and clinical examination

Data regarding smoking history, mental stress, physical activity and health status were obtained through clinical chartings and interviews. Patients were classified according to their smoking status as "nonsmoker" and "smoker". Smokers were further classified as "former smoker" (with subgroups consisted of subjects who ceased smoking more or less than five years ago, respectively) or "current smoker" (with subgroups smoking more or less than 10 cigarettes per day, respectively). Pack-years (PPY) were calculated by multiplying duration of smoking in years and the number of cigarettes smoked per day, and then dividing by 20. Patient histories included information about the subjects' everyday mental stress in terms of intensity and frequency. The final score was calculated as the sum of the mentioned measurements, and categorized as low/medium/high stress [11]. Information about the intensity (low, moderate, hard), frequency (never, less/more than 2 times/week) and type (aerobic/anaerobic) of physical exercise was also recorded.

T2D and chronic periodontitis diagnosis

T2D was diagnosed by measuring glycaemia using oral glucose tolerance test (OGTT), as well as glycated hemoglobin (HbA_1c) values [7]. Nondiabetics exhibited normal parameters on OGTT and $HbA_1c < 6.5\%$. The blood sugar regulation was classified as satisfactory ($HbA_1c \geq 7.5\%$) and poor control ($HbA_1c < 7.5\%$).

A full-mouth clinical examination performed at six sites per tooth was done for each tooth in order to asses the following periodontal parameters: Silness–Löe plaque index (PI), dichotomous bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment loss (CAL). All examinations were performed by two calibrated doctors – one collected the measurements using Williams probe (Hu-Friedy Mfg. Co., LLC, Chicago, IL, USA) and the other recorded the results. Periodontitis was diagnosed if the subject exhibited CAL > 1 mm and PPD > 3 mm at least at three sites in two different quadrants. According to CAL, periodontitis patients were classified into the following three subgroups: patients with mild CP (CAL = 1–2 mm); moderate CP (CAL = 3–4 mm), and severe CP (CAL ≥ 5 mm). Periodontitis was defined as localized/generalized depending on the number of affected sites (more/less than 30%, respectively) [12]. The inflammatory impact from periodontium on general health was calculated using periodontal epithelial surface area (PESA) and periodontal inflamed surface area (PISA) [13]. Calculations were done by using Excel (Microsoft Corporation, Redmond, WA, USA) spreadsheets available at *http://www.parsprototo.info/* using PPD, CAL, recession for PESA, and additionally BOP for PISA on each tooth. Results were presented in mm^2. Patients without clinical signs of periodontitis had PPD < 3 mm and CAL = 0 mm.

Blood sampling and measurements

Venous blood was collected from cubital vein day after periodontal examination, following fasting, in the resting state in the morning, between 7 and 9 a.m., to minimize diurnal variations. Total leukocyte count, plasma concentrations of cholesterol, high and low density lipoproteins, triglycerides, fibrinogen, HbA_1c, sedimentation rate, and fasting plasma glucose (FPG) levels were measured. Sera obtained from blood samples for $TNFR_2$ analysis were immediately frozen at -72°C and stored until use.

The $TNFR_2$ level was measured by the enzyme-linked immunosorbent assay (ELISA) (eBioscience, Human $TNFR_2$ Platinum ELISA, San Diego, CA, USA) as described by the manufacturer. The values were expressed as ng/ml.

Statistical analysis

The sample size calculation using literature based data for $TNFR_2$ level as a reference variable showed that sample size with 25 patients in each group would provide 80% of power and significance level (α) of 5%.

The statistical analysis package SPSS 18.0 (SPSS Statistics for Windows, SPSS, Inc., Chicago, IL, USA) was used. Categorical variables were compared using the chi-square test (χ^2). Numeric data were tested for normal distribution using the Kolmogorov–Smirnov test. For normally distributed data, Student's t-test / One Way ANOVA was used. Non-parametric data were analyzed using the Kruskal–Wallis / Mann–Whitney U-test. Spearman's/Pearson's correlation coefficients were obtained in order to assess the relationship between $TNFR_2$ concentrations and clinical parameters. Linear regression model was used to determine the predictors of $TNFR_2$. Reliability was tested by Cohen's kappa test. The Cohen's kappa score was determined for each periodontal index in order to test intraobserver agreement. Participants with missing data were not included in the study. All reported p-values were two-sided. Differences were considered significant when p-value was <0.05.

RESULTS

Demographic data and clinical measurements

A total of 170 patients were examined. After anamnesis, and clinical and biochemical examinations, 97 were recruited for the study. According to ELISA plate size, we analyzed only 88 samples. Considering that absorbance of three samples were not detectable, results were obtained for 85 patients.

Demographic data and clinical measurements are presented in Table 1. Groups are matched by age and gender. Although the BMIs were 18.0–30.0 kg/m^2, BMI differed between T2D and two other groups. HbA_1c and FPG levels were higher in T2D than in HC and PD groups. The groups were similar by smoking status (χ^2, p > 0.05). Regarding T2D management, 19 subjects used oral antidiabetics, 10 were on insulin therapy and five were on combined insulin therapy.

There was a significant difference in the clinical periodontal parameters between the groups (Table 2). Intergroup analysis revealed differences for PI and CAL between all groups (Mann–Whitney U-test, p = 0.000). BOP and PPD did not differ between PD and T2D groups (Bonferroni > 0.05). Number of present teeth was higher in HC than in other groups (Bonferroni, p < 0.05). PESA and PISA were similar between T2D and PD groups. The kappa score for clinical parameters were 0.5–0.8, representing very good agreement.

TNFR2 concentrations

There was no difference in $TNFR_2$ concentrations between the groups. Average concentration in HC, PD and T2D groups were respectively 4.8 ± 4.680, 4.9 ± 7.366, and 5.2 ± 9.719, expressed in ng/ml. $TNFR_2$ serum concentration of diabetics with and without complications were 6.22 ± 11.94 and 4.22 ± 5.54 ng/ml, respectively.

Relationship between TNFR2 concentrations and periodontal clinical parameters

A significant correlation was observed between CAL and $TNFR_2$ concentrations in PD and CAL, PESA, PISA, and $TNFR_2$ in T2D groups (Table 3). Difference was not observed between the concentration of $TNFR_2$ and extent of periodontitis neither in T2D (p = 0.674, Mann–Whitney) nor in PD group (p = 0.487, Mann–Whitney).

Relationship between TNFR2 concentrations and biochemical parameters

Pearson's correlation coefficient between $TNFR_2$ concentrations and BMI in all patients (r = 0.025, p = 0,822) and each group were positive but nonsignificant (HC group: r = 0.248, p = 0.248; PD group: r = 0.054, p = 0.79; T2D group: r = 0.067, p = 0.708).

According to the smoking status, there was significant difference between $TNFR_2$ concentrations (smokers: 5.6 ± 3.738 ng/ml; non-smokers: 4.4 ± 8.713 ng/ml; p = 0.02,

Table 1. Group demographic and clinical data

Variable	Group			p-value
	Healthy control	Chronic periodontitis	Type 2 diabetes	
Age	42.0 ± 3.709	44.9 ± 12.494	46.1 ± 7.606	0.078a
Gender (male/female) (N/%)	7 (29.2)/ 17 (70.8)	11 (40.7)/ 16 (59.3)	16 (47.1)/ 18 (52.9)	0.390b
Body Mass Index (kg/m2)	22.5 ± 2.625	24.4 ± 3.189	27.0 ± 2.98	0.000a
HbA_1c [(%) mmol/mol]	4.8 ± 0.637 (29.0 ± 6.696)	4.8 ± 0.639 (29.0 ± 7.062)	8.4 ± 2.013 (68.0 ± 22.414)	0.000c
FPG level (mmol/l)	4.7 ± 0.714	4.9 ± 0.753	9.5 ± 2.605	0.000c
Smokers/nonsmokers [N (%)]	2 (8.3)/ 22 (91.7)	9 (33.3)/ 18 (66.7)	9 (26.5)/ 25 (76.5)	0.096b

Results are presented as mean ± standard deviation (SD). Results for discrete measures are presented as percentage (%).
N – number of patients; HbA_1c – glycated hemoglobin; FPG – fasting plasma glucose
[a] ANOVA
[b] Pearson χ^2
[c] Kruskal–Wallis test

doi: 10.2298/SARH1606266M

Table 2. Clinical periodontal parameters

Variable	Group			p-value
	Healthy control	Periodontitis	Type 2 Diabetes	
PI (Silness–Löe)	0.8 ± 0.499	1.5 ± 0.725	2.1 ± 0.815	0.000[a]
BOP (%)	34.8 ± 17.307	59.8 ± 23.646	63.0 ± 29.409	0.000[a]
PPD (mm)	1.9 ± 0.434	2.7 ± 0.544	2.6 ± 0.116	0.048[a]
CAL (mm)	0.0	2.4 ± 1.909	4.1 ± 1.944	0.001[b]
Extent of periodontitis localized/ generalized (%)	N/A	10 (38.5)/ 16 (61.5)	7 (20.6)/ 27 (79.4)	0.128[c]
Number of present teeth	27.3 ± 1.681	21.6 ± 3.974	19.6 ± 3.544	0.000[a]
PESA (mm2)	N/A	1046.2 ± 292.66	976.9 ± 271.84	0.343[d]
PISA (mm2)	N/A	694.7 ± 359.75	720.0 ± 421.79	0.804[d]

Results are presented as mean ± SD. PPD and CAL values presents average of all sites, including affected and unaffected sites.
PI – plaque index; BOP – bleeding on probing; PPD – probing pocket depth; CAL – clinical attachment level; PESA – periodontal epithelial surface area; PISA – periodontal inflamed surface area
[a] One-way ANOVA
[b] Kruskal–Wallis test
[c] Independent samples t-test
[d] Pearson χ^2

Table 3. Pearson's correlation coefficient (r) between periodontal parameters and TNFR2 serum concentrations in periodontitis and type 2 diabetes groups

Group	Clinical parameters		r	p-value
Periodontitis	TNFR2	CAL	-0.46	0.016
		PPD	0.085	0.675
		PESA	-0.357	0.068
		PISA	-0.164	0.414
Type 2 diabetes	TNFR2	CAL	0.363	0.005
		PPD	0.298	0.087
		PESA	0.345	0.046
		PISA	0.578	0.000

PPD – probing pocket depth; CAL – clinical attachment level; PESA – periodontal epithelial surface area; PISA – periodontal inflamed surface area

Kruskal–Wallis) in PD group. In this group, a correlation between PPY and $TNFR_2$ concentrations were observed (r = 0.424, p = 0.028, Spearman's correlation coefficient).

The concentrations of $TNFR_2$ were neither influenced by the levels of self-reported stress (Kruskal–Wallis, p = 0.459) nor by parameters of physical exercise (Kruskal–Wallis, p > 0.05). Spearman's correlation revealed a weak positive but nonsignificant correlation between $TNFR_2$ and HbA_1c concentrations (r = 0.015, p = 0.935), and duration of T2D (r = 0.05, p = 0.781).

In linear regression analysis, none of the parameters (hematological and biochemical data, physical exercise, mental stress, smoking status, clinical periodontal parameters, duration of diabetes) were assessed as univariate predictors.

DISCUSSION

This study investigated serum concentrations of $TNFR_2$ in T2D patients with periodontitis, and compared them with serum $TNFR_2$ levels in nondiabetics with periodontitis and subjects with clinically healthy periodontium. T2D and periodontitis both are chronic inflammatory disorders which share certain pathogenetic mechanisms, have common inflammatory mediators, although they are of different etiological origin. Advanced periodontitis is considered to be a source of inflammatory mediators. On the other hand, low-grade inflammation precedes diabetes. The role of inflammatory mediators derived from chronically inflamed periodontium and its influence on diabetes onset is yet to be determined [14]. Most published studies investigating inflammatory mediators originated from periodontium have been done in systemically healthy subjects. In contrast, studies with diabetic patients did not take into consideration influence of periodontal inflammation, which surely should not be neglected. We did not find differences in $TNFR_2$ serum concentration between the groups. Local production of both receptors is elevated in periodontal pockets > 3 mm, but their levels progressively diverged as the PPD increased, with the $TNFR_2$ level being comparatively lower than $TNFR_1$ [5]. Although local $TNFR_2$ concentration was negatively correlated with PPD and is considered to be protective for periodontal tissues, serum $TNFR_2$ concentrations were not increased after treatment [9]. It is possible that local periodontal production of receptors is not sufficient for a detectable change of serum concentrations. The results of studies dealing with local periodontal production of TNF family are conflicting: although it is almost accepted that diabetes elevates local cytokine production [15], one study showed lower amount of TNFα in gingival crevicular fluid (GCF) in T2D + PD than in PD patients [16]. Elevated concentrations of both receptors are detected in diabetics with complications [17]. In our study we found slightly increased $TNFR_2$ levels in patients with chronic complications of diabetes.

This study tried to determine systemic inflammatory/ infectious burden originating from periodontium using PISA and PESA calculation. There is a limited number of studies investigating PISA–T2D relationship, but all are related to HbA_1c level [18]. As mentioned in the Methods section, PESA and PISA are calculated using PPD, CAL, gingival recession, and BOP. As BOP correlates to PI, we took into consideration all clinical periodontal parameters. Statistical correlation was found between CAL and $TNFR_2$ in T2D and PD groups. Significant correlations between PISA/PESA and $TNFR_2$ were observed in diabetics. In PD group, CAL–$TNFR_2$ correlation was negative. This negative correlation is in agreement with the study that

found negative correlation of local $TNFR_2/TNFR_1$ ratio with PPD, and increasing of this ratio after scaling and root planning [9]. It seems that reduction of inflammation leads to increase of local $TNFR_2$ concentration. The same study also measured receptor serum level before and after the treatment and did not show any differences in serum $TNFR_2$ level due to its long-term stable concentration. Also, measuring the serum TNFα and receptors concentration, Ikezawa et al. [5] found decreased ratio of serum $TNFR_2/TNFR_1$ at periodontitis vs. healthy subjects ($p = 0.051$). This finding also supports a protective role of $TNFR_2$ in periodontal tissues. These findings are in correlation with our results supporting the role of $TNFR_2$ in periodontitis. The $TNFR_2$ is directly involved in TNFα signaling pathways and is considered as prosurvival receptor, which suppresses basal osteoclastogenesis [6, 19]. While TNFα and $TNFR_1$ are overexpressed in gingival tissues at periodontitis, $TNFR_2$ is sporadically found in infiltrating monocyte/macrophage cells and gingival fibroblasts, which may be explained as unsuccessful attempt of gingival tissue to neutralize detrimental effects of TNFα [20]. This in agreement with our results, which support the negative correlation between $TNFR_2$ concentration and CAL in nondiabetic periodontitis patients.

In T2D group, we found significant and positive correlation between CAL/PESA/PISA and serum $TNFR_2$ concentration. Our results lead to the assumption which is in agreement with other studies – that diabetes through extended TNFα/receptors expression changes the inflammatory reaction and thereby response to periodontal pathogens [16, 21]. The aforementioned changes in inflammatory response may lead to exaggerated vulnerability to systematic consequences of local infection. TNFα is implicated in insulin resistance mechanism [10], and serum $TNFR_2$ levels and/or adiponectin concentration are good prediction markers of T2D onset [22]. Increased $TNFR_2/TNFR_1$ ratio is associated with insulin resistance [23]. Almost all studies which explored TNFα level in diabetics did not take into consideration periodontal inflammation. Engebretson et al. [14] considered the periodontal status in T2D patients and demonstrated that severity of chronic periodontitis was associated with increased TNFα level in T2D patients and found a positive correlation between the TNFα serum concentration and CAL.

According to some studies, BMI could influence $TNFR_2$ [24]. We did not find correlation between BMI and $TNFR_2$ in total of 85 patients and separately for each group which is in accordance with most of the studies correlating BMI with TNFα and/or its receptors [10, 14, 17].

TNFα and its receptors serum level could be influenced by emotional stress [25], regular exercise [26], and smoking [27]. According to the results of our study, self-reported evaluation of the aforementioned parameters could not be considered to be an independent predictor of $TNFR_2$ concentration. To the best of our knowledge, this is the first study that took into consideration these factors when examining the influence of periodontitis and T2D on cytokine level.

We observed a higher $TNFR_2$ concentration in smokers in the PD group. This group showed a significant correlation between PPY and $TNFR_2$ concentration. Although a relationship between cigarette consumption and TNFα release from macrophages has been reported [27], results about correlation of smoking and $TNFR_2$ serum concentration are still inconsistent [9].

$TNFR_2$ reflects chronic activation of TNFα and has inducible but relatively stable concentration and may act both as an inhibitor and depot of TNFα [9]. It transduces the most prosurvival signals, buffers TNFα [21], and has certain roles in pathogenesis of peridontitis[19, 28] and diabetes [22, 23]. In our study we could not detect any predictors of $TNFR_2$ concentration using regression statistical models.

CONCLUSION

$TNFR_2$ showed negative correlation with periodontal destruction in nondiabetics, but its dysregulated production and function in T2D may affect this correlation and its potential protective effect. Measuring circulating levels of proinflammatory cytokines is more accurate at animal models, because of the strong influence of environmental factors on expression of these cytokines in humans. Larger and more controlled studies, which take into consideration genetic background, are needed to confirm these findings.

ACKNOWLEDGMENTS

The authors would like to thank Milena Cimbaljević for cooperation in patient recruitment, M.Sc. Biljana Andjelski-Radičević and Aleksandra Stojković (Hematological and Biochemical Laboratory, Faculty of Dental Medicine, Belgrade) for assistance with blood samples.

NOTE

This study was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. 41008.

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Корелација између концентрације рецептора 2 фактора некрозе тумора у серуму и деструкције пародонцијума код болесника са дијабетес мелитусом тип 2: студија пресека

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КРАТАК САДРЖАЈ

Увод Улога фактора некрозе тумора-алфа (*TNFα*) доказана је у патогенези хроничне пародонтопатије (ХП) и дијабетеса мелитуса типа 2 (ДМ тип 2). С обзиром на то да је полуживот *TNFα* веома кратак, рецептор 2 фактора некрозе тумора ($TNFR_2$) користи се као маркер активности *TNFα*.

Циљ рада Циљ овог рада је одређивање концентрације $TNFR_2$ у серуму и корелирање са параметрима деструкције пародонцијума код здравих и испитаника са дијагностикованим ДМ тип 2.

Методе рада У студију је укључено 85 пацијената подељених у три групе: ДМ тип 2 + ХП (ДМ група, $n = 34$), здрави испитаници + ХП (ПД група, $n = 27$) и здраве контроле (ЗК група, $n = 24$). Дијагноза ДМ тип 2 постављена је на основу критеријума СЗО (2013), док је дијагноза ХП постављена на основу критеријума Интернационалне радионице за класификацију стања и обољења пародонцијума (1999). Концентрација $TNFR_2$ мерена је *ELISA* методом.

Резултати Концентрација серумског $TNFR_2$ није се разликовала међу групама (Краскал–Волис, $p = 0,482$). Постоји значајна корелација (Пирсон) између нивоа припојног епитела (НПЕ) и концентрације $TNFR_2$ у ПД групи ($r_p = -0,460$, $p = 0,016$). У ДМ тип 2 групи, статистички значајна корелација уочена је између концентрације $TNFR_2$ и НПЕ ($r_p = 0,363$, $p = 0,005$), као и параметара утицаја инфламације из пародонцијума на системско здравље – *PISA* ($r_p = 0,345$, $p = 0,046$) и *PESA* ($r_p = 0,578$, $p = 0,000$).

Закључак Код пацијената са дијабетесом веће концентрације $TNFR_2$ одговарају већим вредностима НПЕ, *PESA* и *PISA*. Насупрот томе, код системски здравих испитаника са ХП веће вредности НПЕ су повезане са мањим концентрацијама $TNFR_2$, што би могло говорити о потенцијалној заштитној улози овог цитокина на деструкцију пародонцијума. Резултати говоре да дијабетес може утицати на секрецију $TNFR_2$ из пародонцијума.

Кључне речи: хронична пародонтопатија; $TNFR_2$; *TNFα*; дијабетес мелитус тип 2

Примљен • Received: 28/04/2015 **Ревизија • Revision:** 14/07/15 **Прихваћен • Accepted:** 06/11/2015

Comparative effects of riboflavin, nicotinamide and folic acid on alveolar bone loss: A morphometric and histopathologic study in rats

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SUMMARY

Introduction Periodontitis is a chronic inflammatory and osteolytic disease. Vitamin B complex is a class of water-soluble vitamins that play important roles in cell metabolism.

Objective The aim of this study was to evaluate the effects of riboflavin (RBF), nicotinamide (NA), and folic acid (FA) on alveolar bone loss in experimental periodontitis rat model.

Methods Sixty-four male Wistar rats were randomly divided into the following eight groups: Control, Ligated, RBF50 (RBF, 50 mg/kg daily), NA50 (NA, 50 mg/kg daily), FA50 (FA, 50 mg/kg daily), RBF100 (RBF, 100 mg/kg daily), NA100 (NA, 100 mg/kg daily), and FA100 (FA, 100 mg/kg daily). Periodontitis was induced using silk ligature around the right first mandibular molar. After 11 days the rats were sacrificed. Mandible and serum samples were collected. Changes in alveolar bone levels were measured clinically, and periodontal tissues were examined histopathologically. Serum IL-1β (pg/ml) levels were analyzed by using ELISA.

Results Mean alveolar bone loss in the mandibular first molar tooth revealed to be significantly lower in RBF100 group than in the Control group. In the Ligated group, alveolar bone loss was significantly higher than in all other groups. The ratio of presence of inflammatory cell infiltration in the Ligated group was significantly higher than in the Control group. The differences in the serum IL-1β levels between the groups were not statistically significant. Osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups. The osteoblastic activity in the Ligated group, RBF100, and NA100 groups were shown to be significantly higher than those in the Control group.

Conclusion This study has demonstrated that systemic administration of RBF, NA, and FA in different dosages (50–100 mg/kg) reduced alveolar bone loss in periodontal disease in rats.

Keywords: alveolar bone loss; ligature-induced; histomorphometric; micronutrition; vitamin B

INTRODUCTION

Periodontitis is a biofilm-induced chronic inflammatory disease caused by bacterial pathogens and leads to the destruction of the alveolar bone. Although tooth-associated biofilm or dental plaque is required to induce the periodontitis, it is not sufficient to initiate periodontal tissue destruction [1]. Host inflammatory response which is modulated through multiple factors including genetics, smoking, general health, social variables and diet can cause periodontal tissue destruction [1]. Researchers have found strong evidence that macro- and micronutrients could modulate pro- and anti-inflammatory mechanisms effecting individual inflammatory conditions [2]. Some studies have showed that tooth loss is related to poorer nutritional status and poorer quality of life [3, 4]. The association between nutrition intake and periodontal condition has been investigated in many studies. Previously published evidence of the foods and nutrients with antioxidant and anti-inflammatory activity have consistently been linked to a reduced risk of developing periodontal disease such as vitamin C [5], b-carotene [6], vitamin E [7, 8], folic acid [9], and more recently vitamin B_{12} [10]. Therefore, research of micronutritional approaches with many vitamins, antioxidants, and therapeutic agents for periodontal treatment has been going on in recent years. Despite the oral symptoms accompanied by vitamin B complex deficiency [11], it remains unknown whether vitamin B complex is associated with periodontitis.

Vitamin B complex is a class of water-soluble vitamins that play important roles in cell metabolism. The vitamin B complex includes eight different vitamins which differ in their chemical composition and pharmacological properties [12].

Riboflavin (RBF), also known as vitamin B_2, helps to provide good vision, releases energy from the foods, promotes healthy skin. Sources of riboflavin are milk, eggs, mushrooms, whole grains, enriched grains, green leafy vegetables, yeast, liver, and oily fish [13]. Clinical riboflavin deficiency is known as ariboflavinosis, and presents as angular stomatitis, cheilosis, and glossitis. Moreover, it is common in developing country populations [14].

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Nicotinamide (NA), known as vitamin B_3 or niacin, has influence on oxidative stress [15], cellular survival, inflammatory cell modulation [16], metabolic disease[17], and energy management [18]. NA deficiency can lead to fatigue, loss of appetite, pigmented rashes of the skin, and oral ulcerations, while more severe types lead to pellagra, characterized by coetaneous rashes, oral ulcerations, gastrointestinal difficulties, and cognitive loss [19].

Folic acid (FA), known as vitamin B_9, is essential for numerous metabolic functions such as synthesizing, repairing, and methylating DNA; it is also especially important in preventing neural tube defects, for example spina bifida [20]. FA is essential for maturation of oral mucosal epithelium and it depresses the hematologic elements which help prevent and fight against the infectious agents in this section of the mouth. Folate deficiency can lead to diarrhea, megaloblastic anemia, peripheral neuropathy, increased oxidative stress, and periodontal disease [9]. Folate supplementation was hypothesized to prevent or decrease fracture incidence by effecting homocysteine metabolism, but some of these studies had inconsistent data about fracture preventing [21].

Vitamin B complex plays an important role in wound healing, gingival health. Effects of vitamin B complex were mostly investigated in terms of soft tissue healing, but some studies have indicated that vitamin B_{12} [10], vitamin B_9 [22], vitamins B_1, B_2, B_3, B_5, B_6, B_7 reduced the periodontal destruction and tooth mobility [23]. The effects have not been researched comprehensively. The aim of this study was to evaluate these effects in experimental periodontitis rat model, morphometrically and histopathologically.

OBJECTIVE

Vitamin B complex plays an important role in wound healing and gingival health. Effects of vitamin B complex were mostly investigated in terms of soft tissue healing. The aim of this study was to evaluate the effects of RBF, NA, and FA on alveolar bone loss in experimental periodontitis rat model, morphometrically and histopathologically, as these effects have not been researched comprehensively to date.

METHODS

Experimental design

Sixty-four male Wistar rats weighting 250 ± 10 g were used in the study. The animals were kept in temperature-controlled cages (approximately 25°C), exposed to a 24-hour light–dark cycle of equal time, and had access to water and food *ad libitum*. The experimental procedure was approved by the Animal Ethics Committee of Cumhuriyet University School of Medicine. Rats were randomly divided into the following eight groups:

- Non-ligated control (Control) group (n = 8);
- Ligated group (n = 8);
- Riboflavin 50 mg/kg daily (RBF50) group (n = 8);
- Nicotinamide 50 mg/kg daily (NA50) group (n = 8);
- Folic acid 50 mg/kg daily (FA50) group (n = 8);
- Riboflavin 100 mg/kg daily (RBF100) group (n = 8);
- Nicotinamide 100 mg/kg daily (NA100) group (n = 8), and
- Folic acid 100 mg/kg daily (FA100) group (n = 8).

General anesthesia was administered by using ketamine (Eczacıbasi Ilac Sanayi, Istanbul, Turkey) (40 mg/kg). In order to induce experimental periodontitis in Ligated, RBF50, NA50, FA50, RBF100, NA100, and FA100 groups, a 4/0 silk suture (Dogsan Sanayi, Istanbul, Turkey) was placed subgingivally, by the same operator (Karakan NC), around the gingival margin of the right mandibular first molars of the rats. In the Control group, ligature placement was not performed. After application, sutures were checked and loose sutures were tightened again. In the RBF50, NA50, FA50, RBF100, NA100, and FA100 group RBF, NA, and FA (Sigma-Aldrich, Saint Louis, MO, USA) were administered systemically with vehicle solution (physiological saline) by using a gastric gavage, at a rate of 50 mg/kg/d and 100 mg/kg/d [24]. Only physiological saline solution by using gastric gavage was administered in Ligated and Control groups to create sham effect to the rats. Daily systemic treatment with vitamin B groups was continued for 11 days; all rats were sacrificed on the 12th day by using cardiac punction [25]; blood samples were taken and immediately centrifuged at 3,000 rpm for 10 minutes to obtain serum samples, which were stored at -20°C in microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) until used for enzyme-linked immunosorbent assay (ELISA) analysis. The right mandibles of all rats were dissected and fixed in 10% neutral-buffered solution and stained for histomorphometric analysis.

Measurement of alveolar bone loss

Soft tissues of right mandibular region were defleshed manually and cleaned. The jaws were washed, dried and embedded into 1% aqueous methylene blue solution for identifying cement–enamel junction (CEJ) level. Photographs were taken with a stereomicroscope (×25) and transferred to a computer. The alveolar bone loss was measured in three different sites in the right mandibular molar region by a single examiner (Akpinar A), who marked them to identify the samples. The distance from the CEJ to the top of the alveolar bone crest was measured using Vision Image Analysis Software (Clemex, Longueuil, Quebec, Canada). Mean values of measurements were calculated for statistical analysis (Figure 1).

Laboratory assays

Serum samples were assessed by a commercial ELISA kit (Invitrogen, Camarillo, CA, USA) to determine, IL-1β (detection range: 31.3–2,000 pg/ml; sensibility or lower limit of detection: 4 pg/ml of recombinant rat IL-1β) and IL-10

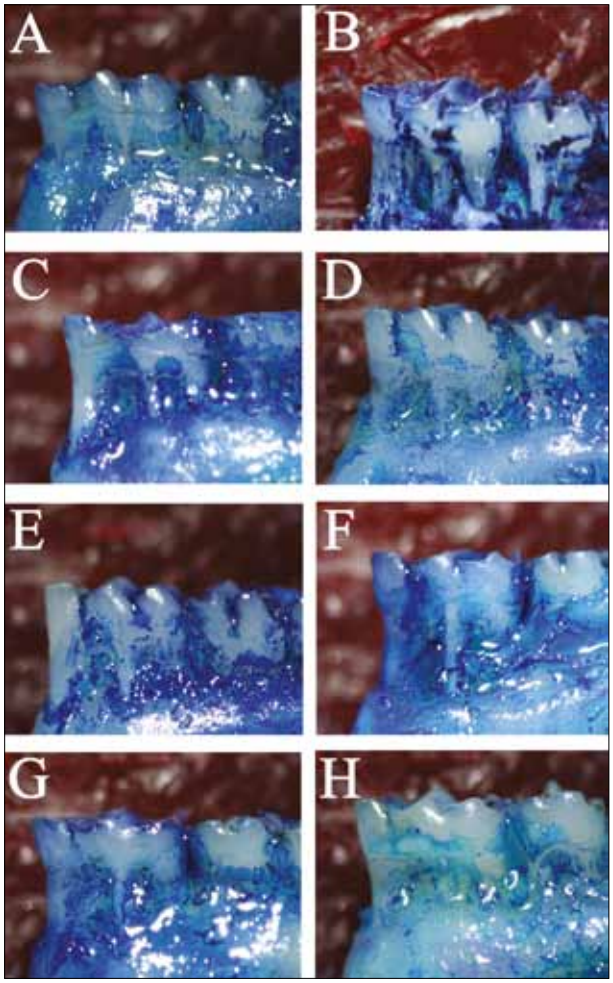


Figure 1. Images of alveolar bone loss in the mandibular first molar in the (A) Control, (B) Ligated, (C) RBF50, (D) RBF100, (E) FA50, (F) FA100, (G) NA50, and (H) NA100 group.

(detection range: 15.6–1,000 pg/ml; sensibility or lower limit of detection: <5 pg/ml of recombinant rat IL-10) in serum samples (samples per group). ELISAs were carried out according to the manufacturer's recommendations; 96-well plates presoaked with appropriate antibodies were used. Standard diluent buffer of 50 µl was added to 50 µl samples; 50 µl of biotin conjugate was added to samples and incubated for two hours at room temperature (37°C). After further incubation at room temperature for two hours, the plates were washed four times, and 100 µl of streptavidin HRP (diluted 1:5,000) was added at room temperature for 30 minutes. The plates were aspirated and washed four times; 100 µl of stabilized chromogen was added and incubated for 30 minutes. The amount of 100 µl of stop solution was added and absorbance of color at 450 nm was measured. The resulting values were expressed in pg/ml. Microwell strips were washed twice with wash buffer. In duplicate 100 µl sample diluents were added to all standard wells, and 96-well plates presoaked with appropriate antibodies were used. After the plates were coated, the samples and standards were added in various dilutions in duplicate and incubated at 4°C for 24 hours. Antibodies anti-IL-1β and anti-IL-10 were then added to the wells. Tetramethylbenzidine substrate solution was added to the wells for 15 minutes and activation of blue color development in the sample was waited for. Color development was stopped when the color of samples turned yellow. The absorbance of the color at 450 nm was measured.

Histopathological and histomorphometric analysis

Histopathologic sample analyses were performed by a single examiner (Göze F), who masked the samples to identify them to each other. Mandible sections were fixed in 10% formalin, and then in 10% formic acid to demineralize the sample. Next, the specimens were dehydrated, embedded in paraffin, and six-µm thickness sections were obtained along the molars in a mesio-distal plane for hematoxylin and eosin described. Alveolar bone and interdental septum were analyzed under light microscopy (Eclipse E 600, Nikon, Tokyo, Japan). Inflammatory cell infiltration (ICI) of the periodontal tissues was scored as follows: not visible ICI (score = 0), slightly visible ICI (score = 1), and dense visible ICI (score = 2). A semi-quantitative scoring was used for determining osteoblastic activity as follows: no activity (0), mild/moderate activity (1), and high activity (2) [26]. Osteoclasts, which are large cells including multiple nuclei near border of the resorption surface, were counted morphologically in the histological assessment

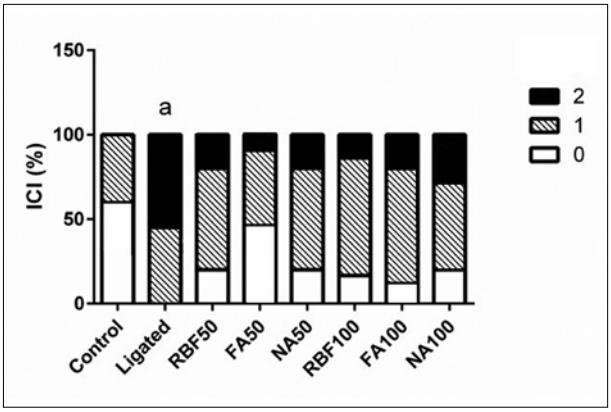
Statistical Analysis

Statistical analyses were performed with SPSS 22.0 for Windows (IBM Corp., Armonk, NY, USA). Kolmogorov–Smirnov test was performed for determining data distribution. Comparisons between the four groups were performed using the Kruskal–Wallis test. Two independent group comparisons were performed using Mann–Whitney U-test. The data were presented as mean ± standard deviation and $p < 0.05$ was considered statistically significant. The ratios of the presence of ICI and osteoblastic activity were analyzed using a χ^2 test.

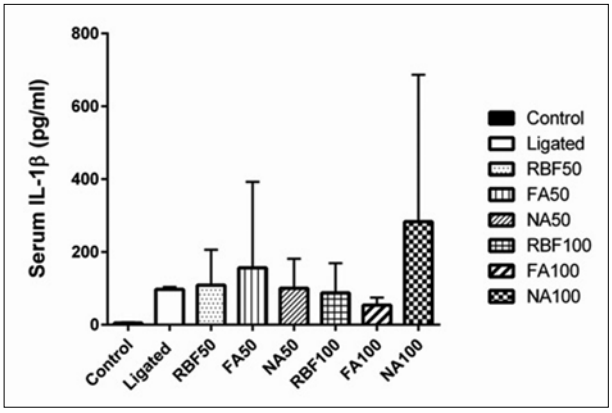
RESULTS

The presence of silk ligature around the first right molars of the rats induced an inflammatory response and periodontitis. The ratio of presence of ICI in the Ligated group ($p < 0.05$) was significantly higher than in the other groups. The ICI in the RBF50, FA50, and NA50 groups and RBF100, FA100, NA100 groups were similar and there was no significant difference between these groups ($p > 0.05$) (Graph 1).

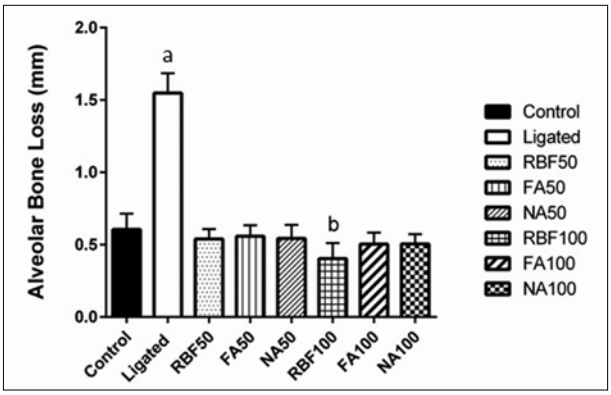
Mean alveolar bone loss measurement with stereomicroscope in the mandibular first molar tooth was revealed to be significantly lower in the RBF100 group than in the Control group ($p < 0.05$). In the Ligated group, alveolar bone loss was significantly higher ($p < 0.05$) compared to all the other groups (Graph 2). The differences in the serum IL-1β levels between the groups were not statistically significant ($p > 0.05$) (Graph 3). Serum IL-10 levels of all



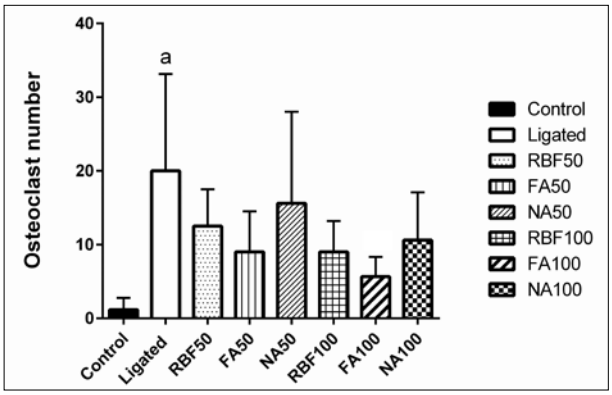
Graph 1. Frequencies (%) of inflammatory cell infiltration (ICI) in groups: (a) Ligated group was revealed as statistically more significant regarding ICI than the Control group ($p < 0.05$).



Graph 3. Mean serum IL-1β levels with standard deviation bars of the groups; there was no statistical significance between the groups ($p > 0.05$).



Graph 2. Mean alveolar bone loss and standard deviation bars in Control, Ligated, RBF 50, FA50, NA50, RBF100, FA100 and NA100 groups: (a) significant bone loss in Ligated group compared to that of other groups ($p < 0.05$); (b) in the RBF100 group, alveolar bone loss was significantly lower than that in the Control group ($p < 0.05$).



Graph 4. Mean osteoclast numbers in the groups; the osteoclast numbers of all the groups were lower than that in the Ligated group, but this difference was not statistically significant ($p > 0.05$); osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups ($p < 0.05$).

the groups were under the detection range, hence they were not represented in the data.

Graph 4 shows the osteoclast numbers of the groups. The osteoclast numbers of all the groups were lower than the Ligated group, but this difference was not statistically significant ($p > 0.05$). Levels of osteoclasts that were observed in the Ligated group were significantly higher than those of the Control and FA100 groups ($p < 0.05$).

It was revealed that osteoblastic activity in the Ligated group, RBF100, and NA100 groups was significantly higher than that in the Control group ($p < 0.05$). There was no significant difference in term of osteoblastic activity between RBF50, NA50, FA50, and FA100 groups ($p > 0.05$) (Figure 2).

DISCUSSION

The understanding of the nature of periodontal disease causation and pathogenesis has changed dramatically, and as a result the approach of periodontal treatment started evolving 30 years ago, from blocking inflammation to moderating it [27]. There are many studies in literature that refer to links between periodontal disease and dietary intake. Nutrients derived from diet perform as antioxidants, co-enzymes in energy production and metabolic processes and components of tissue structures that keep the body's system functioning properly and maintain good overall health, including oral health. Thus we investigated the effects of different dosages of RBF, NA, and FA on periodontal bone loss in rats and found that the vitamins increased osteoblast activity, and reduced the osteoclast numbers and alveolar bone loss.

In one study, authors researched vitamin B complex supplementation on periodontal wound healing in humans [11]. Patients were given 50 vitamin B complex tablets and 50 placebo tablets for 30 days after the surgery. At the end of the study Vitamin B supplemented subjects demonstrated significantly superior clinical attachment level (CAL) gains, but not in plaque index (PI), gingival index (GI), bleeding on probing (BOP), and BANA test values.

Erdemir and Bergstrom [28] investigated relationship between FA and vitamin B_{12} serum levels on the one hand, and smoking on the other, in patients with chronic periodontitis. They concluded that mean PI, GI, and CAL were significantly more increased in smokers than in non-smokers, although FA levels of smokers were lowered by 26% in comparison with that of non-smokers. In another study authors tried to evaluate the effects of folate mouthwash on periodontium in experimental gingivitis model

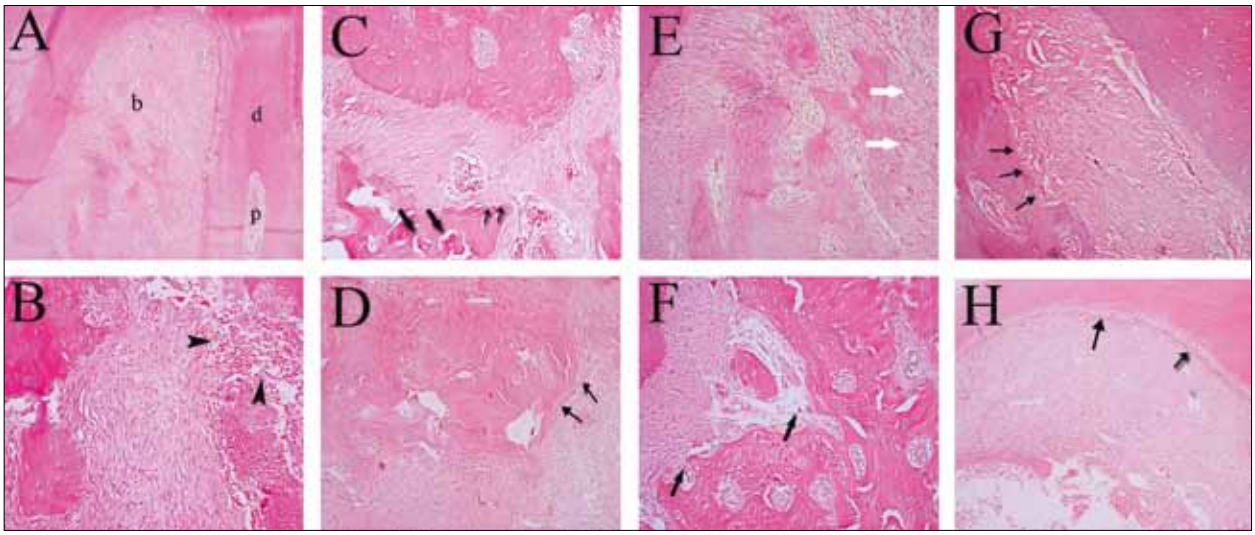


Figure 2. Histopathological sections of mandibular first molar tooth in all the groups (hematoxylin and eosin staining; original magnification: A: ×10; B: ×40; C: ×20; D: ×10; E: ×20; F: ×20; G: ×40; H: ×20): (A) normal mandible showing alveolar bone (b), pulp (p), and dentin (d); (B) in the Ligated group after 11 days, dense inflammatory infiltration area (arrow); (C) RBF50 group showing osteoblastic activity border of alveolar bone (narrow arrows) and ruffled osteoclast border with multiple nuclei (wide arrows); (D) RBF100 group revealed dense osteoblastic activity (arrows); (E) FA50 group, osteocytes in the lacunas (white arrows); (F) FA100 group showing osteoclastic activity (arrow); (G) NA50 and (H) NA100 groups, showing increased osteoblastic activity (black arrows), osteocytes (white arrow), and reduction in osteoclast number, respectively.

[29]. At the end of the study they concluded that the folate mouthwash did not affect plaque accumulation or clinical signs of gingivitis. According to data from the national health and nutrition examination survey 2001/02, serum folate levels are important indicators for periodontal health in older adults and may help maintain oral health properly [9]. Mohammadi et al. [22] aimed to evaluate the protective effect of FA on cyclosporine-induced bone loss in rats. Consequently, they suggested that FA may have a preventive role against cyclosporine adverse effects to bone in rats. In a recent study performed with diabetic rats, authors observed that alveolar bone loss, number of inflammatory cells, and nuclear factor kappa-B ligand positive cells were more decreased in streptozotocin in the NA group than in the streptozotocin group [30]. In our study the results showed that RBF, FA, and NA significantly prevented alveolar bone loss which had been initiated by using ligature on experimental periodontitis model in rats.

There are many experimental periodontitis models which have been applied on rats, such as using ligature on molar teeth, dietary manipulation, or introduction of pathogenic microorganisms. Placing a ligature is a highly presumable model for determining effects of different drugs or agents against periodontal tissues. According to studies, placement of a ligature around the molar teeth of rats leads to appearance an inflammation beneath the ligature [31] and most intense bone loss was achieved by day 11 after ligature placement [32]. We used ligature-induced periodontitis model in our study, rats were sacrificed on day 12 and ligature placement on the first molar tooth caused a significant amount of bone loss.

In this study the dosages were arranged according to studies listed below. No evidence of RBF toxicity was based on limited human and animal studies [33]. Bertollo et al. [34] assessed antinociceptive and anti-inflammatory activities of RBF in different experimental models with different dosages, i.e. 25, 50, and 100 mg/kg. In an experimental rat model, the authors used 100 mg/kg dosage of RBF to evaluate its antioxidant effect [35]. In another study RBF was administered at 30 and 100 mg/kg by oral gavage to test its protective effect on hepatic injury [36]. The risk of toxicity from FA is low, because folate is a water-soluble vitamin and is regularly removed from the body through urine. Different dosages of FA have been used in many animal studies. In an experimental study on mice, 400–1,200 nmol/kg dosages of FA were used [37]. The dose of FA could be chosen to evaluate its efficiency – a 100-fold dose of its acceptable daily dose, which is considered to be 15 mg/day for a 70-kg healthy adult [38]. In general, NA is given in doses ranging 40–250 mg/day orally when NA deficiency occurs [13]. The median lethal dose for subcutaneous administration of NA in rats is 1.68 g/kg, hence the therapeutic index of NA is wide, but at very high doses it causes reversible hepatotoxicity in animals and humans [39]. No clinical signs, abnormal behaviors, or infections were observed in rats of any group. In our study, 50 mg/kg and 100 mg/kg dosages of RBF, FA, and NA were used on rats. Both of the dosages were effective on alveolar bone loss, although 100 mg/kg dosage of these vitamins revealed more effective results compared to 50 mg/kg dosage; however, these findings are not statistically significant.

IL-1β plays a key role in periodontal pathogenesis and induces the synthesis and secretion of different mediators such as prostaglandins, chemokines, and other cytokines. IL-1β exacerbates inflammation and alveolar bone resorption. A development of systemic infection in rats might have increased the levels of IL-1β. Apart from the serum cytokine analysis, gingival homogenates analysis would be performed to eliminate systemic inflammation effect. In our study, serum levels were the highest in the NA100 group, but the difference between the groups was not sig-

nificant – although NA has an antioxidant effect that may prevent alveolar bone loss in spite of increased IL-1β level.

CONCLUSION

Within its limitations, this study states that administration of systemic RBF, FA, and NA with different dosages increased osteoblast activity, decreased osteoclast numbers, and diminished alveolar bone loss in experimental periodontitis in a ligature-induced rat model. Considering this data, RBF, FA, and NA, which are different kinds of vitamin B complex, may provide additional benefits to periodontal therapy for preventing bone loss. More research is needed on effects of vitamin B complex on periodontal bone healing.

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Упоредни утицај рибофлавина, никотинамида и фолне киселине на алвеоларни губитак коштане масе: морфометријска и хистопатолошка студија на пацовима

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КРАТАК САДРЖАЈ

Увод Пародонтопатија је хронична инфламаторна и остеолитичка болест. Комплекс витамина Б је класа витамина растворљивих у води који играју важну улогу у ћелијском метаболизму.

Циљ рада Циљ овог истраживања је био да се процени утицај рибофлавина (РБФ), никотинамида (НА) и фолне киселине (ФК) на алвеоларни губитак коштане масе у експерименталном моделу пародонтопатије код пацова.

Методе рада Шездесет четири мужјака пацова соја вистар насумично су подељена у следећих осам група: контролна, лигатурна, РБФ50 (РБФ, 50 *mg/kg* дневно), НА50 (НА, 50 *mg/kg* дневно), ФК50 (ФК, 50 *mg/kg* дневно), РБФ100 (РБФ, 100 *mg/kg* дневно), НА100 (НА, 100 *mg/kg* дневно) и ФК100 (ФК, 100 *mg/kg* дневно). Пародонтопатија је изазвана везивањем свилене нити око првог десног мандибуларног кутњака. После 11 дана пацови су жртвовани. Сакупљени су узорци доње вилице и серума. Клинички су утврђене промене нивоа алвеоларне коштане масе, док су пародонтална ткива испитана хистопатолошки. Нивои *IL-1β* (*pg/ml*) серума анализирани су тестом *ELISA*.

Резултати Показало се да је средњи губитак алвеоларне коштане масе у првом мандибуларном кутњаку знатно нижи у групи РБФ100 у односу на контролну групу. У лигатурној групи алвеоларни губитак коштане масе био је значајно виши него у осталим групама. Проценат присуства инфилтрације инфламаторних ћелија у лигатурној групи био је знатно већи у односу на контролну групу. Разлике у нивоима *IL-1β* у серуму између група нису биле статистички значајне. Остеокласти уочени у лигатурној групи били су знатно виши од оних у контролној и групи ФК100. Остеобластична активност у лигатурној, групи РБФ100 и групи НА100 била је значајно већа него у контролној групи.

Закључак Ова студија је показала да системска примена РБФ, НА и ФК у различитим дозама (50–100 *mg/kg*) смањује губитак алвеоларне коштане масе код пародонтопатије код пацова.

Кључне речи: губитак алвеоларне коштане масе; индуковано лигатуром; хистоморфометријски; микронутриционизам; витамин Б

Примљен • Received: 08/05/2015 **Ревизија • Revision:** 14/10/15 **Прихваћен • Accepted:** 20/01/2016

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):280-287
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1606280S
UDC: 615.466.099

Cytotoxicity investigation of a new hydroxyapatite scaffold with improved structural design

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SUMMARY

Introduction Biodegradable porous scaffolds are found to be very promising bone substitutes, acting as a temporary physical support to guide new tissue regeneration, until the entire scaffold is totally degraded and replaced by the new tissue.

Objective The aim of this study was to investigate cytotoxicity of a synthesized calcium hydroxyapatite-based scaffold, named ALBO-OS, with high porosity and optimal topology.

Methods The ALBO-OS scaffold was synthesized by the method of polymer foam template. The analysis of pore geometry and scaffold walls' topography was made by scanning electron microscope (SEM). The biological investigations assumed the examinations of ALBO-OS cytotoxicity to mouse L929 fibroblasts, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor (MTT) and lactate dehydrogenase (LDH) tests and inverse phase microscopy.

Results The SEM analysis showed high porosity with fair pore distribution and interesting morphology from the biological standpoint. The biological investigations showed that the material is not cytotoxic to L929 cells. Comparison of ALBO-OS with Bio-Oss, as the global gold standard as a bone substitute, showed similar results in MTT test, while LDH test showed significantly higher rate of cell multiplication with ALBO-OS.

Conclusion The scaffold design from the aspect of pore size, distribution, and topology seems to be very convenient for cell adhesion and occupation, which makes it a promising material as a bone substitute. The results of biological assays proved that ALBO-OS is not cytotoxic for L929 fibroblasts. In comparison with Bio-Oss, similar or even better results were obtained.

Keywords: hydroxyapatite; cytotoxicity; MTT; LDH

INTRODUCTION

Bone is a dynamic and highly vascularized tissue which has significant self-healing capacity for the repair of small fractures. However, bone grafts are needed to provide support, fill bone defect, and enhance biological repair in larger ones. Due to source limitation of autogenic bone grafts and immunologic rejection problem of natural bone grafts, bone tissue engineering technique has become a crucial strategy for permanent repair of bone defects in current tissue transplantation methodologies and grafting [1, 2, 3].

Hence, 3D biodegradable porous structures well-known as scaffolds are found to be a promising solution because they act as a temporary physical support to guide new tissue regeneration until the entire scaffold is totally degraded and replaced by the new tissue. Ideally, these temporary scaffolds should be porous in order to accommodate cell growth and facilitate both tissue regeneration and vascularization [4, 5, 6]. Furthermore, they should also be biocompatible, mechanically strong, and have a biodegradation rate similar to the cell/tissue growth rates. Three more properties should be fulfilled: (i) the material should establish a stable direct connection with patient's bone, both structural and functional, (ii) the material should be osteoinductive, and (iii) it should be osteoconductive, for recruitment of mesenchymal progenitor cells and guided bone deposition on the scaffolds surface.

Scaffold has to favor cell attachment, proliferation and differentiation, bone growth, and *in vivo* revascularization. It should also possess mechanical properties matching those of target tissue, designed geometrically and functionally to mimic native extracellular matrix environment as much as possible [7–10]. A ceramic carrier designed to mimic bone structure hierarchy covered by polymer thin films with appropriate properties is probably one of the best solutions for the above requested scaffold construction.

ALBO-OS scaffold was developed as innovative product following the aforementioned requirements. It is a composite of calcium hydroxyapatite (CHA) and poly-lactide-co-glycolide (PLGA) with very high porosity and pronounced topography of the inner walls. PLGA (coating CHA in ALBO-OS) belongs to the family of synthetic biodegradable polyesters commonly used in tissue engineering, as it satisfies the requirements of mechanical

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properties and convenient processing [11, 12]. It has favorable degradation rate that matches the rate of healing of damaged bone tissue [13]. This composite was made in an attempt to enhance the scaffold surface for various cell activities, including cell adhesion, proliferation and release of cytoplasmic metabolites, changes of the cell shape, density of their occupations, etc.

Presented *in vitro* study was aimed at investigating the biological response (cytotoxicity and cell adhesion) of fibre-like cells to ALBO-OS. The potential of such scaffold was compared with Bio-Oss through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor (MTT) and lactate dehydrogenase (LDH) tests; cell line of mouse fibroblasts L929 was used for investigation of biological response to the implanted material.

OBJECTIVE

The aim of this study was to investigate cytotoxicity of the synthesized scaffold on the base of calcium hydroxyapatite and poly(lactic-co-glycolic acid) (PLGA), with improved structural design, on the cell line of mouse fibroblasts L929 using MTT and LDH assays and inverse phase microscopy.

METHODS

Scaffold preparation

The synthesis of scaffold named ALBO-OS consisted of three stages: synthesis of CHA powder, production of CHA granules, and deposition of a thin film of PLGA on the surface of the granules.

CHA powder was synthesized hydrothermally from approximately stoichiometric mixture of $(NH_4)_2HPO_4$ and $Ca(OH)_2$, by procedure given in our previous investigations [14, 15]. The procedure, in brief, consisted of the following: 500 ml of a 2.32 cmol aqueous solution of $(NH_4)_2HPO_4$ was poured into 500 ml of a 3.02 cmol aqueous solution of $Ca(OH)_2$, under vigorous stirring. The mixture's pH value was adjusted to 7.4, and it was autoclaved at 150°C under a pressure of 5×10^5 Pa for 8 h. After the hydrothermal treatment, the precipitate was decanted, dried at 80°C for 48 h, washed with deionized water, and ultracentrifuged. In the next step, a mixture of 5 g of hydrothermally synthesized CHA and 1.5 g of poly(ethylenevinyl acetate)/poly(ethylene vinyl versatate) (PEVA/PEVV) was made and further processed in the autoclave at 120°C for 2 h. The obtained CHA powder was further used for production of CHA granules.

The CHA powder was mixed with water to form ceramic slurry. To enhance the wetness of the slurry and its rheological properties, 2% of PVA was added. Polyurethane (PU) foam with adequate pore size distribution was then dipped into the slurry followed by gently squeezing the foam several times to allow the slurry to penetrate the foam and the excess slurry to be squeezed out. Compressed air through an air gun was used to avoid a blockage of pores. The ceramic slurry-coated PU foam was left to dry at room temperature and was then heated in an oven at 600°C to burn out the PU foam, and then sintered at 1,200°C for 4 h. The obtained porous CHA compact was further disintegrated into granules of suitable size.

The final step in ALBO-OS synthesis was the deposition of PLGA thin film on the surface of CHA granules. PLGA pellets (50:50, M = 45,000–70,000; Durect Corporation, Cupertino, CA, USA) were dissolved in chloroform to obtain 1% w/w solution which was further poured over CHA granules. After the solvent evaporation, thin PLGA film was formed at the granules' surface [16].

Scaffold characterization

For structural analysis of CHA granules, as the main component of ALBO-OS, the methods of X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) were used. For XRD analysis, Philips PW 1050 diffractometer (Koninklijke Philips N. V., Amsterdam, the Netherlands) with Cu-Kα1-2 lamp was used, and the data were collected in the 2 θ range from 9° to 67°, in steps of 0.055°, and with exposure time of 2 seconds per step. For FTIR analysis, spectrometer 380 Nicollet FTIR (Thermo Electron Corporation, Waltham, MA, USA) was used. FTIR spectra were taken in the spectral range 4,000–400 cm^{-1}.

Scanning electron microscopy (SEM) was used to analyze the microstructure of pore walls of CHA granules. Sample preparation for SEM was performed by steaming with gold in BALZERS, and the samples were observed under a JSM-5300 microscope (JEOL USA, Inc., Peabody, MA, USA).

Total porosity of the synthesized CHA scaffolds was determined by using the following equations: bulk density (ρB) = weight of the sample divided by volume of the sample; theoretical density of the CHA (ρ_0) = 3.16 g/cm^2; relative density (R.D.) = $(\rho B/\rho_0) \times 100\%$; and total porosity = 100% - R.D. The dimensions and the weight of each sample were measured and recorded using the weight of displaced liquid (water) with measured quantity of the sample. Three identical specimens were used to determine the total porosity.

Nano-porosity of the CHA scaffold was estimated by high-pressure mercury intrusion porosimeter (Carlo Erba Porosimeter 2000 with Milestone 100 Software System). The porosimeter operates in the pressure interval 0.1–200 MPa, enabling estimation of pores in interval 7.5–15,000 nm.

Biological examination

Biological samples

Study included three different materials (ALBO-OS, Bio-Oss, and control) and three independent experiments (three biological replicates). In each experiment, five wells were analyzed for each material, which makes 15 samples in total, for each material.

Cell culture

The mouse fibroblasts L929 were grown in appropriate containers, in a culture medium (DMEM) with addition of 10% fetal calf serum (FCS), L-glutamine (3 mM), penicillin (100 IU/ml) and streptomycin (100 mg/ml). After supplementation, pH of the medium solution was adjusted to 7.2 using a bicarbonate buffer. Thereafter, the solution was filtered through a filter with a pore size of 0.22 μm. Cells were grown at the temperature of 37°C in air with 5% CO_2.

To optimize the growing conditions of cells, experimental curves of cell growth were defined, depending on the growth time. The cells were monitored using alamarBlue (Thermo Fisher Scientific, Waltham, MA, USA) assay (at 570 nm) after two, 24, 48, 72, and 96 hours. The growth curves after seeding 500, 1,000, 2,000, 4,000, and 8,000 cells were investigated. Based on the growth curves, the time required to double the cell number was determined and on its basis the optimal number of cells for experiments was determined. That number was 6,000 cells/well in the 96-well plate, for indirect toxicity testing, and 100,000 cells/well in the six-well plate for direct toxicity testing [17, 18, 19].

MTT test

For the test of indirect cytotoxicity (MTT test) the extracts were obtained by suspending the granules of ALBO-OS in culture medium (DMEM with10% FCS) at a concentration of 0.02 g/ml and incubated at 37°C for one, 24, or 72 hours with constant agitation. After incubation, extract solutions were centrifuged for 10 minutes at 2,000 rpm, decanted and filtered through a 0.22 μm filter (to eliminate any solid particles of the material). pH values of the obtained solutions were determined and they were used for MTT test.

For MTT test, L929 cells were seeded in 96-well cell culture plates in 100 μl growth medium at concentration of 6,000 cells/well. After 24 h of culture in a humidified atmosphere with 5% CO_2 at 37.1°C, medium was removed and replaced with 100 μl of previously prepared extract dilutions (obtained after one-, 24-, or 72-hour contact of medium and ALBO-OS). Extract of Geistlich BioOss (as a global gold standard) obtained only after 72-hour contact of BioOss with culture medium was used for comparison of cell viability on these two materials. Cells cultured in growth medium served as controls. After 72 h of incubation with each extract, the cell culture was treated with 20 μl/well of MTT (5 mg/ml in PBS) and incubated for further 4 h in humidified atmosphere with 5% CO_2 at 37.1°C. Then, MTT was removed and 100 μl 10% SDS in 0.01 M HCl was added in order to dissolve the formazan crystals. The next day, the optical density (OD) was read on ELISA multiwell microplate reader at 570 nm [17, 18, 19].

LDH test

To determine direct cytotoxicity, a thin (300 μm – 1 mm) ALBO-OS coating of the granules was deposited on round polypropylene flakes, which were further fixed to 6-well plates and sterilized with γ-rays (25 kGy, 48 h). BioOss granules prepared in the same way were used for comparison with ALBO-OS. Then, the optimal number of L929 cells (100,000 per well) was added. Further, plates were incubated for one, two and seven days in humidified atmosphere with 5% CO_2 at 37.1°C. The number of viable cells was estimated after physical lysis. The cells were lysed by alternate freezing and defrosting in three cycles, then centrifuged and LDH level was determined from the obtained supernatant. Supernatant aliquots of each well were transferred to a new 96-well plate and the LDH quantified using an enzymatic kit (Sigma Aldrich, St. Louis, MO, USA). Number of cells was determined by the level of LDH using previously constructed calibration curve. The optical density was read on microplate reader at 490 nM.

Statistical analysis

The results are presented as mean ± standard deviation (SD). The differences between groups (independent samples) were analyzed using One-way Analysis of Variance (ANOVA) and Student's t-test. Statistical analysis was performed using SPSS statistical software version 15.0 (SPSS, Chicago, Illinois). All p-values less than 0.05 were considered to be significant.

Phase contrast microscopy

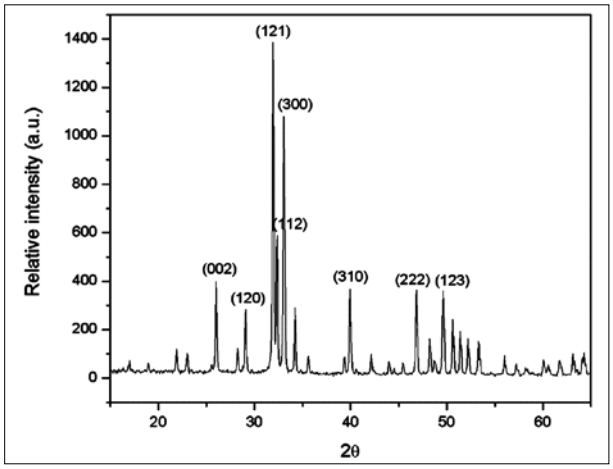
Cells were observed under phase contrast microscope (Leica Microsystems, Wetzlar, Germany) after contact with the extracts of ALBO-OS for 24, 48, and 72 h. Their morphology and the difference in cell density compared to controls (cells cultivated in culture medium, for 24, 48, and 72 h also) were evaluated [17, 18, 19]. The percentage of survived cells in ALBO-OS extracts was obtained by analysis of contrast phase microscopy images for each well in each experiment (15 images). The results are presented as mean percentage ± standard deviation of percentage for each group.

RESULTS

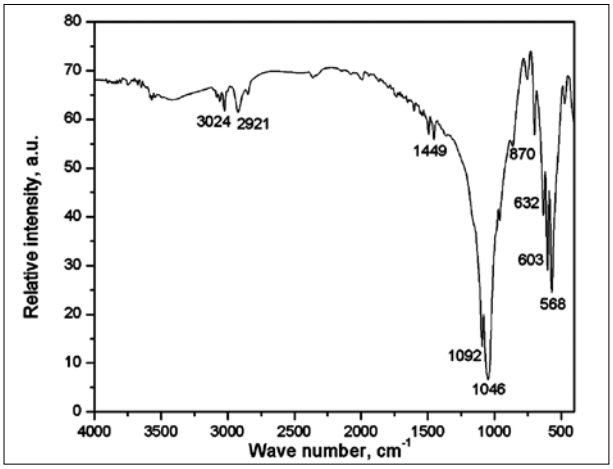
Scaffold granules characterization

The obtained XRD spectrum (Graph 1) showed that the phase composition of CHA scaffold granules corresponds to carbonate calcium hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (JCPDS 9–432). All characteristic diffraction peaks were present: (002) at $2\theta = 25.98°$, (120) at $2\theta = 29.03°$, (121) at $2\theta = 31.87°$, (300) at $2\theta = 33.07°$, (310) at $2\theta = 39.94°$, (222) at $2\theta = 46.82°$ and (123) at $2\theta = 49.54°$.

FTIR spectrum of CHA granules (Graph 2) showed the bands characteristic for CHA. The bands around 1,092, 1,046, 603, and 568 cm^{-1} correspond to PO_4^{3-} group. Vibrations of OH^- groups were detected at about 632 cm^{-1}. Vi-



Graph 1. XRD pattern of CHA scaffold granules



Graph 2. FTIR spectra of CHA scaffold granules

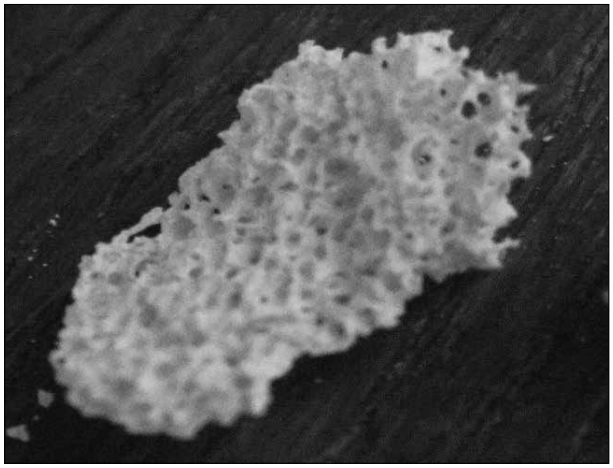


Figure 1. Enlarged photograph of the CHA compact

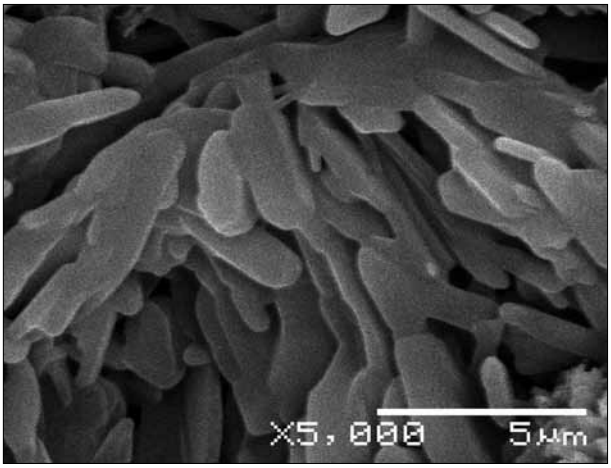


Figure 2. SEM: Typical appearance of the inner walls of CHA scaffold granules at 150,000× magnification

brations of CO_3^{2-} group at 1,449 and 870 cm^{-1} indicate the presence of carbonate groups in the apatite structure that partially replace OH^- ions. Based on this, it was identified that the carbonate apatite type B had been obtained, which is the most active form of carbonated apatite (type B predominates in the bones of young people, while the A type predominates in the bones of elderly people). The bands at 2,921 cm^{-1} and 3,024 cm^{-1} correspond to the ν(C-H) symmetric and asymmetric (respectively) stretching vibrations of intercalated PEVA/PEVV at the surface of CHA particles. A broad band between 3,150 cm^{-1} and 3,500 cm^{-1} can be attributed to the OH stretching vibrations, which belong to various hydroxyl groups present in the sample.

Porous CHA compacts had very porous 3D macrostructure (Figure 1). The pores were cylindrical and interconnected, with a diameter of 0.1–1 mm, while most of them have a diameter of 0.2–0.3 mm. The average scaffold porosity was 87 ± 8%. Scaffold granules obtained by scaffold disintegration were from 300 μm to 1 mm.

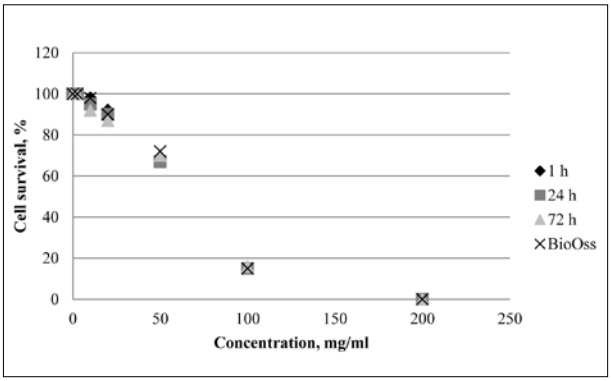
The structure of the inner walls of CHA scaffold granules is shown on SEM micrograph (Figure 2). A well-defined internal geometry could be observed with the elongated grains of a mean diameter 0.5–1.5 μm, and length 1.7–4 μm.

Investigations of the nano-structure of the scaffold walls, by mercury porosimetry, showed the presence of nano-pores with diameters of 30–400 nm, with prevailing pores in the range of 100–200 nm.

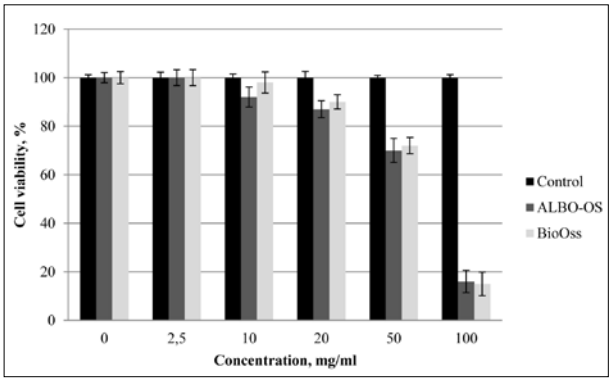
Indirect cytotoxicity

MTT test

The results of MTT test are presented as the percentage of viable L929 fibroblasts in the presence of ALBO-OS extracts at different concentrations (obtained after ALBO-OS degradation in the culture medium after one, 24, and 72 hours) compared to the controls, which represent a pure cell culture of L929 cells without ALBO-OS (Graph 3). Concerning cell survival relative to the concentration of the extract, we noticed the following: for concentration of 200 mg/ml (100% extract), the cytotoxic effect was total (the number of survived cells was negligible); for concentration of 100 mg/ml (two times diluted extract) survival rate was about 15%; for concentration of 50 mg/ml (four times diluted 100% extract) survival was 60–70%, and for concentrations up to 20 mg/ml, the percentage of survival was 80–100%, regardless of the contact time with the cell extract. Similar results were obtained for BioOss. BioOss extract was obtained after a 72-hour degradation in culture



Graph 3. Survival of cell culture of L929 mouse fibroblasts in the presence of ALBO-OS extract at different concentrations, for the extracts obtained after exposure of culture medium to ALBO-OS for one, 24, and 72 hours, and in the presence of the extract of BioOss; survival in the control group was 100%. Results are given as mean values of three independent experiments with five wells in each (n = 15). Standard deviations (SD) were between ± 5 (for higher concentrations) and ± 15 (for lower concentrations).



Graph 4. MTT viability assay of L929 mouse fibroblasts incubated with extracts of ALBO-OS and BioOss (obtained after exposure of culture medium to ALBO-OS/BioOss for 72 hours). Data are expressed as percentage over the control cells, considering the control group as 100% (cells without exposure to materials). Results are given as mean values of cell viability of three independent experiments with five wells in each ± SD (n = 15).

medium and the same concentrations were used as in the case of ALBO-OS.

As it is evident from Graph 3, cell survival was only slightly dependent on the contact time between the growth medium and the material, while it was highly dependent on the extract concentration.

In order to define the difference between ALBO-OS and BioOss more precisely, only results obtained in the presence of ALBO-OS and BioOss extracts obtained after 72 h were compared (Graph 4). Statistically significant difference between them was noticed only for concentrations of 10 and 20 mg/ml ($p < 0.05$).

Phase contrast microscopy

The morphology of cells that were in contact with extracts of ALBO-OS for 24, 48, or 72 h, were observed by phase contrast microscopy (Figures 3a, 3b, and 3c). An increase of cytotoxicity with the increase of time the medium and ALBO-OS are in contact could be observed (Figure 3a). Observing the contact of cells with the ALBO-OS extract obtained after one hour of contact with the medium, it can be seen that the cell morphology was similar while their density was lower than that in the control group (that was the case only in contact with the culture medium). When the extract obtained after a 24-hour contact was used for cell cultivation, cell density was also lower, compared to the control, and morphology was changing from elongated forms, typical for fibroblasts, to spheroidal form. In the case of extract obtained after 72 hours, cell number was significantly lower than in the control group and cells were spherical in shape.

From Figure 3b it is obvious that the cytotoxicity increased with the increase of contact time of the culture medium with ALBO-OS. Observing the cells cultured in the ALBO-OS extract obtained after one hour of contact with the medium, cell morphology was similar, while their density was lower than in the control group. In the case of the extracts obtained after 24 and 72 hours, cell density was significantly lower than in the control group, and the spherical morphology was observed. There was no significant difference between the cells that were in contact with the extract of ALBO-OS obtained after 24 and 72 h.

The cytotoxicity continued to grow with the increase of contact time between the culture medium and ALBO-OS (Figure 3c). In contact of the cells with the extract of ALBO-OS obtained after one hour, the cell morphology was similar, while their density was lower than in the control group. When the extract obtained after 24 and 72 hours were used, cell density was significantly lower than in the control group and the morphology was spherical. A significant difference was observed in the density of cells that were seeded in the extract of ALBO-OS obtained after 24 and 72 hours of contact with the culture medium.

It is evident from Graph 5 that the number of viable L929 cells slightly decreased when they were exposed to the products of ALBO-OS degradation over a period of 24, 48, and 72 hours, i.e. cytotoxicity slightly increased with the increase of contact time between the cells and the ALBO-OS extracts. The ratio between the density of survived cells in three different ALBO-OS extracts (obtained after one-, 24-, and 72-hour contact of ALBO-OS and medium) and control (expressed as percentage) showed mild decrease with the increase of contact time between ALBO-OS and medium (Graph 5).

Comparatively observing the presented results, the test material ALBO-OS showed quite similar results from the standpoint of cytotoxicity on L929 fibroblast culture as the Geistlich BioOss (Geistlich Pharma North America Inc., Princeton, NJ, USA) gold standard (Graph 3).

Direct Cytotoxicity

Cell proliferation, shown in Graph 6 as the number of cells adhered to the surface as a function of time, showed clearly that the number of cells grows during the entire period of ALBO-OS contact with the cells. The initial cell number in the well was 100,000. On the first day of the experiment

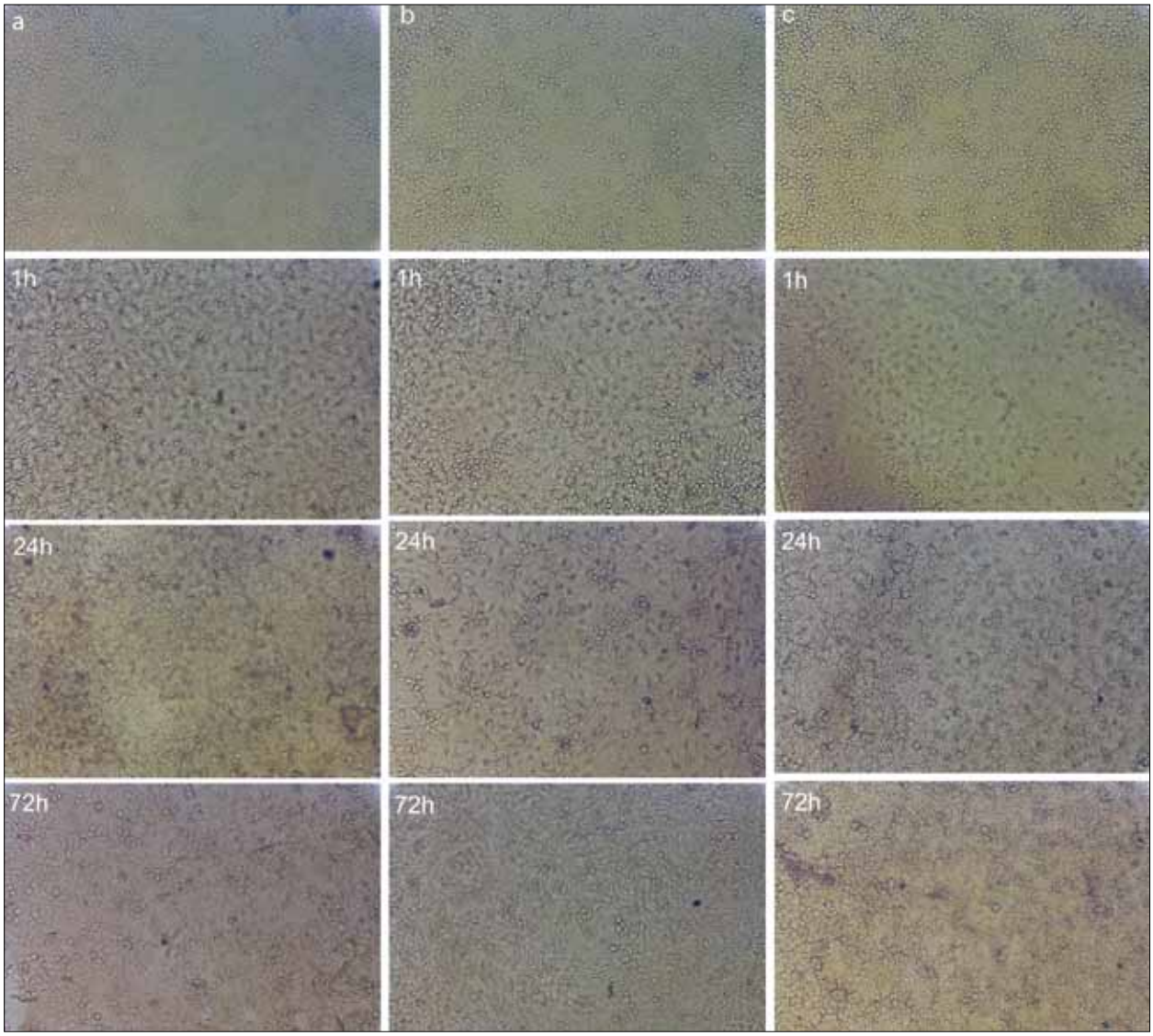
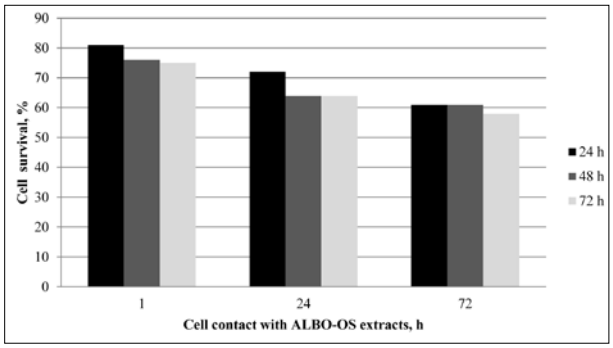
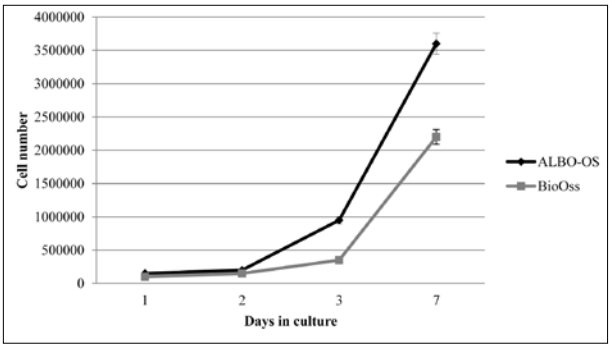


Figure 3. Phase contrast microscopy images of mouse fibroblasts L929 treated for: a) 24 h, b) 48 h, and c) 72 h, with extracts obtained after contact of culture media with ALBO-OS for one, 24 and 72 hours (magnification 100×)



Graph 5. Percentage of survived cells in ALBO-OS extracts (obtained after contact of medium and ALBO-OS for one, 24, and 72 hours) compared with survived cells in control, attained from contrast phase microscopy images obtained after 24-, 48-, and 72-hour contact with cells. For each sample 15 images were analyzed. SDs were between ± 5 and ± 10.



Graph 6. The number of L929 cells adhered on ALBO-OS and BioOss. Data are expressed as mean values ± SD of three independent experiments with five wells in each (n = 15).

the number of cells in the presence of ALBO-OS did not significantly increase, but from the second day of the experiment the cells were extensively multiplied and their number was growing exponentially, so that it was 3.6×10^6 cells per well on the seventh day. Obviously, ALBO-OS did not show cytotoxicity in direct contact with the L929 fibroblast cell culture because the number of cells grew steadily over the entire period of contact. Statistically significant difference between ALBO-OS and BioOss was noticed after two days ($p < 0.001$).

DISCUSSION

High average ALBO-OS scaffold porosity (nearly 87%) and its macrostructure, as it is shown in Figure 3a, mainly built from cylindrical and interconnected pores with a diameter of 200–300 μm, is very suitable for convenient adhesion of bone cells. The other very important element for cell adhesion and proliferation is very specific nanotopology of scaffold pore walls, with properly distributed hills and valleys, which enables the attachment of cell filopodia, as it is shown in Figure 3b.

Considering the results of MTT assay (Graph 3), it is evident that cell survival was highly dependent on the extract concentration, while it was just slightly dependent on the extraction time. The results obtained for ALBO-OS and BioOss for extraction time of 72 hours were very similar, with statistically insignificant difference. Similar results, for systems with PLGA alone or a component of a composite with CHA, were obtained in other investigations, showing the cytotoxicity increase with the extract concentration, caused mainly by the decrease in pH after PLGA degradation to PGA and PLA [20–23].

Phase contrast microscopy showed that the cytotoxicity of ALBO-OS slightly increased with the increase of contact time between cells and ALBO-OS extracts, while the increase of its cytotoxicity was more pronounced for longer extraction time when cell morphology became slightly changed (Figure 3 and Graph 5).

LDH assay showed that after the second day of the experiment the cells were already significantly multiplied, growing exponentially over the entire period of contact. This was also confirmed in previous investigations of the same material (ALBO-OS) on dental pulp stem cells, where LDH test showed favorable effect of the material on the cell proliferation [21]. The comparison of the number of cells grown on ALBO-OS and BioOss showed that in first two days, when cells were in the adaptation phase, the difference was not so significant although the number of cells grown on ALBO-OS was about 1.5 times higher. After three days, significant difference could be noticed (the number of cells on ALBO-OS was about 2.7 times higher than on BioOss), while after seven days of cultivation, the cell number on ALBO-OS was only 1.6 times higher. The difference in cell number on ALBO-OS and BioOss was particularly expressed after three days of cultivation. This can be assigned to shorter adaptation period of cells to ALBO-OS, due to its synthetic origin, while BioOss is of natural origin, containing remained collagen fibres, which induces corresponding immune response. The results for hydroxyapatite-PLGA composite scaffolds obtained in investigations of other researchers also showed the increase of cell number with contact time, but the data are not completely comparable because the increase of cell number on ALBO-OS was significantly faster [24, 25].

CONCLUSION

The paper describes synthesis of hydroxyapatite scaffold with surface modified using PLGA thin film, signed as ALBO-OS. The phase analysis of ALBO-OS was made by XRD and FTIR, while its geometrical structure and appearance of thin walls of porous body were investigated by SEM. The macroscopic structure showed a high degree of interconnected pores with a diameter of 0.1–1 mm. Specific morphology, shown by SEM, indicated that ALBO-OS probably has great potential as a synthetic bone substitute.

Biological investigations, using MTT and LDH tests and inverse phase microscopy, confirmed that this material is not cytotoxic for fibroblast cells L929. The MTT test showed similar results for ALBO-OS and Bio-Oss, while LDH test of cell viability showed significantly better results for ALBO-OS, probably due to its synthetic origin.

ACKNOWLEDGEMENT

This work is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Project No. 172026).

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Испитивање цитотоксичности носача на бази хидроксиапатита с унапређеним структурним дизајном

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КРАТАК САДРЖАЈ

Увод Порозни биодеградабилни носачи су се показали као добри заменици кости јер делују као привремена физичка потпора за усмерену регенерацију ткива док се читав носач потпуно не разгради и замени новим ткивом.

Циљ рада Циљ овог рада је био да се испита цитотоксичност носача ткива на бази калцијум-хидроксиапатита, високе порозности и оптималне топологије, названог *ALBO-OS*.

Методе рада Заменик кости *ALBO-OS* је синтетисан методом матрице направљене од полимерне пене. Геометрија пора и зидова носача анализиране су помоћу скенирајуће електронске микроскопије (СЕМ). Биолошка истраживања изведена су испитивањем цитотоксичности *ALBO-OS*-а на мишјим фибробластима *L*929 помоћу *MTT* и *LDH* тестова и фазно контрастне микроскопије.

Резултати СЕМ анализа је показала велику и равномерну порозност и занимљиву морфологију са биолошког становишта. Биолошка истраживања показала су да материјал није цитотоксичан. Поређењем материјала *ALBO-OS* и *Bio-Oss*, који је, глобално, златни стандард међу заменицима кости, добијени су слични резултати на *MTT* тесту, док су резулати *LDH* теста показали значајно већи број ћелијских деоба у контакту са *ALBO-OS*-ом.

Закључак Дизајн носача са становишта расподеле величине пора и топологије је веома погодан за адхезију и насељавање ћелија, због чега има велики потенцијал као заменик кости. Резултати биолошких тестова су показали да *ALBO-OS* није цитотоксичан за *L*929 фибробласте. У поређењу са материјалом *Bio-Oss*, добијени су слични или бољи резултати.

Кључне речи: хидроксиапатит; цитотоксичност; *MTT*; *LDH*

Примљен • Received: 22/06/2015 **Ревизија • Revision:** 22/10/15 **Прихваћен • Accepted:** 26/01/2016

Srp Arh Celok Lek. 2016 May-Jun;144(5-6): 288-292
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1606288V
UDC: 616.428-006-089.844

Tensor fascia lata flap is a workhorse for defects after inguinal lymph node block dissection

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SUMMARY

Introduction Enlarged inguinal lymph nodes very often present a site of metastatic disease. Inguinal lymph node block dissection is a demanding procedure, which usually requires at least one of reconstructive modalities. Among different reconstruction options we selected the tensor fascia lata (TFL) musculocutaneous flap.

Objective The paper aims at presenting a series of inguinal block dissections, followed by immediate reconstruction, using the TFL flap, and evaluation of tumor type, flap dimension, complication rate and the duration of hospital stay.

Methods We present a consecutive case series of 25 conducted block dissections. The defects were reconstructed using TFL flap, because of the extent and site of the tissue defects, reliability of the flap, and potentially primarily infected exulcerated tumors.

Results The reconstruction was successful in all cases, the incidence of surgical complications was 16%, no further complications, such as lymphedema or gait disturbances, were noted. Primary skin tumors were predominant (13 cases), followed by genitalia tumors (four cases). The male sex was more frequently affected (14 vs. 11 cases).

Conclusion Having in mind that TFL presents as a flap of adjustable size, length, shape, and volume, with negligible donor site morbidity, and after comparing of our results to those of other authors, we advise broader use of TFL flap. As a reliable flap, not too difficult to harvest, with a low complication rate, it must be taken into consideration regarding the benefits for the patient, and, on the other hand, the surgery cost and duration.

Keywords: inguinal block dissection; reconstruction; tensor fascia lata flap

INTRODUCTION

Enlarged inguinal lymph nodes could be a site of primary disease (infection, Hodgkin or non-Hodgkin lymphoma), but more often they represent the site of secondary (metastatic) disease. Primary malignancy can usually be found on genitalia, perineum, buttocks, lower abdominal wall, anus (below the pectinate line), the thigh and the leg. There are two groups of inguinal lymph nodes – superficial and deep [1]. The inguinal dissection in metastatic disease should be properly performed to achieve optimal local control and minimize recurrence rate [2, 3].

Routinely, lymph node dissection is performed under general anesthesia, and consists of ablation of superficial and deep lymph nodes of the groin. In cases of extranodal spread, with skin metastases, a skin excision should be additionally performed, and such surgical procedure is named block dissection. This surgery leads to excessive soft tissue coverage deficiency and exposure of vital structures. Those facts underline the need for immediate reconstruction, which is performed according to the general rule of the "reconstructive ladder." Most often, the direct closure cannot be achieved. Exposure of the femoral vessels and nerves exclude the use of skin grafts. The reliable choice for immediate reconstruction would be the use of local flaps such as tensor fascia lata (TFL) flap [4], inferior based rectus abdominis flap [5, 6], or anterior thigh flap [6]. Some authors even recommend the prophylactic use of TFL in cases of ilioinguinal dissection [7]. However, postoperative complications are frequently reported, such as distal flap necrosis, or even, in some cases, compartment syndrome. Also, a controversy exists on the flap's safe dimensions to prevent such complications [8].

OBJECTIVE

We present a consecutive case series including 25 patients with inguinal block dissection and immediate reconstruction using the TFL flap. We evaluated the tumor type, flap dimensions, complication rate and the duration of hospital stays.

METHODS

This study was performed in the Clinic for Plastic and Reconstructive Surgery, Clinical Center Niš, Serbia. Over the period of 24 months from March 2012 to the end of March 2014, 25 TFL flaps were used for reconstruc-

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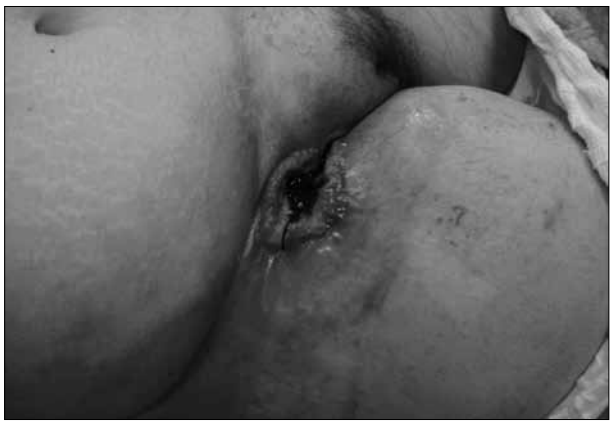

Figure 1. Metastatic disease of cervical cancer in the right groin

Figure 2. After block dissection, the tensor fascia lata flap was raised and donor site was closed primarily

tion of large groin defects following inguinal block dissection. The block dissection was accomplished by performing an excision of the skin affected by the metastatic disease (Figure 1), followed by ablation of underlying superficial and deep lymph nodes. All patients underwent primary reconstruction using TFL flap (Figure 2), and the active suction drain was routinely placed.

RESULTS

In our study we registered male predominance (14 vs. 11) and average age of the patients was 59.4 years. The primary site was the skin (squamous cell carcinoma or melanoma) in 13 cases, external genitalia in four cases, cervical (PVU) in three, large bowel in two cases. In three patients the location of the primary tumor was unknown.

All cases of block dissection also included the harvesting of the large saphenous vein. The defect size was between 12 × 20 cm and 15 × 25 cm. TFL flap was raised in retrograde manner and the flap size always achieved the defect requirements. Suction drain was in all cases removed on the fourth day. The complication rate and gender distribution is presented in Table 1.

The donor site was directly sutured in all the cases, with additional split-thickness skin grafting in five cases. There

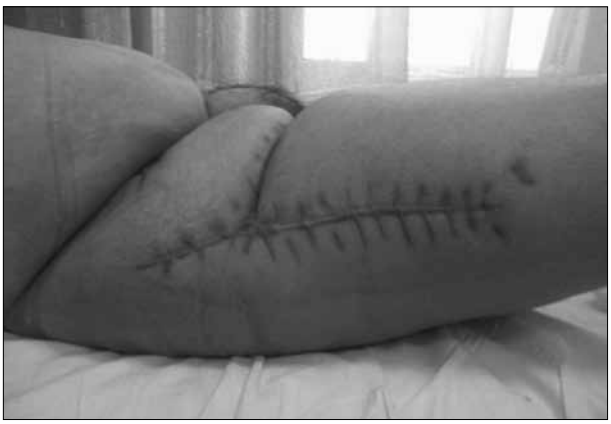

Figure 3. Postoperative result after three months

Table 1. Incidence of postoperative complications and gender distribution

Complication	Number of cases (n = 25)	
	Male patients % (n)	Female patients % (n)
Seroma/hematoma	4 (1)	4 (1)
Partial flap loss	4 (1)	0 (0)
Infection	0 (0)	0 (0)
Wound dehiscence	4 (1)	0 (0)
Cases without complications	44 (11)	40 (10)

were no significant donor site complications, apart from partial skin graft loss in one case.

Wound dehiscence and partial flap loss were secondary treated under local anesthesia.

Hospital stay was from six to 12 days, average being 10 days. After the wound healing we conducted a surgical primary follow-up (a two-month period) (Figure 3).

DISCUSSION

Reconstruction of large tissue defects has to be vigorously planned. There are several options to obtain the tissue continuum. According to the reconstructive ladder, the simplest choice would be the direct closure of the wound. The next step should be the reconstruction using split-thickness or full-thickness skin grafts. Because of the extent of the surgical procedure, and also the exposure of vital structures and postoperative treatment, these techniques could not have been used. The reconstruction was conducted by using the pedicled TFL flaps.

A variety of muscle and skin flaps have been described for the reconstruction of large groin defects, e.g. sartorius, rectus abdominis, rectus femoris, gracilis, abdominal skin flaps and TFL flap [9]. Potential disadvantages, as mentioned in the literature, would be the following: abdominal weakness, bulging or hernia (the use of rectus abdominis muscle flap) [10], lateral thigh paresthesia (the use of anterior thigh flap) [11], significant knee weakness (rectus femoris muscle flap) [12], large defect of the donor site and excessive bulkiness on the recipient site (use of muscular flaps in general) [12, 13, 14]. The consensus which flap represents the best suitable choice does not exist;

nevertheless, the use of local instead of free flaps, if possible, remains justified [15].

Tensor fascia lata flap is a myocutaneous flap, and as such many authors suggest it for coverage of large groin defects. It is based on the ascending branch of the lateral circumflex femoral artery, branch of the profunda femoris artery. The TFL muscle is a thin, flat muscle, with a single dominant vascular pedicle (Type I flap by Kormack Lamberty). The flap showed great success with relatively low donor site morbidity, compared to other flaps [13, 14, 16]. The advantages of the TFL flap would be the following: the involvement of well-vascularized tissue composed of thin sensate skin, thin subcutaneous tissue, and muscle, including large amount of durable fascia; long arch of rotation, and broad coverage area of up to 600 cm^2. The flap can be designed into the desired shape and volume, and it leaves very little functional deficiency. The muscle tissue, among others, possesses an important potential to aid the infection eradication. Thus, the TFL flap can also be used as an aid in handling of various infectious conditions, such as the exposure of osteomyelitic focus or infected prosthetic vascular graft [17].

The TFL flap-raising is not very demanding and the early postoperative radiotherapy can be promptly started as healing is fast, and hospital stay is not too long.

Some authors suggest the preservation of the great saphenous vein during the superficial groin dissection. This modification was retrospectively reviewed for metastatic vulvar cancer. A significantly reduced rate of cellulitis [18], wound dehiscence, and chronic lymph edema was found, and among other things it was shown that ligation of great saphenous vein did not significantly impact the development of mentioned complications, including the limb lymphedema formation, even on long-term follow-up [19]. In terms of vascular anatomy, as suggested by other authors, the vertical incision of the region should be placed 2 cm distal to the inguinal ligament to maintain the vascular supply, when possible, in order to prevent the tissue devascularization and consequent tissue necrosis [20].

Because of the oncological rule, great saphenous vein was harvested in all 25 cases in our study, but we did not register any significant lymphedema.

There are several possible complications expected after inguinal block dissection followed by primary TFL flap reconstruction. Partial flap loss, seroma/hematoma formation, infection or dehiscence could be expected. In our study there were two cases of seroma/hematoma formation, one case of partial flap loss, and one case of wound dehiscence. There was no clinically detectable functional morbidity like knee instability or gait disturbances in any of the cases in this study.

There are several predictors in terms of postoperative complications. The total number of removed lymph nodes presents the individual predicting factor for any complication, whereas the predicting factors for wound infection are AJCC stage, age, inguinal lymph node dissection followed by sartorius flap reconstruction, or surgery before 2008 [21]. Other authors presented to a certain extent similar findings: the direct association between the risk of grade 2 or higher (Clavien–Dindo classification) complications' occurrence, and body mass index, sartorius muscle transposition, and bilateral dissection [22].

The complications' rate is slightly lower comparing to other studies in which the TFL flap was used, probably because of the limitations of the study in terms of the number of cases and absence of preoperative tumor infections. In the literature diverse complications' rates were mentioned: partial flap necrosis of TFL flaps (0–16%), seroma formation (around 0–15%), wound dehiscence (up to 30%); infection rate ranged in some studies from somewhat similar to our results up to 24% of all cases. Hospital stay ranged from 10 to 16 days [22–26].

The opinion on use of surgical adhesives remains rather open. In some cases, for using one particular adhesive, as reported, a reduction of postoperative wound related complications, and thus the reduction of need for revision surgery, was clearly noted, whilst using another adhesive was, despite the initial promising results, slightly unsatisfactory [27].

The TFL flap presents a trustworthy and resourceful reconstruction option, which is undoubtedly less time-consuming, specifically for reconstruction of regions such as around the trochanter major, groin, lower abdomen, perineum, and around the ischial bone [28]. The use of free flaps in certain cases is clearly justified, particularly when harvesting local flaps is not possible. However, the vascular anastomosis is always at risk of thrombosis, especially in malignancy patients. The anastomosis is usually within the field of radiotherapy. In general, free flaps are more complicated for harvesting, operations last longer, and success of the surgery can be uncertain.

CONCLUSION

Presenting as a flap of adjustable size, length, shape and volume, with negligible donor site morbidity, and after comparing of our results to those of other authors, we advise the broader use of the TFL flap. Inguinal block dissection is the standard treatment of malignant deposits in the inguinal region involving skin. Wide local excision demands reconstruction according to the principles of plastic surgery. Tensor fascia lata local flap based on a single known vascular pedicle is a reliable flap, not too difficult to harvest, with a low complication rate, which must be taken into consideration regarding the benefits for the patient on the one hand and, on the other, the surgery cost and duration, as well as hospital stay costs.

doi: 10.2298/SARH1606288V

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Режањ тензора фасције лате је решење за ткивне дефекте након ингвиналне блок дисекције

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Увод Увећани лимфни чворови често представљају место метастатске болести. Ингвинална блок дисекција је захтеван захват, након којег је најчешће неопходан бар један од реконструктивних модалитета. За реконструкцију дефекта одабран је тензор фасција лата мишићнокожни режањ.
Циљ рада Циљ рада је приказ серије ингвиналних блок дисекција праћених непосредном реконструкцијом режњем тензора фасције лате, као и процена типа тумора, величине режњева, броја и врсте компликација, као и дужине болничког лечења.
Методе рада Представљена је серија од 25 случајева блок дисекције. Дефекти су реконструисани режњем тензора фасције лате, који је одабран због величине и локализације дефекта, поузданости режња, као и због постојања потенцијално примарно инфицираних егзулцерисаних тумора.
Резултати Реконструкција је спроведена успешно код свих лечених пацијената. Инциденца хируршких компликација износила је 16%. Одложене компликације попут лимфедема или поремећаја ослонца нису забележене. Примарни кожни тумори су били најчешћи (13 случајева), праћени туморима гениталија (четири случаја), доминантно се радило о мушким пацијентима (14 вс. 11).
Закључак Као режањ прилагодљиве величине, дужине, облика и запремине, са занемарљивим морбидитетом на донорском месту, и након поређења са резултатима других аутора, препоручујемо чешћу употребу режња тензора фасције лате. Овај поуздани режањ, не превише компликован за препарацију, мора бити размотрен, узимајући у обзир корист за пацијента, као и, са друге стране, трошкове и трајање саме операције.
Кључне речи: ингвинална блок дисекција; реконструкција; режањ тензора фасције лате

Примљен • Received: 03/03/2015 **Ревизија • Revision:** 15/02/16 **Прихваћен • Accepted:** 07/04/2016

Results of open tibial fracture treatment using external fixation

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SUMMARY

Introduction Open lower leg fractures are the most common open fractures of the locomotor system and their treatment is associated with a number of complications.

Objective The aim of the paper was to present the results of the treatment of 68 patients with open lower leg fractures, as well as the complications that accompany the treatment of these fractures.

Methods In the analyzed group, there were 45 (66.18%) men and 23 (33.82%) women. The majority of patients – 33 (48.53%) of them – were injured in motor vehicle accidents, whereas 24 (35.29%) patients sustained injuries due to falls from heights. In two (2.94%) patients the cause of open tibial fractures was gunshot injuries. In the analyzed group, there were 18 (26.47%) type I open fractures, 21 (30.88%) type II open fractures, 19 (27.94%) type IIIA open fractures, seven (10.29%) type IIIB open fractures, and three (4.41%) type IIIC open fractures.

Results The tibial shaft fracture healed without serious complications in 50 (73.53%) patients, whereas in 18 (26.47%) patients we observed some complications. Nonunion was found in 10 (14.71%) patients, osteitis in four (5.88), malunion in two (2.94%) patients. Milder complications such as soft tissue pin tract infection developed in 13 (19.12%) patients, infection of the open fracture wound soft tissue was observed in four (5.88%) patients.

Conclusion Basic principles in the treatment of open lower leg fractures in this study are thorough primary open fracture wound treatment followed by the delayed wound closure, stable fracture fixation using unilateral external skeletal device, proper antibiotic treatment and tetanus prophylaxis. The results correlate with similar studies.

Keywords: open lower leg fractures; treatment; external skeletal fixation; postoperative complications

INTRODUCTION

Open lower leg fractures are the most common open fractures and account for 63% of all open fractures of the locomotor system [1]. As high energy traumas, they are usually the result of a motor vehicle accident [2].

Treatment of open lower leg fractures involves the primary management of an open fracture wound, fracture fixation, antibiotic therapy, tetanus prophylaxis, and delayed wound closure. One of the most important procedures in fighting infection is the primary surgical management of the open fracture wound, followed by the removal of avital tissues from the wound, i.e. wound debridement. Primary management of the open fracture wound is an important factor for the prevention of both aerobic and anaerobic infections (osteitis, gas gangrene, and tetanus) [3]. Treatment of open lower leg fractures includes a number of complications, such as infection of the open fracture wound, deep bone infection (osteitis), delayed healing, malunion, nonunion, and loss of extremity. The aim of the treatment of open tibial fractures was to ensure healing and promote restoration of the injured extremity function, which enables patients to return to their work activities and daily routines [4].

In modern traumatology, there is still a debate on the choice of the open tibial fracture fixation method and surgical management of damaged sheaths of the lower leg soft tissue [5, 6].

OBJECTIVE

The aim of the paper was to present the results of the treatment of 68 patients with open lower leg fractures using external skeletal fixation. The patients were treated during a four-year period at the Clinic of Orthopedics and Traumatology, Clinical Center Niš, Serbia.

METHODS

The results of the treatment of 68 patients with open lower leg fractures have been analyzed. The patients were treated at the Clinic of Orthopedics and Traumatology, Clinical Center Niš, from January 1, 2005, until January 1,

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2009. After surgical care, the patients were followed up for 16–24 months.

In the analyzed group there were 45 (66.18%) men and 23 (33.82%) women. The youngest patient was 14, and the oldest 82 years of age. The mean age of patients was 46.7 years.

Analyzing the etiology of injury, we found that the majority of patients – 33 (48.53%) of them – were injured in motor vehicle accidents, whereas 24 (35.29%) patients sustained injuries due to falls from heights. In the analyzed group, seven (10.29%) patients sustained agricultural injuries (timber falls, tractor overturns, etc.), two (2.94%) patients sustained sports injuries, whereas two (2.94%) sustained gunshot injuries.

For the staging of open fractures, Gustilo classification, introduced in 1976 and subsequently modified in 1984, was used [7].

In the analyzed group, there were 18 (26.47%) type I open fractures, 21 (30.88%) type II open fractures, 19 (27.94%) type IIIA open fractures, seven (10.29%) type IIIB open fractures, and three (4.41%) type IIIC open fractures.

With regard to the type of injury, an isolated injury of the tibial shaft was found in 38 (55.88%) patients, whereas 30 (43.22%) patients had multiple injuries, including the open lower leg injury.

The treatment of patients with open tibial fractures included profuse irrigation and primary management of the open fracture wound, followed by the reposition of bone fragments and external skeletal fixation using the Mitkovic external skeletal fixator, an original unilateral device (Figures 1–5). The open fracture wound was left open and delayed wound closure was performed (by primary wound closure, secondary closure, or some of the plastic surgery methods used for the management of soft tissue defects) (Figure 6). Primary amputation was performed in two patients, one of whom had a comminuted fracture as he had been run over by a truck, whereas forefoot amputation was performed in another patient previously injured by a roller. Tibial shaft fracture was treated by external skeletal fixation.

In patients with open tibial fractures, antibiotic therapy was administered. Regularly, the combination of the 3rd- and 4th-generation cephalosporins and aminoglycoside (amikacin) was administrated as a first step. In type I and II open fractures, antibiotic therapy is continued 48–76 hours after sustaining an injury, and in type III fractures it can be administered up to 120 hours after sustaining an injury and performing primary debridement [8]. Anti-tetanus prophylaxis is given to all patients with open fracture, according to the protocol.

In heavily contaminated wounds, especially with soil, there is a risk of serious infection caused by anaerobic bacteria, the cause of gas gangrene. In these cases, besides the regular antibiotics combination, metronidazole and clindamycin were administered.

Figure 1. An open fracture in the distal lower leg (A) and extensive soft tissue injury (B)

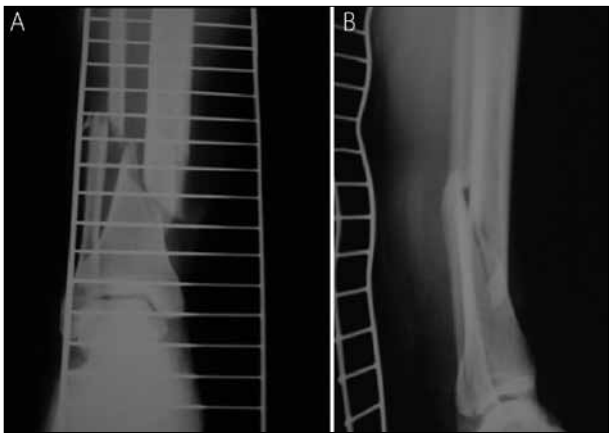

Figure 2. The antero-posterior (A) and lateral (B) X-rays demonstrate spiral fractures in the distal tibial and fibular shaft with dislocation of fragments

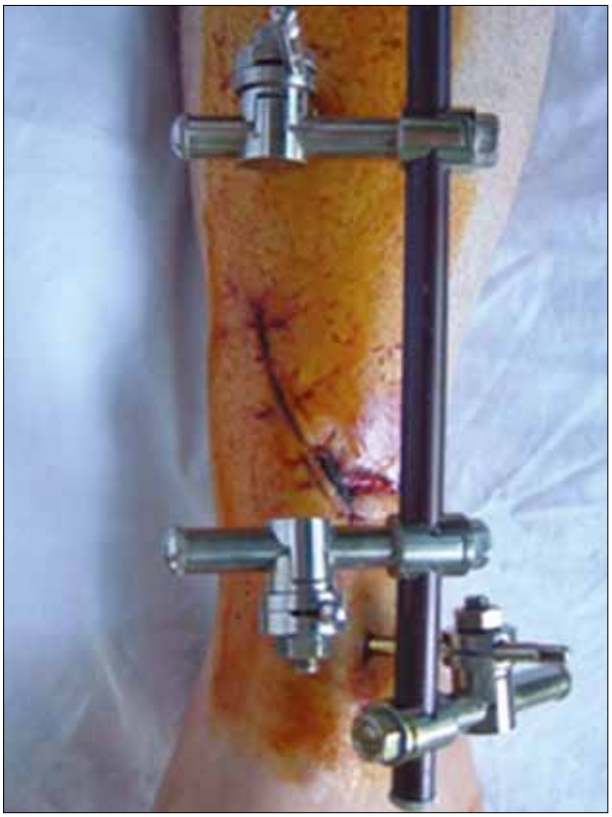

Figure 3. Upon admission, after short preoperative preparation, the following procedures were performed: operative treatment, management of the open fracture wound, reposition of fragments, and fracture stabilization using an external skeletal fixator. The open fracture wound was closed by secondary suture. Part of the wound which could not be sutured was left open to heal spontaneously.

doi: 10.2298/SARH1606293G

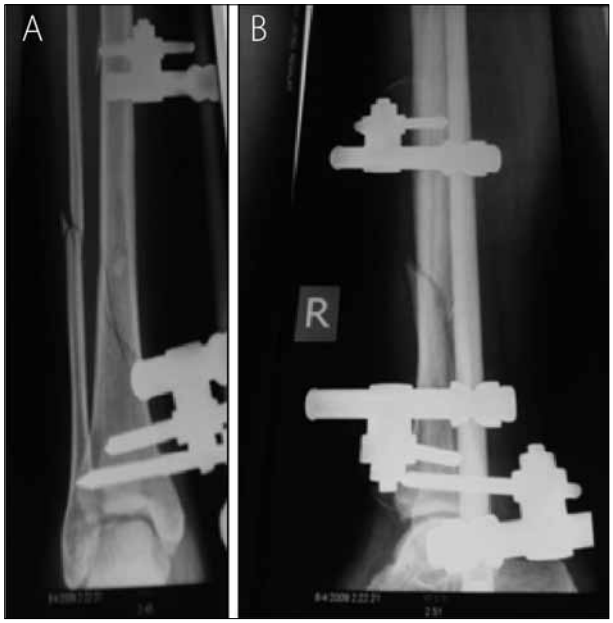

Figure 4. The antero-posterior (A) and lateral (B) X-rays show well-positioned and stabilized fractures of both lower leg bones with external skeletal fixation of tibia

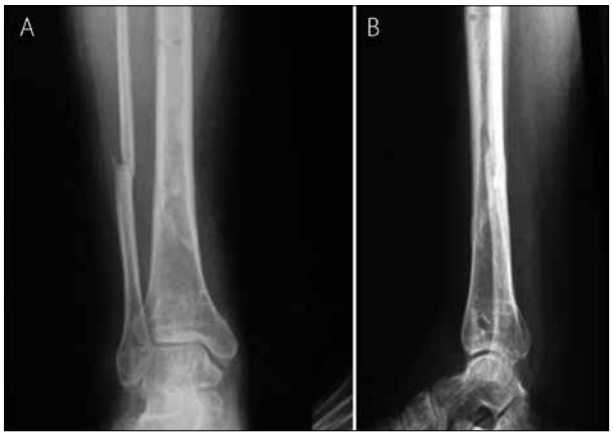

Figure 5. Four months after sustaining an open fracture and undergoing surgical treatment, the antero-posterior (A) and lateral (B) X-rays show the healed fracture of the tibial shaft

Externa skeletal device was removed after complete fracture healing was achieved. Weight bearing was applied depending of radiological signs of bone healing and physiotherapy was promoted as soon as possible (Figures 5 and 7).

The following postoperative complications were followed up: soft tissue infection of the open fracture wound, pin site infection, chronic osteitis, nonunion, malunion, and limb amputation.

RESULTS

Analysis of the treatment of open lower leg fractures using primary wound management and external fixation has demonstrated that tibial shaft fracture healed without serious complications in 50 (73.53%) patients, whereas in 18 (26.47%) patients we observed the complications which required additional surgical care.

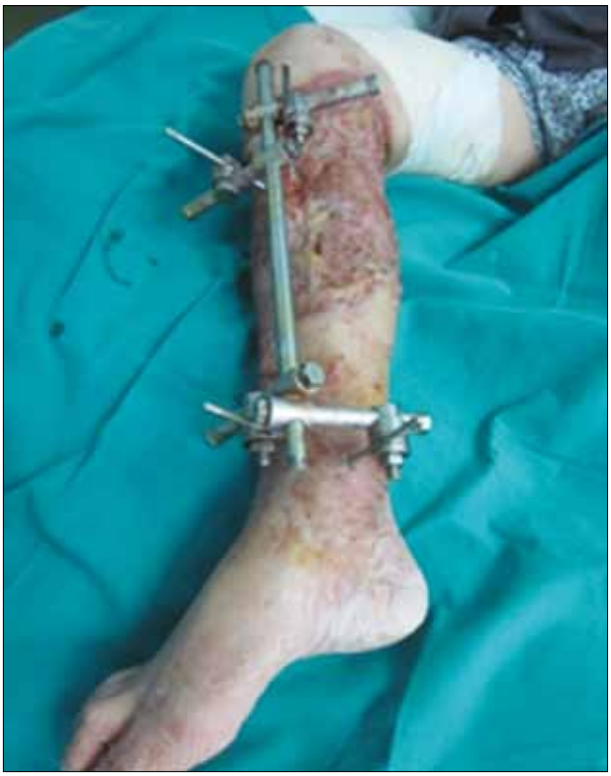

Figure 6. Good early result of the soft tissue defect coverage using free Thiersch's skin graft taken from the thigh of the same leg

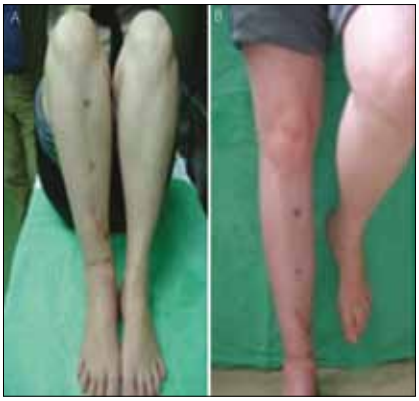

Figure 7. The status of the leg after physical therapy; there is a good range of motion in the knee and ankle joints (A); the leg that was operated on is stable, and the patient is fully weight bearing (B).

Average healing time of open lower leg fractures was 22 weeks.

Nonunion (both septic and aseptic pseudoarthrosis) was found in 10 (14.71%) patients, osteitis in four (5.88%), malunion in two (2.94%) patients; amputation was performed in one (1.47%) patient. For the treatment of pseudoarthrosis, the Ilizarov apparatus was applied in four (5.88%) patients, whereas the Mitković compression-distraction device was utilized in six (8.82%) patients.

In two (2.94%) patients, the cause of malunion was pin loosening. In one patient, the correction of angular deformity was done, with good functional result; however, the second patient didn't want to undergo the correction of the deformity.

Three patients with type IIIC open fractures were studied. Lower leg amputation was performed in one patient

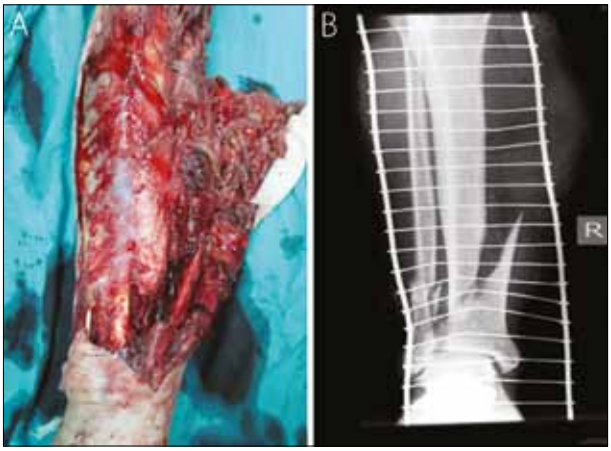

Figure 8. Patient aged 74 years was injured while tilling his garden, when he sustained a severe comminuted fracture of the right lower leg, Gustilo type IIIC (A). The X- ray of the right lower leg shows comminuted fractures of tibia and fibula in the distal lower leg, with dislocation of bone fragments (B).

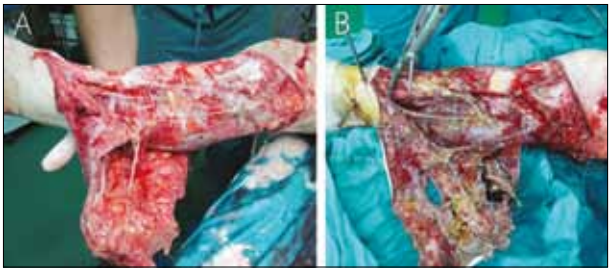

Figure 9. After short preoperative preparation, profuse irrigation of the open fracture wound was done, and all foreign bodies were removed (A). After the reposition of the fracture, external fixator pins were placed first in the distal fragment in the convergent fashion to achieve good stability of the fragment (B).

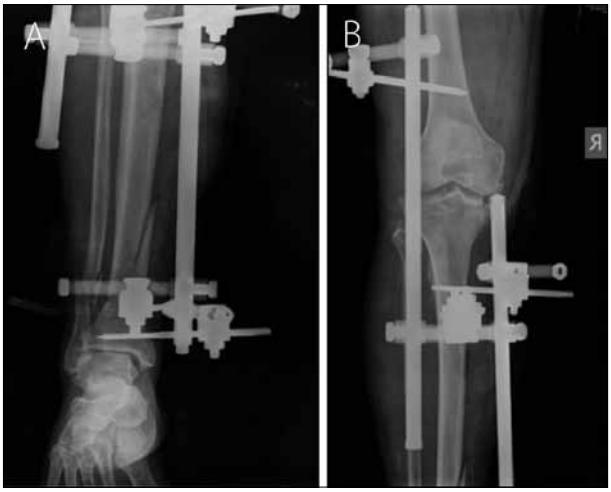

Figure 10. (A) The X-ray demonstrates the distal fracture of the lower leg, after the reposition and fixation using external skeletal fixation; (B) proximal extension of the external fixation device was applied for knee joint bridging to stabilize the tibial medial condyle fracture by ligaments distraction.

who had been run over by a truck and sustained severe type IIIC fracture. Another patient of the group, who had been injured by a roller, sustained concomitant crush injury of the forefoot and amputation was performed. Open tibial fracture of the same lower leg was treated with external fixation. Tear of posterior tibial artery was closed with end-to-end sutures. However, this leg remained shorter

Figure 11. State of the lower leg after primary management of the open fracture wound and external skeletal fixation. Soft tissue defect affects two thirds of the anterior lower leg.

by 2.5 cm. Third patient with the IIIC fracture needed comprehensive treatment including direct suturing of the anterior tibial artery laceration and soft tissue coverage procedures (Figures 8–11).

Milder complications such as soft tissue pin tract infection developed in 13 (19.12%) patients; infection of the open fracture wound was observed in four (5.88%) patients. The treatment of soft tissue pin tract infection included daily dressing of the wound and antibiotic therapy, whereas the management of the infection of the open fracture wound required daily wound dressing, additional surgical wound care and antibiogram-based therapy.

Distribution of the complications in relation to Gustilo classification of open fractures is shown in Table 1. The presence of complications was similar in type I and II fractures. In type III group, nonunion was found in almost one third (27.6%) and pin site infection in 37.9% of patients.

Osteitis developed in three type III fractures (10.3%). In type IIIA, this complication was found in one patient, and in two patients with type IIIB fracture, which is almost one third of the group (28.6%).

Both patients in group IIIC with preserved legs sustained nonunion, which makes 66.5% of the whole group, but in fact it is 100%, because the third patient's leg was primarily amputated (Table 1).

DISCUSSION

The treatment of open lower leg fractures should be considered an emergency, and it includes the following procedures: thorough wound irrigation, wound foreign body removal, primary treatment of the open fracture wound, fracture stabilization, antibiotic therapy, tetanus prophylaxis, and delayed wound closure. Primary treatment of the open fracture wound should be done as soon as possible after injury (certainly within six hours), as rapid development of microorganisms will contaminate the open fracture wound. Primary surgical care of the open fracture wound is one of the most important steps in the fight against infections, both aerobic and anaerobic (gas gangrene and tetanus). It includes the removal of damaged

doi: 10.2298/SARH1606293G

Table 1. The presence of complications in relation to the type of fracture by Gustilo classification.

Gustilo type	n	Nonunion	Malunion	Osteitis	Wound infection	Pin site infection
I	18	1 (5.5%)	1 (5.5%)	-	-	1 (5.5%)
II	21	1 (4.8%)	-	1 (4.8%)	-	1 (4.8%)
III	29	8 (27.6%)	1 (3.4%)	3 (10.3%)	4 (13.8%)	11 (37.9%)
IIIA	19	2 (10.5%)	1 (5.3%)	1 (5.3%)	2 (10.6%)	5 (26.3%)
IIIB	7	4 (57.1%)	-	2 (28.6%)	2 (28.6%)	4 (57.1%)
IIIC	3	2 (66.7%)*	-	-	-	2 (66.7%)
Total	68	10 (14.71%)	2 (2.94%)	4 (5.88%)	4 (5.88%)	13 (19.12%)

*There was one amputation as a primary treatment of IIIC fracture

skin, subcutaneous fat, fascia, and muscles, as well as removal of small periosteum bone fragments. Debridement of the open fracture wound can be repeated within 24 or 48 hours, and the aim is to remove the necrotic tissue. The debridement is followed by fragment reposition and external skeletal fixation [8].

External skeletal fixation is a standard method for the stabilization of all open lower leg fractures except for type I open fractures, when internal fixation can be applied as well. External skeletal fixation provides good biomechanical conditions for the management of open lower leg fractures, enables good approach and care of the wound, and doesn't disturb the movements of the knee and ankle joints [9].

The problems frequently encountered in external skeletal fixation are soft tissue and bone infections around the external fixator pins, especially when patients wear the device longer than six months [10, 11]. In the series of 171 open fractures treated by external skeletal fixation, Edwards et al. [10] registered 50 (29.24%) soft tissue pin tract infections and four (2.33%) cases of local osteitis developed around the pins. In the series of 101 open tibial fractures treated with external fixation, Marsh et al. [11] observed 39 (38.61%) complications related to pins, 10 of which had to be replaced, and emphasized a low percentage (6%) of deep bone infection at the fracture site.

Naveed et al. [12] reported results of treatment of 60 patients with types II, IIIA and IIIB open fractures, who were treated with primary management of open fracture wound and external fixation. Three to five days after the use of skeletal fixation, the external fixator was removed and final fixation using the Ilizarov apparatus was performed. In patients with type II open fracture and the majority of patients with type IIIA open fracture, delayed wound closure was done. Healing was observed in all the patients. Mean time of fracture healing was 22.24 weeks. The most common complication was pin site infection. Therapy outcome was estimated minimum one year after the exclusion of all mobility aids, applying the Tucker criteria. Forty-eight (80%) patients had excellent results, 10 (16.7%) patients had good results, whereas satisfactory results were observed in two (3.3%) patients. None of the treated patients had poor results [12].

Soni et al. [13] reported their results using Gustilo classification. In a 15-year period, 18 patients with type IIIC open fractures were followed up. There were 15 men and three women in the analyzed group. Mean age was 30.7 years and mean Mangled Extremity Severity Score was 6.9 (3–10). In total, 15 limbs were salvaged, whereas three were amputated (two primary and one delayed amputation). In four patients, fractures were stabilized using external skeletal fixation; internal fixation was applied in 12 patients. Wound infection was observed in seven, and nonunion in four patients, which required further surgical care. Delayed healing was more commonly observed in the distal tibia fractures when they were associated with the posterior tibial artery lesion. After completion of treatment, 39% of patients could not return to their job [13].

In the reference literature, there are still controversial data related to the treatment of patients with open lower leg fractures associated with the major blood vessel injuries. However, the studies published in the last two decades have shown that salvaged limb provides better quality of life and lower treatment costs in spite of additional surgical care when compared to amputation. A long-term goal of the treatment of the open tibial fracture associated with major blood vessel injuries is to enable patients to return to their daily activities and professional work [14, 15].

In the current traumatology, the primary intramedullary fixation of type I, II, and IIIA open fractures, with proper wound debridement, is gaining popularity [16]. The role of intramedullary fixation in the treatment of type IIIB open fractures is still controversial. Intramedullary fixation in type IIIB open fractures is associated with higher percent of infection and nonunion. Joshi et al. [17] observed infection in 10.7% of cases after the use of intramedullary fixation for the treatment of open fractures, in spite of a thorough debridement and adequate coverage by soft tissues.

An adequate alternative method for the treatment of severe open fractures is a delayed intramedullary fixation after the external skeletal fixation. However, the intramedullary fixation, after the application of the external skeletal fixation of an open tibial fracture, can be associated with increased percentage of infection if the pin site infection was present [18, 19].

Nonunions – both septic and aseptic pseudoarthroses – were observed in 10 (14.71%) patients of the analyzed groups. Papaioannou et al. [20] reported a 10% nonunion rate of type II and III open fractures using the external fixation method. They found that the major problems in the treatment of open lower leg fractures with external fixation, besides nonunion, are pin site infection and malunion.

Golubović et al. [8] published in 2008 results of the delayed wound closure. After surgical care and external skeletal fixation, the open fracture wound wasn't primarily closed, but left open. It was closed when there were

no signs of infection, using a delayed, secondary closure or some of the plastic surgery methods (fasciocutaneous, microvascular flap), which depends on soft tissue defects.

The treatment of type IIIB open tibial fracture is a major challenge and it needs aggressive debridement, adequate fixation, and early flap coverage of soft tissue defect. The flaps could be either nonmicrovascular, which are technically less demanding, or microvascular, which demand steep learning curve and are available in only a few centers. Kamath et al. [21] concluded that open fracture of the tibia which needs flap coverage should be treated with high priority of radical early debridement, rigid fixation, and early flap coverage. The study included 151 cases of Gustilo Anderson type IIIB open tibial fractures which needed flap coverage for soft tissue injury. Ninety-four cases were treated in the acute stage by debridement; fracture fixation and early flap coverage were performed within 10 days. Thirty-eight cases were treated between 10 days to six weeks in the subacute stage. The remaining 19 cases were treated in the chronic stage after six weeks. The soft tissue defect was treated with various nonmicrovascular flaps depending on the location of the defect. A majority of these wounds can be satisfactorily covered with local or regional microvascular flaps.

Franken et al. [22] recommend that all patients with large soft-tissue defects of the lower leg after an open tibial fracture should be initially treated with a local, musculocutaneous flap whenever possible. If the location or size of the defect makes local reconstruction impossible, free flaps remain the only possibility for reconstruction.

Early intravenous antibiotic therapy should be started immediately after sustaining an injury [23]. Right after the admission of a patient with an open lower leg fracture, benzylpenicillin is administered intravenously in the dose of 4,000,000–6,000,000 i.u. per four hours through intravenous infusion, together with an aminoglycoside (amp. amikacin 1 g/24 h). If the wound is contaminated with soil, than, in addition to the aforementioned antibiotics, metronidazole and clindamycin should be included to prevent the occurrence of gas gangrene. This therapy is administered in duration of three days, when benzylpenicillin is replaced with a third- or fourth-generation cephalosporin. Cefazolin, which covers gram-positive bacteria, should be given for all open fractures. Aminoglycosides, which cover gram-negative bacteria, are obligatory in cases of all open fractures with extensive soft tissue injuries and contamination. As penicillin covers the anaerobes, it is indispensable in cases where there is a risk of wound contamination by these organisms (e.g. *Clostridium perfrigens*), which is common in agricultural injuries when wound is contaminated with soil. If benzylpenicillin is not available, a third- or fourth-generation cephalosporin should be administered. In this study, cephalosporins of third generation in combination with aminoglycosides were used as a first step in all patients. The duration of the treatment depended on the type of the fracture and injury circumstances.

CONCLUSION

Treatment of lower leg fractures includes profuse wound irrigation, removal of all foreign bodies, debridement of avital tissues, fracture stabilization using the external skeletal fixation, early reconstruction of soft tissue defects, antibiotic and tetanus prophylaxis, and physical therapy. Open lower leg fractures are associated with a number of complications, the most important of which are pseudoarthrosis (both septic and aseptic) and osteitis. In the analyzed group of open fractures, there were 10 (14.71%) patients with pseudoarthrosis of the tibia and four (5.88%) patients with osteitis. Abiding by the basic principles of the treatment of open lower leg fractures provides limb salvage and good functional result of the injured limb.

ACKNOWLEDGEMENT

This paper is part of the project No. III 41017, "Virtual Human Osteoarticular System and its Application in Preclinical and Clinical Practice," funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Резултати лечења отворених прелома потколенице спољном скелетном фиксацијом

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КРАТАК САДРЖАЈ

Увод Отворени преломи потколенице спадају у групу најчешћих отворених прелома локомоторног система и њихово лечење прати читав низ компликација.

Циљ рада Циљ рада је био да се прикажу резултати лечења 68 болесника са отвореним преломом потколенице, као и компликације које прате лечење ових прелома.

Методе рада У анализираној групи било је 45 (66,18%) мушкараца и 23 (33,82%) жене. Највећи број испитаника 33 (48,53%) повређен је у саобраћајној несрећи, док је 24 (35,29%) повређено при паду. Код два (2,94%) испитаника узрок отвореног прелома потколенице било је стрелно рањавање. У анализираној групи било је 18 (26,47%) отворених прелома I степена, 21 (30,88%) II степена, 19 (27,94%) III*A* степена, седам (10,29%) III*B* степена и три (4,41%) III*C* степена.

Резултати Преломи потколенице су срасли без тежих компликација код 50 (73,53%) испитаника, док су код 18 (26,47%) испитаника регистроване компликације. Несрастање прелома регистровано је код десет (14,71%) испитаника, остеитис код четири (5,88%), зарастање прелома у лошој позицији код два (2,94%) испитаника. Лакше компликације, мекотивна инфекција око клинова спољног скелетног фиксатора развила се код 13 (19,12%) испитаника, а инфекција меких ткива у пределу ране отвореног прелома код четири (5,88%) испитаника.

Закључак Опсежна примарна хируршка обрада ране отвореног прелома потколенице и њено одложено затварање, стабилизација прелома спољним скелетним фиксатором, антитетанусна заштита и адекватна антибиотска терапија представљају основне принципе у лечењу отворених прелома потколенице у овој студији. Приказани резултати корелирају са сличним студијама.

Кључне речи: отворени преломи потколенице; лечење; спољна скелетна фиксација; постоперативне компликације

Примљен • Received: 16/04/2015 **Ревизија • Revision:** 26/11/2015 **Прихваћен • Accepted:** 26/11/2015

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):300-306
ОРИГИНАЛНИ РАД / ORIGINAL ARTICLE
DOI: 10.2298/SARH1606300K
UDC: 618.146:[616.988:578.827(497.113)

Human papillomavirus infection prevalence in female university students in Novi Sad, Serbia

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SUMMARY

Introduction Cervical cancer, attributed to human papillomavirus (HPV) infection, represents the fourth most common and lethal cancer in Serbian women, and the second most common cancer in women aged 15–44.

Objective The aim of the study was to determine the presence of high-risk and low-risk HPV types in population of unvaccinated female university students in Novi Sad, Serbia, and to evaluate possible risk factors for HPV infection.

Methods Sample consisted of 250 young women (19–26 years of age) attending outpatient clinics for screening gynecological examination. All participants in the study completed a specially designed anonymous questionnaire. For the detection of HPV DNA, two commercial kits – High Risk HPV Real-TM and Low Risk HPV 6/11 Real-TM (Sacace Biotechnologies, Como, Italy) were used. Thirty positive samples were retested by GenoFlow HPV Array Test (DiagCor Bioscience Incorporation Limited, Hong Kong, China).

Results The overall prevalence rate of HPV was 61.6%. The most common HPV types in the present study were as follows: HPV 16, 31, 51, 52, and 18. Female students with only one sexual partner had significantly lower chance of having HPV infection. Other variables describing lifestyle did not show statistical significance.

Conclusion The present paper provides data on the prevalence of high- and low-risk HPV genotypes among university students in Novi Sad. Obtained results indicate the need for educational activities on sexually transmitted infections, including HPV, together with promotion of healthy lifestyles. According to our results, bivalent and quadrivalent prophylactic vaccines have the potential to prevent over 50% of infections. Percentage of protection with a second-generation prophylactic nonavalent vaccine would be more than 80%.

Keywords: human papillomavirus; real-time PCR; students

INTRODUCTION

Human papillomavirus (HPV) is one of the most prevalent sexually transmitted infections in the world. The important consequence of this infection is cervical cancer (CC), the fourth most common cancer among women worldwide (15%) and the second most common in developing countries [1]. According to the latest data from GLOBOCAN (2012) study, the incidence of cervical cancer in Serbia is 23.8 per 100,000 inhabitants, ranking Serbia fourth among European countries – after Romania, Lithuania, and Bulgaria. Currently, with 1,501 new cases and approximately 609 deaths per year, cervical cancer is the fourth most common and lethal cancer in Serbian women, while in the group of women aged 15–44 it is in second place [2].

HPV infection is common in young sexually active population. Large number of newly acquired HPV infections are transient, but certain percentage of infections persist, and the persistent infections are involved in the development of cervical cancer and its immediate precursor lesions [3]. However, it is also well known that the development of cervical cancer involves complex interactions among various factors. Although viral infection is necessary for neoplastic transformation, evidence suggests that host and environmental co-factors also play a significant role [4].

Primary prevention of HPV infection, and thus prevention of its sequelae, is important for public health [5]. Early detection of HPV infection in the population of young, sexually most active women, together with the introduction of prophylactic vaccines, represent effective prevention of cervical cancer.

OBJECTIVE

The main objective of this study was to determine the presence of most common high-risk (HR) and low-risk (LR) HPV types in the population of unvaccinated female university students from Novi Sad, Serbia. The second objective was to gain insight into lifestyle habits proven to be risk factors for acquiring HPV infection.

METHODS

Samples

Sample consisted of 250 young, unvaccinated women (age 19–26 years; mean age 22.5 years) who gave consent for participation in the study.

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Subjects attended gynecological outpatient clinics for gynecological examination as part of a required health evaluation for students, at the Department of Gynecology, Institute for Health Protection of Students, Novi Sad.

All potential participants in the study were informed of the aims of the project by trained interviewers before gynecological evaluation. Informed consent was obtained from all individual participants included in the study. They were told that their participation is voluntary and that they have a full right to withdraw from the study at any point. Only after all the information was provided, subjects completed a specially designed anonymous questionnaire.

The questionnaire contained 24 multiple-choice questions regarding the demographic and educational characteristics, lifetime habits (tobacco smoking, oral contraceptive use, condom use), and other potential risk factors related to sexual habits (age at first intercourse, number of sexual partners). Additional questions were asked on history of sexually transmitted infections, knowledge about HPV infections, frequency of visits to a gynecologist and family history of cancer.

Speculum examination was performed by gynecologists. In addition to smear taken for the conventional cytology, a smear for HPV testing was taken and placed in a container containing preservation solution (Copan Diagnostics Inc., Murrieta, CA, USA). After the collection and transport of samples for HPV DNA assay, the samples were examined for the presence of individual HPV genotypes.

Ethical Committee of the Institute of Public Health of Vojvodina approved the study protocol.

Nucleic acid isolation

Cervical swab samples were preserved in specimen transport medium (Copan Diagnostics Inc.). HPV DNA isolation protocol was carried out according to manufacturer's instructions (Sorb-A DNA extraction kit, Sacace Biotechnologies, Como, Italy). DNAs were extracted from an aliquot of 100 µL specimen. The extracted DNA was eluted in 100 µl elution buffer and stored at -20°C.

Nucleic acid amplification

HPV DNA detection and genotyping was performed by real-time polymerase chain reaction (PCR) at the Center of Virology, Institute of Public Health of Vojvodina. Twelve high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) were determined in the endocervical swabs with HPV High-Risk Typing Real-TM kit, (Sacace Biotechnologies). Regarding the validity of the test used, the authors relied on the certificate provided by the manufacture. The certificate ensured satisfactory clinical validity of the used test in relation to hybrid capture method in terms of sensitivity and specificity, according to the international rules. The comparison was performed by the manufacturer.

Viral E7 gene was amplified using multiplex primers and four TaqMan probes. To prove the HR HPV, multiplex reaction was performed in a volume of 13 µl. The process of amplification was performed in 45 cycles under the following conditions: 15 minutes at 95°C, 20 seconds at 95 °C, and 60 seconds at 60°C.

Two LR HPV types (6, 11) were determined in the endocervical swabs with HPV 6/11 Real-TM kit (Sacace Biotechnologies). Viral L1 gene was amplified using the primers and TaqMan probes. Reproduction of target DNA for the detection of LR HPV was performed in a volume of 25 µl. The process of amplification is performed in 45 cycles under the following conditions: 15 minutes at 95°C, 20 seconds at 95°C, and 60 seconds at 60°C.

GenoFlow test

The GenoFlow Array Test kit (DiagCor Bioscience Incorporation Limited, Hong Kong, China) uses biotin-labeled primers and specific probes to detect 33 common HPV types (18 HR HPV types and 15 LR types). The extraction DNA (Sorb-A DNA extraction kit, Sacace Biotechnologies) was mixed with PCR reagent mix and DNA *Taq* polymerase provided with the GenoFlow test kit, and PCR-amplified using the thermocycling condition stated in the manual. The amplicons were genotyped using flow-through hybridization according to manufacturer's instructions. Retesting was done on 30 samples previously genotyped by HPV High Risk Typing Real-TM kit, (Sacace Biotechnologies) with GenoFlow HPV Array Test kit (DiagCor Bioscience Incorporation Limited).

Statistical analysis

Data were statistically processed by two-way analysis of variance (ANOVA), for each variable. Analysis of variance and LSD-tests were performed on data transformed by arcsine transformation in order to meet normal distribution of frequencies. The results were considered significant if the probability of test was less than 0.05. For statistical analysis of data, STATISTICA 12 (StatSoft Inc., Tulsa, OK, USA) statistical package was used [6].

RESULTS

Demographic characteristics of the study population

A total of 250 non-vaccinated, young women (mean age of 22.5 years, age range 19–26 years) were enrolled in the study from September 2012 to December 2014. The group of University of Novi Sad students mostly consisted of students from Vojvodina (90%), and only a small part (10%) was from other parts of Serbia. Almost all of young women were single 240 (96%), nine (3.6%) were married, and one (0.4%) was divorced. Seven of them had at least one full-term pregnancy. Nine (3.6%) of the participants had previous abortion.

HPV Type Distribution

Overall prevalence of HPV DNA in a sample of young females was 61.6% (154/250). Among positive samples, oncogenic HPV types were detected in 96.1% (148/154), and LR HPV types in 28.6% (44/154).

Infections with a single HPV type was detected in 49.4% (76/154) participants, while most co-infections (23.4%; 36/154) involved two HPV genotypes; multiple infections with three HPV types were found in 17.5% (27/154) of the participants; four or more HPV genotypes were found in 9.7% (15/154) of the cases (Table1).

Our analysis involved determination of 12 highly oncogenic HPV types, type 16 being the most frequent with 48.1% (74/154), followed by types 31, 51, and 18. Two low-oncogenic types were detected – HPV type 6 was the predominant one, with 27.3% (42/154), while HPV 11 was considerably rare, with 3.9% (6/154) (Table 2).

Vaccinal HPV types 16/18 were present in 54.5% (84/154) of the cases; types 16/18/6/11 in 64.2% (99/154), and HPV types 6/11/16/18/31/33/45/52/58 included in the new nonavalent prophylactic vaccine were present in 87% (134/154) of the cases, both as single infections and in combination with other HPV types (Table 3).

Comparison between HPV High Risk Typing Real-TM kit (Sacace Biotechonologies) and GenoFlow HPV Array Test kit (DiagCor Bioscience Incorporation Limited)

In the total of 30 samples, the comparison between the kits showed complete agreement in 89.3%, partial agreement in 7.1%, and disagreement in 3.6% of the samples. By applying the GenoFlow test, additional HR HPV types were detected: HPV 73 (5/30 cases); HPV 66/68 (4/30), HPV 81 (4/30); HPV 53 (2/30); HPV 71, 82, and 83 (1/30). From LR HPV types, the following additional types were detected: HPV 26/84 (5/30 cases), HPV 40/61 (5/30); HPV 43/44 (4/30); HPV 54/55 (2/30); HPV 42 (1/30).

Overall HPV DNA prevalence and factors associated with HPV infection

In our study, about 22.8% of the young women had a history of smoking, approximately for 4.9 years and 12.2 cigarettes per day. Out of 250 young women, 35.2% reported condom use, 26.4% use of oral contraceptives, 12.8% reported a history of sexually transmitted infections, and 20.9% of the participants had condylomata. Approximate age of first sexual intercourse was 17.7 years. Average number of sexual partners in the previous two years was two. Significant differences were found among HPV DNA positive and negative women only for variables associated with number of sexual partners in the previous two years ($p = 0.009$). Women who had one sexual partner in the previous two years showed lower rates of HPV infection compared to women who had two or more sexual partners.

Table 1. Distribution of single, double, and multiple HPV infections (n = 154)

HPV infection	n (%)
Single HPV infection	76 (49.4)
Co-infection with 2 HPV types	36 (23.4)
Co-infection with 3 HPV types	27(17.5)
Co-infection with 4 HPV types	15 (9.7)

HPV – human papillomavirus

Table 2. Frequency of 12 high-risk HPV and two low risk genotypes in university students in Novi Sad (n = 154)

HPV genotypes		n (%)
High-risk	16	74 (48.1)
	31	28 (18.8)
	51	28 (18.8)
	52	24 (15.6)
	18	19 (12.3)
	56	15 (9.7)
	59	13 (8.4)
	33	12 (7.8)
	58	11 (7.1)
	45	8 (5.2)
	39	7 (4.5)
	35	5 (3.2)
Low-risk	6	42 (27.3)
	11	6 (3.9)

Table 3. Frequency of vaccinal HPV types (n = 154)

Vaccinal HPV types	N (%)
16/18	84 (54.5)
6/11/16/18	99 (64.2)
6/11/16/18/31/33/45/52/58	134 (87.0)

Other variables, as tobacco use ($p = 0.955$), oral contraception method used ($p = 0.806$), condom use ($p = 0.293$), and history of sexually transmitted infections were not statistically significant ($p = 0.825$) (Table 4).

A summary of variables which describe individual health care are provided in Table 5. Our research shows that 60% of the young women had knowledge about HPV infection and sources of information were internet sites and primary care physicians. Eighteen percent of the interviewed women had undergone an HPV test in the recent past. Of all the participants, 94% had regular gynecological check-ups, while 6% of the women went to a gynecologist irregularly.

DISCUSSION

Among 250 non-vaccinated university students in Novi Sad, Serbia (19–26 years of age, with mean age of 22.5 years), the overall HPV prevalence was 61.6%.

Numerous studies focused on the prevalence of HPV in women show that the highest percentage of infection is present in youngest women, i.e. < 25 years. HPV infection in women has been consistently found to have a peak just after the onset of sexual activity, usually starting from 15 years of age [7], reaching prevalence of up to 80% in some populations [8]. Results obtained in this study regarding the frequency of HPV infection in women 19–26 years

Table 4. Characteristics of life style and sexual habits in relation to the presence of HPV

Variables	% of HPV-positive women (n = 154)	% of HPV-negative women (n = 96)	p-value (F-test)
Tobacco users	23.1[a]	22.6[a]	0.955
Oral contraceptive users	25.4[a]	26.8[a]	0.806
Condom users	31.6[a]	38.8[a]	0.293
First sexual intercourse before 17 years of age	49.9[a]	55.2[a]	0.412
One sexual partner in previous two years	57.3[b]	31.8[a]	0.009*
≥ 2 sexual partners in previous two years	42.7[b]	68.2[a]	
Previously reported STDs	12.1[a]	12.9[a]	0.825

* statistically significant difference
[a, b] Labels of homogenous groups: values with the same letter do not differ significantly at level α = 0.05

Table 5. Personal health habits and knowledge about HPV in relation to the presence of HPV

Variables	% of HPV-positive women (n = 154)	% of HPV-negative women (n = 96)	p-value (F-test)
Regular visit to gynecologist	95.5[a]	93.5[a]	0.652
Papa test	87.9[a]	81.3[a]	0.125
Family history of cancer	28.9[a]	37.4[a]	0.255
Knowledge about HPV	61.1[a]	58.7[a]	0.707
Recent HPV analysis	18.6[a]	16.4[a]	0.586

[a] Labels of homogenous groups: values with the same letter do not differ significantly at level α = 0.05

of age are in accordance with data found in neighboring countries. Grozdanov et al. [9] tested a group of 95 18- to 29-year-old women in Bulgaria and detected HPV DNA in 63% of samples, while in Romania, Moga et al. [10] reported the highest HPV prevalence in women of age < 25, being 54.93%. The authors further state that prevalence decreases with age. A study on a group of 37 young women (age < 29) in Montenegro detected HPV prevalence of 53% [11]. Differences in prevalence depend of geographic areas, diversity of population samples, type of the test used for DNA HPV detection, and other factors [12]. Most common HPV types in the present study were HPV 16, 31, 51, 52, and 18, which is in accordance with the data of Bruni et al. [13] stated in the report provided by the Information Centre on HPV and Cancer. A significant feature of the present study is high prevalence of HPV 16, meaning that nearly one half (48.1%) of HPV DNA-positive young women had infection with HPV 16. This result is similar to the findings of other authors from the region [14, 15, 16]. HPV 18 was detected less frequently and ranked fifth, similarly as in other countries in women of the same age group [14, 17]. Also, considerably high percentage of multiple infections was noticed (49.4%), which is in accordance with the results from neighboring countries [9, 10, 15]. Multiple infections may lead to the increased risk of viral persistence and neoplastic progression [18].

Detection and monitoring of HPV infection is an important step in prevention of cervical cancer development, and incorporation of a broader HPV DNA genotype range into screening programs would be desirable. Our pilot study on 30 samples retested by GenoFlow HPV Array Test method showed high agreement (89.3%) with the real-time PCR test (Sacace Biotechonologies) routinely used in our laboratory. GenoFlow HPV Array Test offered a broader range of genotypes determination (33 vs. 14 genotypes), and indicated a relatively high frequency of some genotypes not covered by a conventional real-time PCR tests. Some of the detected genotypes have been detected for the first time in Serbia. By applying the GenoFlow test, we observed that additional HR HPV types (53, 66, 68, 71, 73, 81, 82) are present in population of young women in our region. As for the LR HPV, besides genotypes 6 and 11, we detected the presence of HPV 26, 40, 42, 43, 44, 54, 55, 61, and 84. According to these preliminary results, even higher prevalence rate could be postulated, especially of LR HPV types.

From the public health perspective, in countries like Serbia, where resources for screening are scarce, population of university students could be a clever choice for gaining the epidemiological insight into HPV genotype prevalence. Taking into account great variety of circulating genotypes in this age group, even a limited number of samples could give a valuable data on the circulating genotypes for the entire population.

Routine HPV vaccination programs are implemented in 22 out of 31 EU/EEA countries [19]. In addition to the two vaccines against HPV infection approved previously by the Food and Drug Administration (FDA) – quadrivalent (HPV4) Gardasil (2006; Merck & Co., Inc., Kenilworth, NJ, USA) and bivalent (HPV2) Cervarix (2009; GlaxoSmithKline, London, UK) [20] – FDA recently approved a nonavalent vaccine Gardasil9 (HPV9) (December 2014, Merck & Co., Inc.) [21]. Cervarix (HPV2) and Gardasil (HPV4) both protect against HPV types 16 and 18. In addition, HPV4 protects against HPV types 6 and 11. HPV9 offers protection against five additional HPV types: 31, 33, 45, 52, and 58, which cause approximately 20% of cervical cancers and are not covered by previously FDA-approved HPV vaccines. The HPV9 vaccine has a potential to prevent approximately 90% of cervical, vulvar, vaginal, and anal cancers [22].

Coverage rates vary in different EU countries and range between 17% in Luxembourg and 84% in Portugal [19]. Although both HPV2 and HPV4 vaccines are registered in Serbia, vaccination programs have not

yet been implemented. It should be emphasized that in our study, vaccine-covered HPV types 16/18/6/11 were present in 64.2% (99/154) of the cases, and HPV types included in the new nonavalent prophylactic vaccine (6/11/16/18/31/33/45/52/58) were identified in 87% of the cases (134/154), both as single infections and in combination with other HPV types [23].

It has been shown that among healthy young women, most of the HR HPV infections tend to be cleared without treatment and become undetectable without clinical consequences. However, some infections persist and a subset of persistent infections may progress to cervical intraepithelial neoplasia or invasive cancer [24]. Our intention was to gain insight into the lifestyle, sexual habits, and knowledge of HPV infections, the factors of significance for the health of young women in our region which correlate with the frequency of acquiring HPV infections.

Habit of tobacco use is rather frequent in women from our region (22.8%). However, this factor was not associated with HPV DNA positivity in the present study. This is not surprising, since only the persistency was proven to be associated with heavy smoking [25, 26]. About 26.4% of the young women in our study used oral contraceptives, most of them reporting the period of one year. Research suggests that long-term use of oral contraceptives could increase the risk of cervical cancer by up to fourfold in women with HPV infection [27]. This variable is presently not associated with HPV DNA positivity, but similarly to tobacco use it may contribute to persistency of HPV infection and the increased risk for development of precancerous cervical lesions and cancer in the future.

The results of the statistical analysis demonstrated that HPV infection was associated with some of the parameters of sexual behavior, such as average age of the first intercourse and number of sexual partners in previous two years (Table 4). Nielsen et al. [28] found that lifetime number of sexual partners was the main risk factor for HR HPV infection. In our research, the average age of the first intercourse among young women was 17.7 years. Only women who had one partner in the previous two years had statistically lower chance of HPV infection. It is well known that monogamous lifestyle can reduce the risk of infection and consequently the occurrence of precancerous lesions. Other variables describing sexual habits including condom use and history of sexually transmitted infections were not statistically significant in the present study. The absence of any significant association between HPV and past sexually transmitted diseases might be accounted for by the fact that most women are reluctant to disclose the details of previously sexually transmitted diseases, as well as the number of sexual partners. Recent studies suggest that condom use provides only partial protection against the transmission of genital HPV types, the effectiveness being up to 70% due to the fact that HPVs can infect portions of skin not covered by condom [29]. Recommendations for the use of condoms are still given, in order to reduce the risk of transmission of HPV warts and spread to other parts of healthy skin and mucous membranes, as well as to prevent transmission of other sexually transmitted diseases.

According to our questionnaire, 94% of the participants had the habit of regular gynecological check-ups. This percentage is much higher than the one for general population and can be attributed to the fact that we surveyed female university students. The 2013 Ipsos survey (Ipsos Strategic Marketing, Belgrade, Serbia) showed that the percentage of women of fertile age in Serbia who had visited a gynecologist in the previous year was 47.5%. Differences in this respect exist by region, type of settlement, education, and financial situation of the household [30].

Literature data show different levels of knowledge about HPV infections among young population worldwide. While only 17.7% of students from Nigeria (368 female students, aged 16–29 years) have some knowledge on HPV infection, an international study showed that higher percentage of young adults in the USA (62%), the UK (44%), and Australia (40%) are informed about HPV infection [31]. Results from the present study indicate that 40% of the respondents have no knowledge on HPV, and that 82% of them have never submitted themselves to an HPV test.

CONCLUSION

The present work provides data on the prevalence of HR and LR HPV genotypes and potential risk factors for HPV infection among university students in Novi Sad, Serbia. The results obtained in this study indicate the need for educational activities on sexually transmitted infections, including HPV. In countries such as Serbia, where HPV vaccination programs have not yet been implemented, and resources for HPV screening programs are scarce, university students could be a target population group for the HPV testing and awareness raising, together with the promotion of healthy lifestyles. Undertaken at this moment in their lives, the activities will most probably make a long lasting effect on individual, as well as reproductive health of the entire population.

Furthermore, our data suggest that currently licensed prophylactic vaccines, if applied, have the potential to prevent approximately 50% of infection, while percentage of protection with a second-generation prophylactic nonavalent vaccine would be more than 80%. However, a wider-scale HPV screening with a test for broader genotype range is needed as a starting point in prevention actions, as well as a better algorithm for cancer diagnosis and adequate treatment.

ACKNOWLEDGMENT

This study was funded by the City of Novi Sad, City Administration for Health, in the period 2012–2014, Projects No. XII-51-46-3/2012, XII-51-46-51/2013, and XII-51-52-7/2014.

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Учесталост инфекције хуманим папилома вирусом код студенткиња у Новом Саду у Србији

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КРАТАК САДРЖАЈ

Увод Рак грлића материце, проузрокован ХПВ инфекцијом, представља четврти најчешћи летални карцином код жена у Србији и други најчешћи малигнитет у групи жена од 15 до 44 године.

Циљ рада Циљ рада је био да да се одреди присуство *HR* и *LR* ХПВ типова у популацији невакцинисаних студенткиња Универзитета у Новом Саду и процене могући фактори ризика за ХПВ инфекцију.

Методе рада Узорак се састојао од 250 младих жена (старости 19–26 година) укључених у студију током систематских гинеколошких прегледа. Све учеснице су попуниле специјално дизајниран анонимни упитник. За детекцију ХПВ ДНК коришћена су два комерцијална кита: *High Risk* ХПВ-*real-TM* и *Low risk* ХПВ 6/11 *real-TM* (*Sacace Biotechnologies, Italy*). Тридесет позитивних узорака је ретестирано употребом *GenoFlow* ХПВ *Array Test* (*DiagCor*, *Bioscience*).

Резултати Укупна стопа учесталости ХПВ инфекције је износила 61,6%. Најчешћи типови ХПВ у овој студији били су: ХПВ 16, 31, 51, 52 и 18. Студенткиње са само једним сексуалним партнером су имале сигнификантно мању шансу за стицање ХПВ инфекције. Остале варијабле које описују начин живота нису показивале статистичку значајост.

Закључак Презентовани рад пружа податке о учесталости високо и нискоризичних генотипова ХПВ међу студенткињама Унивезитета у Новом Саду. Добијени резултати указују на потребу едукације о сексуално преносивим инфекцијама, укључујући и ХПВ, заједно са промоцијом здравих стилова живота. Према нашим резултатима, бивалентна и четворовалентна профилактичка вакцина би могле да спрече преко 50% инфекција. Проценат заштите применом деветовалентне вакцине износио би преко 80%.

Кључне речи: хумани папилома вирус; *real-time PCR*; студенти

Примљен • Received: 27/04/2015 **Ревизија • Revision:** 14/07/15 **Прихваћен • Accepted:** 18/11/2015

Detection of carbapenemase genes in *Klebsiella pneumoniae* isolates

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SUMMARY

Introduction *Klebsiella pneumoniae* is one of the leading causes of serious hospital-acquired infections worldwide among *Enterobacteriaceae* species. It is the most common producer of carbapenemases in many parts of the world.

Objective The aim of the study was to determine which enzymes were responsible for resistance to carbapenems in *Klebsiella pneumoniae* strains isolated at the Centre of Microbiology of Public Health Institute of Vojvodina.

Methods A total of 29 *Klebsiella pneumoniae* non-duplicated strains resistant to at least one carbapenem isolated from clinical samples of hospitalized patients between November 1st 2013 and April 30th 2014 were studied. The species identification and susceptibility were done using VITEK 2 (bioMérieux, Marcy-l'Étoile, France) system. Phenotypic conformation of carbapenemase production was done by double-disc synergy test. PCR technique was performed for detection of genes encoding production of carbapenemases (bls_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48}).

Results Isolates of *Klebsiella pneumoniae* resistant to at least one carbapenem showed positive on double-disc synergy test between meropenem and dipicolinic acid. All strains positive in phenotypic test contained bla_{NDM} gene. In isolates resistant only to ertapenem, neither production of carbapenemases nor presence of genes encoding these enzymes were detected. Among these isolates, nine produced extended-spectrum β-lactamase.

Conclusion The presence of NDM metallo-β-lactamase was determined in all *Klebsiella pneumoniae* isolates resistant to at least one carbapenem.

Keywords: *Klebsiella pneumoniae*; carbapenem-resistance; NDM β-lactamase

INTRODUCTION

Enterobacteriaceae are one of the leading causes of both community- and hospital- acquired serious infections in humans that are associated with increased morbidity and mortality rates and healthcare costs. *Escherichia coli* and *Klebsiella pneumoniae* are responsible for the majority of these infections including urinary tract infections, septicemia, pneumonia, peritonitis, meningitis, and device-associated infections [1]. They are often multidrug resistant (MDR), and infections caused by these strains are a formidable problem to clinicians because therapeutic options are often very limited [2]. Carbapenems had been antibiotics of choice for the treatment of severe infections caused by MDR strains until 1990s, when the first carbapenemase producer in *Enterobacteriaceae* was identified [3]. After that, other types of enzymes that hydrolyze carbapenems were detected and reported worldwide [4]. Emergence and spread of carbapenemase-producing *Enterobacteriaceae* further reduced options for effective treatment of these infections.

Resistance to carbapenems is a result of production of enzymes that belong to the following three classes of β-lactamases: Ambler class A, B, and D β-lactamases [5]. Among class A β-lactamases, the most clinically significant is *Klebsiella pneumoniae* carbapenemase (KPC) [6]. Class B β-lactamases or metallo-β-lactamases are mostly of the IMP, VIM, and most recently of the New Delhi metallo-β-lactamase (NDM) type [7]. The most important types of class D are OXA-48-like enzymes [8]. Most of these enzymes were identified in MDR *Klebsiella pneumoniae*, but distribution and prevalence vary widely in different regions [9].

OBJECTIVE

The aim of this study was to determine which enzymes were responsible to resistance to carbapenems in clinical isolates of *Klebsiella pneumoniae*.

METHODS

The study was conducted at the Center for Microbiology of Public Health Institute of Vojvodina. A total of 29 non-duplicated strains of *Klebsiella pneumoniae* resistant to at least one carbapenem were isolated from clinical

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samples including blood (nine), urine (nine), wound swab (eight), and tracheal aspirate (three) of patients hospitalized on the various wards of Clinical Centre of Vojvodina and Institute of Child and Youth Health Care of Vojvodina between November 1st 2013 and April 30th 2014. Isolated strains were identified using VITEK 2 Compact system, GN cards (bioMérieux, Marcy-l'Étoile, France). To determine susceptibility to antimicrobial drugs and production of extended-spectrum β-lactamase (ESBL) VITEK 2 Compact system, AST-GN71 and AST-N240 cards were used according to Clinical and Laboratory Standards Institute guideline. Susceptibility to fosfomycin was tested by Etest strip (AB Biodisk, Solna, Sweden) using European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (2013) [10, 11]. All isolates were tested for the presence of the most prevalent enzymes (KPC, VIM, NDM, OXA-48) and genes responsible for the resistance to carbapenems (Table 1). Phenotypic confirmation of carbapenemase production and interpretation of results were done by double-disk synergy test according to manufacturer's instructions and EUCAST guideline [12], using tablets containing meropenem (10 μg), cloxacillin, dipicolinic acid, boronic acid (Rosco Diagnostica Neo-Sensitabs, Taastrup, Denmark). Enhancement of the zone of inhibition in the area between meropenem disc and the inhibitor-containing disc was considered to be a positive result. Detection of genes encoding production of carbapenemases using PCR technique was performed as follows: extraction of total bacterial DNA was performed in a 2 mL tube containing 1.5 mL of bacteria in a tryptic soy broth culture. It was centrifuged at 13,000 rpm for five minutes, followed by discarding of the supernatant, and the pellets were suspended with 200 μL DNAse/RNAse free water (Gibco, Invitrogen, Waltham, MA, USA). The suspension was vortexed briefly, then heated at 100°C for five minutes, and cooled at 4°C for five to 10 minutes. Mixture was then centrifuged for five minutes at 13,000 rpm, and 150 μL of supernatant was transferred to a new 1.5 mL tube and was ready for use.

PCR reaction was performed with MasterCycler personal (Eppendorf, Hamburg, Germany). The final reaction volume was 25 μL with 8 μL DNAse/RNAse free water (Gibco, Invitrogen), 12.5 μL PCR Master Mix M752 (Promega, Fitchburg, WI, USA) and 0.5 μL of each primer. PCR reaction of four genes was performed as two separate multiplex reactions. First reaction included primers for bla_{NDM} and bla_{KPC}, and second one included primers for bla_{OXA-48} *like* and bla_{VIM}. PCR cycling conditions for *ndm* and *kpc* reaction were one cycle at 95°C for five minutes, 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle at 72°C for three minutes, and holding stage at 4°C. PCR cycling conditions for *oxa48-like* and *vim* reaction were one cycle at 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 60 seconds, followed by one cycle at 72°C for three minutes, and holding stage at 4°C. The PCR-amplified products were analyzed by 2% agarose (Fisher Scientific International, Inc., Hampton, NH, USA) gel electrophoresis and stained with ethidium bromide. Images were documented by a BioDocAnalyze (Biometra, Gottingen, Germany) system.

Table 1. Primers for detection of genes encoding carbapenemase production

Primer name	Sequence
*bla*KPC Fw	ATGTCACTGTATCGCCGTCT
*bla*KPC Rw	TTTTCAGAGCCTTACTGCCC
*bla*VIM Fw	GATGGTGTTTGGTCGCATA
*bla*VIM Rw	CGAATGCGCAGCACCAG
NDM-For	GGGCAGTCGCTTCCAACGGT
NDM-Rev	GTAGTGCTCAGTGTCGGCAT
OXA48A	TTGGTGGCATCGATTATCGG
OXA48A	GAGCACTTCTTTTGTGATGGC

Table 2. Susceptibility of *Klebsiella pneumoniae* to antimicrobial drugs

Antimicrobial drug	*Klebsiella pneumonia*	
	No. of isolates tested	No. (%) of resistant isolates
Amoxicillin/clavulanic acid	29	29 (100)
Tazobactam/piperacillin	29	29 (100)
Ceftazidime	29	29 (100)
Ceftriaxone	29	29 (100)
Cefotaxime	29	29 (100)
Cefepime	29	29 (100)
Imipenem	29	15 (51.7)
Meropenem	29	15 (51.7)
Ertapenem	29	29 (100)
Gentamycin	29	22 (75.8)
Amikacin	29	20 (68.9)
Cotrimoxazole	29	29 (100)
Ciprofloxacin	29	28 (96.5)

Table 3. Susceptibility to colistin, fosfomycin, and tigecycline of *Klebsiella pneumoniae* isolates resistant to all carbapenems tested

Antimicrobial drug	*Klebsiella pneumoniae*
	No. of resistant/tested isolates
Colistin	0/15
Fosfomycin	1/15
Tigecycline	13/15

RESULTS

Table 2 shows results of susceptibility testing of 29 *Klebsielle pneumoniae* isolates resistant to at least one carbapenem.

Among carbapenems, ertapenem showed lower activity than meropenem and imipenem. Percentage of isolates resistant to ertapenem was higher compared to other carbapenems tested. All *Klebsiella pneumoniae* isolates were resistant to eight of 13 antimicrobial agents tested (Table 2).

All isolates of *Klebsiella pneumoniae* resistant to all carbapenems tested were susceptible to colistin, only one isolate was resistant to fosfomycin, while 13 of 15 isolates were resistant to tigecycline (Table 3).

Isolates of *Klebsiella pneumoniae* resistant to all carbapenems tested showed an enhancement of the zone of inhibition in the area between meropenem disc and the disc containing dipicolinic acid that indicated a metallo-β-lactamases production (Figure 1).

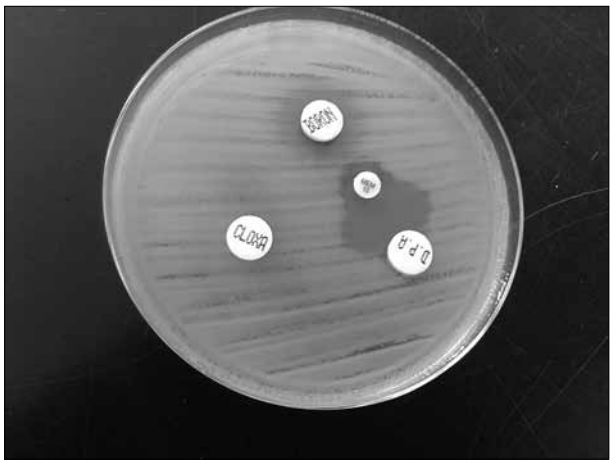

Figure 1. Positive Double Disc Sinergy Test. The enhancement of the zone of inhibition in the area between meropenem disc and the disc containing dipicolinic acid.

Figure 2. Detection of bla_{NDM} gene in *Klebsiella pneumoniae* isolates

All strains positive in phenotypic confirmation test carried bla_{NDM} gene encoding NDM β-lactamase detected by PCR technique (Figure 2).

In isolates resistant only to ertapenem, neither production of carbapenemases nor presence of genes encoding these enzymes were detected. Among these isolates, nine out of 14 were ESBL producers.

DISSCUSION

Klebsiella pneumoniae is one of the leading causes of serious hospital-acquired infections worldwide. Emergence of carbapenemase resistant isolates is of particular concern because infections caused by these strains are associated with therapeutic failure and high mortality rates. According to the European Survey on Carbapenemase-Producing *Enterobacteriaceae* working group report, *Klebsiella pneumoniae* has been the most common producer of carbapenemase in majority of European countries. KPC is the most frequently detected enzyme [13]. The first KPC-producing strain was isolated from clinical specimen in North Carolina in 1996 [14]. Subsequently, KPC producers have been reported worldwide causing outbreaks in United States, many European countries, China, and South America [2, 5, 15, 16, 17]. The most important and particularly problematic carbapenemase in the Ambler class B, with increasing detection in many geographic areas, is the metallo-β-lactamase NDM. It was first isolated in Sweden in 2008, from an Indian patient with urinary tract infection previously hospitalized in New Delhi [7].

Emergence and spread of carbapenemase producers were reported all over the world. Types of enzymes and their frequency of detection differ from country to country. In this study, in all *Klebsiella pneumoniae* strains resistant to at least one carbapenem tested for the presence of the most common carbapenemase, only NDM metallo-β-lactamase was detected. Strains producing this enzyme were isolated in many European countries, but their distribution was not as wide as that reported for KPC-β-lactamase [2]. The largest number of NDM positive isolates was reported in the United Kingdom. Most of these cases had a direct connection to the Indian subcontinent (i.e. India, Pakistan, and Bangladesh). Fewer NDM producers were detected in Denmark, Belgium, France, Austria, Italy, and Germany, which originated most probably from Balkan countries and the Middle East. According to surveillance report of European Center for Disease Prevention and Control, NDM-producing *Enterobacteriaceae* were isolated from persons who had received medical care in Serbia (Kosovo and Metohija), Montenegro, and Bosnia and Herzegovina [18]. Some data indicate that Balkan states and the Middle East are, in addition to the Indian subcontinent, endemic reservoirs for NDM producers [19, 20]. Several isolates were detected in some other countries such as the USA, Canada, China, Japan, and Israel, related to unknown origin [2].

Our results suggest that ertapenem was not appropriate carbapenem for detecting NDM β-lactamase producers because all isolates resistant only to ertapenem were negative in phenotypic confirmation and genotypic tests. Other authors also found low specificity of ertapenem in their studies [6, 21]. Carbapenem resistance is mainly the result of carbapenemase production, but other mechanisms, such as overproduction of ESBL and AmpC β-lactamase associated with porin loss, may cause decreased susceptibility to carbapenems [6, 22, 23]. In this study, among 14 *Klebsiella pneumoniae* isolates resistant only to ertapenem, ESBL production was determined in nine strains.

Carbapenemase resistant *Enterobacteriaceae* are usually resistant to a wide range of antimicrobial drugs. Plasmids carrying genes encoding carbapenemases often carry high number of genes that confer high level of resistance to other antibiotics and can be easily transferred to other bacterial strains and species [9, 20, 24].

The majority of carbapenem resistant *Klebsiella pneumoniae* isolates in this study were MDR, which is in agreement with other reports [9, 19, 20]. All isolates were resistant to ß-lactam antibiotics (except meropenem and imipenem) and cotrimoxazole, and only one isolate was susceptible to ciprofloxacin. Lower but still high percentage of resistance to gentamycin and amikacin (75.8% and 68.9%, respectively) was detected. These results are similar to those of other investigations [25, 26]. The most effective antibiotic against all *Klebsiella pneumoniae* resistant to all

carbapenems tested in this study was colistin, followed by fosfomycin. Resistance to tigecycline was detected in almost all isolates tested. Many authors reported very good activity or low percentage of resistance to colistin and fosfomycin. None of the studies reported as high resistance to tigecycline as we have found in our isolates [25, 26].

Treatment of infections caused by MDR *Klebsiella pneumoniae* is a formidable therapeutic problem because there are few potentially effective antibiotics, such as colistin, tigecycline, fosfomycin, and aminoglycosides [27]. Several studies found higher rate of positive outcomes in patients when combination therapy with two or three antibiotics, including colistin, tigecycline, and fosfomycin, was used [28, 29]. Therefore, combination therapy for the treatment of severe infections caused by MDR isolates is recommended.

CONSLUSION

According to the results obtained, presence of NDM metallo-β-lactamase in all *Klebsiella pneumoniae* isolates resistant to at least one carbapenem was determined. Almost all isolates were multidrug resistant. In order to prevent emergence and spread of multidrug resistant species, it is mandatory to identify carbapenemase-producing isolates and determine mechanisms of resistance to carbapenems in routine laboratory work.

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Доказивање гена резистенције на карбапенеме код изолата *Klebsiella pneumoniae*

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КРАТАК САДРЖАЈ

Увод *Klebsiella pneumoniae* је један од водећих узрока тешких болничких инфекција из фамилије *Enterobacteriaceae* широм света. Она је најчешћа ентеробактерија која продукује карбапенемазе у многим географским подручјима.

Циљ рада Циљ рада био је утврђивање ензима одговорних за резистенцију на карбапенеме *Klebsiella pneumoniae* изолованих у Центру за микробиологију Института за јавно здравље Војводине.

Методе рада Испитивањем је обухваћено 29 примоизолата *Klebsiella pneumoniae* резистентних најмање на један антибиотик из групе карбапенема добијених из клиничких узорака болнички лечених пацијената у периоду 1. новембар 2013 – 30. април 2014. године. Идентификација изолата и испитивање осетљивости на антимикробне лекове вршени су применом Витек 2 система. *Double Disc Synergy Test* коришћен је за фенотипску потврду продукције карбапенеметаза. *PCR* методом доказивано је присуство најчешћих гена који кодирају продукцију карбапенеметаза (bls_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48}).

Резултати Сви изолати *Klebsiella pneumoniae* резистентни најмање на један карбапенем испољили су синергизам између меропенема и дипиколиничне киселине у фенотипском потврдном тесту. Код свих изолата позитивних у овом тесту доказано је присуство bla_{NDM} гена, док код изолата резистентних само на ертапенем није потврђена продукција карбапенемаза нити присуство гена који кодирају њихову синтезу. Бета-лактамазе проширеног спектра доказане су код 9 од 14 изолата резистентних само на ертапенем.

Закључак Присуство ензима *NDM* метало-*β*-лактамазе доказано је код свих изолата *Klebsiella pneumoniae* резистентних најмање на један карбапенем. Продукција бета-лактамаза проширеног спецтра доказана је код 9 од 14 изолата резистентних само на ертапенем.

Кључне речи: *Klebsiella pneumoniae*; резистенција на карбапенеме; *NDM β*-лактамаза

Примљен • Received: 13/05/2015 **Прихваћен • Accepted:** 05/06/2015

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):312-314
ПРИКАЗ БОЛЕСНИКА / CASE REPORT
DOI: 10.2298/SARH1606312K
UDC: 616.839.1-001-07; 615.217.2.035

The role of apraclonidine in Horner's syndrome – A case report

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SUMMARY

Introduction Horner's syndrome is an interruption of the sympathetic nervous system at any point along its course between the hypothalamus and the orbit. Horner's syndrome is classically presented as an ipsilateral miosis, subtle ptosis, and facial anhidrosis. Pharmacologic testing is very useful in the diagnosis of Horner's syndrome as it could help to localize the lesioned neuron in the sympathetic pathway, suggesting an etiology.

Case Outline We present a case report of a 41-year-old woman who reported right eyelid drooping immediately after operation of sympathetic chain schwannoma. We performed apraclonidine test for the diagnosis of Horner's syndrome, which produced mydriasis on the affected eye, while there was no significant change of the normal eye. Based on the clinical presentation of anisocoria and one-sided ptosis, and previous medical history of surgical removal of the mediastinal tumor, the patient was diagnosed with a right-sided, partial Horner's syndrome.

Conclusion Timely recognition, exact localization of the lesioned neuron, and referral for urgent imaging studies are important for ophthalmologists in order to prevent and treat life-threatening conditions. Besides its diagnostic value in Horner's syndrome, topical apraclonidine could correct ptosis for the sake of esthetics or when ptosis reduces the superior visual field.

Keywords: Horner's syndrome; apraclonidine test; sympathetic chain schwannoma

INTRODUCTION

Horner's syndrome (HS), also known as oculosympathetic palsy, was first described by Johann Friedrich Horner, as an interruption of the sympathetic nervous system at any point along its course between the hypothalamus and the orbit [1, 2]. The interruption of the sympathetic fibers could be central (between the hypothalamus and the fibers' exit from the spinal cord) or peripheral (in the cervical sympathetic chain, at the superior cervical ganglion, or along the carotid artery) [3]. HS is classically presented as an ipsilateral miosis, subtle ptosis, and facial anhidrosis [4]. Pharmacologic testing is very useful in the diagnosis of HS as the pupillary response to different pharmacological agents could help localize the lesioned neuron in the sympathetic pathway and identify the level of involvement (i.e. preganglionic or postganglionic), suggesting an etiology [1]. Localizing the lesion is very important because preganglionic lesions are associated with a higher incidence of malignancy and need rapid investigations. Pharmacological tests traditionally consist of cocaine testing with hydroxyamphetamine localization, but recently apraclonidine testing has become a pertinent alternative [1]. Appropriate evaluation of HS and timely determination of its etiology may allow for a potentially life-saving intervention [5].

We had the opportunity to observe a patient who developed HS as a consequence of mediastinal tumor. Clinical, imaging, and pharmacologic studies are presented.

CASE REPORT

We present a case report of a 41-year-old woman who reported right eyelid drooping immediately after operation of sympathetic chain schwannoma two years earlier. She was diagnosed with a mediastinal tumor by routine chest radiography. A mass was found in the right side of the superior mediastinum. Magnetic resonance imaging revealed a homogenous mass in the same location at the level from Th1 to Th3, which was suspected to be neurogenic. Surgical resection of the tumor was performed and the tumor was completely removed. The histopathological examination showed schwannoma characteristics. Immediately after the surgical intervention, the patient developed ptosis and miosis on the right side. No recurrence of the tumor was observed in two years after operation. Her ptosis and miosis remain until present day.

She was referred to the University Eye Clinic, Clinical Center of Serbia, in Belgrade, because of the drop of the eyelid on the right eye, and different size in pupils. She had been aware of her ptosis for the previous two years, but she hadn't sought ophthalmological assistance. On admission, her visual acuity was 20/20 on both eyes. Her extraocular motilities

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Figure 1. The photograph shows the patient with longstanding right-side Horner's syndrome; right ptosis and miosis.

Figure 2. The same patient following the instillation of 0.5% drop of apraclonidine in each eye – note the reversal of anisocoria and complete resolution of ptosis

was normal, with no signs of restriction or double vision or pain. Confrontational visual fields were normal except in her right eye, with defect in the upper half of the visual field. When her right upper eyelid was lifted, visual field was full-to-finger-count. Slit lamp examination of the anterior segment and intraocular pressure testing gave results within normal limits. Her pupils in the light measured 3 mm in the right eye, and 4 mm in the left eye. In the dark, the anisocoria increased with the pupils measuring 3 mm and 6 mm, respectively. There were no signs of the afferent pupillary defect, and both pupils reacted to light well. Also, her accommodative pupillary responses were normal. Ophthalmic examination showed ptosis of the upper lid on the right eye. Interpalpebral fissure widths were measured at 8 mm in the right eye, and 11 mm in the left, which confirm the right eye lid ptosis (Figure 1). As she was hysterectomized, no signs of ipsilateral anhidrosis were found, nor reported by the patient. She also denied a history of dry eyes. The patient's fundus examination provided normal findings. Subsequently we performed the apraclonidine test for the diagnosis of HS. We instilled one drop of 1% apraclonidine into each eye. After 20 minutes, instillation of apraclonidine into the affected eye produced mydriasis (Figure 2), while there was no significant change of the normal eye. Based on the clinical presentation of anisocoria and one-sided ptosis, and previous medical history of surgical removal of the mediastinal tumor, the patient was diagnosed with a right-sided, partial HS. As our patient was not keen to undergo the lid surgery for ptosis, we suggested the use of apraclonidine for ptosis relief. We monitor this patient on a four-month basis as long as no new symptoms and signs occur.

DISCUSSION

A thorough cognition of the sympathetic nervous system is critical in detecting the underlying pathology of HS. The oculosympathetic pathway has a long and tortuous route from the hypothalamus prior to innervation of the ocular structures [4]. Preganglionic lesions affect the neurons that travel from the brainstem to the spine, and from the spine to the superior cervical ganglion, while the postganglionic lesions affect the fibers from the superior cervical ganglion [6]. Preganglionic HS indicates a serious underlying pathology and is associated with high incidence of malignancy. Postganglionic HS has primarily benign causes, such as vascular headache [7], while painful HS suggest the possibility of internal carotid artery dissection [4].

In the past, pharmacologic diagnosis of HS with the topical use of 5% or 10% cocaine and hydroxyamphetamine solution has been the gold standard [8]. However, cocaine has several drawbacks. It is a controlled substance that must be prepared by individual pharmacies for local use and has become difficult to obtain for a wide range of practitioners [6]. Higher concentrations of cocaine solutions (i.e. 10%) could compromise the corneal epithelium [1]. An innovative pharmacologic alternative has recently been suggested [4]. Apraclonidine drops (approved for glaucoma; off-label application for HS) are now replacing cocaine in diagnosing HS, as topical apraclonidine is readily available and widely used [9, 6].

Patients with HS develop denervation hypersensitivity of α1 receptors on the iris dilator muscle. Instillation of one drop of either 0.5% or 1% apraclonidine in each eye result in mydriasis of the affected pupil, often dramatically [9]. This response was seen in eyes with preganglionic or postganglionic lesions. On the other hand, in the normally innervated eyes, apraclonidine produces little or no pupil dilation. Also, reversal of ptosis is reported in some patients after 0.5–1% apraclonidine installation [5, 6].

Garibaldi et al. [10] demonstrated the effect of apraclonidine on anisocoria and ptosis in HS by installing one drop of 0.5% apraclonidine in both eyes of three patients who presented with acute HS. Apraclonidine results in a slight mydriasis of the affected side and no change of the unaffected side (a reversal of the anisocoria), and in some patients a mild upper lid retraction. Morales et al. [6] also found the apraclonidine to be a safe and readily available alternative to cocaine for the diagnosis of HS.

In our case, in-office pharmacologic testing helped us to ascertain the diagnosis of HS based upon the patient's history of surgical intervention and presenting signs. HS is a frequent complication of the surgical resection of schwannoma that originates from the cervical sympathetic chain. This complication should probably be discussed with patients during the perioperative counseling [11].

The preferred surgical intervention for patients with ptosis from HS is either a mullerectomy or an external levator aponeurotic advancement [12]. For the patients

who are not keen to go through the lid surgery, 0.5% apraclonidine drops could induce pharmacologic reversal of HS-related ptosis, as it was suggested by several authors [10, 13]. In most patients with HS (87%), apraclonidine caused significant lid elevation. Yet, this effect was not specific to HS, because near one half of normal, control eyes had the same effect. The lid elevation effect can be useful in long-term symptomatic relief of patients with HS, affected by their ptosis [14].

In most cases, patients with HS tolerate well the associated mild ptosis. They also have no visual disturbance. Timely recognition, exact localization of the lesioned neuron, and referral for urgent imaging studies are important for ophthalmologists in order to prevent and treat life-threatening conditions. Persistent ptosis could be repaired by lid surgery or by topical apraclonidine. Besides its diagnostic value in HS, topical apraclonidine could correct ptosis for the sake of esthetics or when ptosis reduces the superior visual field.

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Улога апраклонидина код Хорнеровог синдрома – приказ болесника

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Увод Хорнеров синдром је прекид симпатичког нервног система на било ком делу свог тока од хипоталамуса до орбите. Хорнеров синдром се типично приказује као ипсилатерална миоза, умерена птоза и анхидроза. Фармаколошко тестирање је веома значајно у постављању дијагнозе Хорнеровог синдрома јер утврђивање локализације оштећења на симпатичком путу може да сугерише на етиологију.
Приказ болесника Приказујемо случај 41-годишње жене која наводи пад горњег капка на десном оку убрзо након операције шванома симпатичког ланца. Извели смо апраклонидински тест, који је довео до мидријазе на захваћеном оку, док на нормалном оку није било одговора. Према клиничкој презентацији анизокорије и једностране птозе, увидом у претходну медицинску документацију хируршког уклањања медијастиналног тумора, постављена је дијагноза десностраног Хорнеровог синдрома.
Закључак Благовремено препознавање, тачна локализација оштећеног неурона и хитно упућивање на снимања су кључне за офталмологе у циљу превенције и лечења по живот опасних стања. Локални апраклонидин, поред дијагностичког значаја код Хорнеровог синдрома, може да коригује птозу било због естетских разлога, било због смањења горњег видног поља.

Кључне речи: Хорнеров синдром; апраклонидински тест; шваном симпатичког ланца

Примљен • Received: 12/08/2015 **Прихваћен • Accepted:** 05/04/2016

Recurrent malignant otitis externa with multiple cranial nerve involvement: A case report

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SUMMARY

Introduction Necrotizing otitis externa is a rare but conditionally fatal infection of external auditory canal with extension to deep soft tissue and bones, resulting in necrosis and osteomyelitis of the temporal bone and scull base. This condition is also known as malignant otitis due to an aggressive behavior and poor treatment response. Early diagnosis of malignant otitis is a difficult challenge. We present an illustrative case of necrotizing otitis externa and suggest some strategies to avoid diagnostic and treatment pitfalls.

Case Outline A 70-year-old patient presented with signs of malignant otitis externa, complicated by peripheral facial palsy. Adequate diagnostic and treatment procedures were performed with clinical signs of resolution. The recurrence of malignant infection had presented three months after previous infection with multiple cranial nerve neuropathies and signs of jugular vein and lateral sinus thrombosis. An aggressive antibiotic treatment and surgery were carried out, followed by substantial recovery of the patient and complete restoration of cranial nerves' functions.

Conclusion Necrotizing otitis externa is a serious condition with uncertain prognosis. The suspicion of malignant external otitis should be raised in cases of resistance to topical treatment, especially in patient with predisposing factors. Evidence-based guideline for necrotizing otitis externa still doesn't exist and treatment protocol should be adjusted to individual presentation of each patient.

Keywords: external ear; pseudomonas infection; facial paralysis; skull base; osteomyelitis

INTRODUCTION

Necrotizing otitis externa (NOE) is a rare but conditionally fatal infection of external auditory canal with extension to the deep soft tissue and bones, resulting in necrosis and osteomyelitis of the temporal bone and scull base. This condition is also known as malignant otitis due to aggressive behavior and poor treatment response.

It usually affects elderly individuals with a serious comorbidity, such as diabetes or immunocompromised patients. *Pseudomonas aeruginosa* is identified in the culture of external auditory canal in great majority of cases, although other microorganisms such as *Staphylococcus aureus*, *Klebsiella oxytoca*, *Proteus mirabilis* or *Aspergillus* species may cause a serious form of NOE [1]. Fungal infection should be suspected in all cases with no clinical signs of improvement after adequate anti-*Pseudomonas* treatment [2, 3].

Insidious course of the disease is responsible for the diagnostic difficulties that may be encountered in the early stages of the infection, further causing delay of appropriate treatment and occurrence of complications. Advanced stages of necrotizing otitis are associated with severe neurological disorders characterized by multiple cranial nerve neuropathies and intracranial extension [4]. Facial nerve paralysis is the indicator of stylomastoid foramen involvement and is usually the first neurological sign of disease progression [5].

We present an interesting case of NOE and suggest some strategies to avoid diagnostic and treatment pitfalls.

CASE REPORT

A 70-year-old male patient presented with severe otalgia, ear canal suppuration and peripheral facial palsy that were treated for six weeks with systemic and local antibiotic therapy in another institution. The patient suffered from diabetes for years and had a good glucoregulation, optimized with therapy prescribed by endocrinologist.

By close inspection of the patient, signs of peripheral paralysis of facial nerve (House–Brackmann scale, grade III) and vaguely bordered tumefaction below auricle were noticed. Otomicroscopy revealed extensive skin edema of external ear canal that detained ear drum visualization. Granulation tissue was observed at the bottom of the canal, along with thick inflammatory secretion that showed the presence of *Candida* species during culturing. Pure tone audiometry depicted severe mixed hearing loss on the affected side and severe sensorineural hearing loss on the other side.

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The parameters of inflammation (erytrocyte sedimentation rate 140, C-reactive protein 110 mg/L) were greatly increased during laboratory testing and ultrasound examination of the neck revealed multiple reactive lymph nodes up to 10 mm in size. CT scan showed inflammatory changes and osteolysis of bony portion of external auditory canal and mastoid process (Figure 1).

The patient was treated with high-dose antibiotic therapy (combination of third-generation cephalosporins and clindamycin), followed by surgery. A cortical mastoidectomy was performed along with excision of the extremely hypertrophic and granulomatous skin of external hearing canal. Histological analysis of meatal skin, middle ear mucosa and bony septa revealed signs of chronic inflammation and bone necrosis and calcification. The patient was discharged from the hospital after six weeks of systemic antibiotic therapy with clinical signs of recovery. During a two-month follow-up, otomicroscopy finding was normal and general condition significantly improved.

However, three months after the discharge, the patient's condition worsened. Fever and weakness were followed by neurological disorders such as facial palsy, swallowing and speech difficulties, as well as taste disorders. The patient also had severe otalgia and purulent discharge from the external auditory canal; however, this time, swab analysis results came positive to ciprofloxacin-resistant strain of *Pseudomonas aeruginosa*. Neurological status revealed impaired function of VII, IX, and X cranial nerves.

CT scan and MR imaging showed devastating osteomyelitis of the petrous pyramid of the temporal bone, otomastoiditis and signs of jugular vein and lateral sinus thrombosis (Figure 2). Multidisciplinary treatment was carried out by an otorhinolaryngologist, neurologist, neurosurgeon, vascular surgeon, and infectologist. Intensive antibiotic therapy with vancomycin, ceftazidime, and metronidazole combined with antimycotics gave positive results on the condition of the patient, decreasing the levels of inflammatory parameters after seven weeks of treatment. Histological analysis and findings during revision surgery of the middle ear and mastoid cavity showed no signs of active inflammation. The patient presented substantial postoperative recovery that was verified on CT scan. During a six-month follow-up the patient's hearing improved and cranial nerves functions were completely restored.

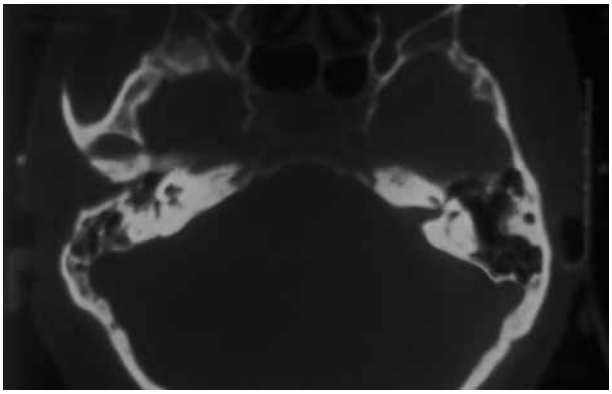

Figure 1. CT scan: Inflammatory changes involving osseous portion of external auditory canal and mastoid process

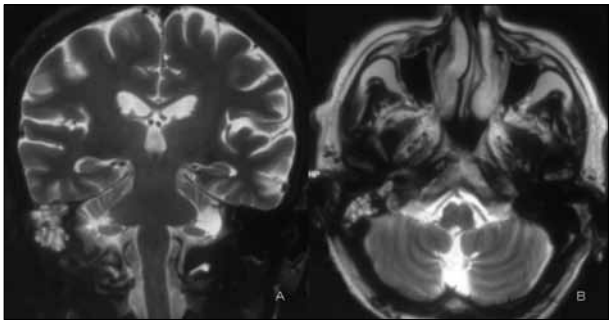

Figure 2. MR imaging: purulent collection involving skull base (A – coronal view; B – axial view)

DISCUSSION

Early diagnosis of NOE is a difficult challenge. Various authors suggest diagnostic criteria and treatment recommendations, although evidence-based guideline for necrotizing otitis externa still doesn't exist. The set of criteria for diagnosis of NOE established by Levenson et al. [6] is frequently cited in literature and is based on characteristic clinical findings, the isolation of *Pseudomonas aeruginosa* in aural secretion and clinical settings of diabetes. More than two thirds of authors agree that presentation of otitis externa refractory to treatment is a major diagnostic criterion [7]. Jacobsen and Antonelli [8] reported 6.9 months as the mean time necessary to make a diagnosis of NOE, and history or clinical presentation overlapping with benign otitis externa were identified as primary reasons for diagnostic delay.

Pseudomonas aeruginosa is isolated from aural drainage in about 95% of NOE cases [9]. Necrotizing otitis requires an intensive and prolonged antimicrobial therapy, although role of topical antipseudomonal therapy is controversial. Recurrent topical use of quinolones repels local signs of infection, but also it increases the risk of negative cultures or development of resistant strains. Our patient was formerly treated with ciprofloxacin ear drops in another institution, and during the first hospitalization at our clinic we were unable to isolate *Pseudomonas aeruginosa* in ear canal secretion. On the other hand, ciprofloxacin-resistant strain of *Pseudomonas aeruginosa* was identified as the causative agent during the second hospitalization. This resistance may be explained by prolonged antibiotic treatment and local use of ciprofloxacin ear drops. Le Clerc et al. [10] reported that 25% of *Pseudomonas aeruginosa* strains isolated in NOE patients are resistant to ciprofloxacin. Also, an increasingly implicated organism in NOE is methicillin-resistant *Staphylococcus aureus*, which may be found in 15% of cases [11]. In addition, topical antibiotic therapy may affect the incidence of mycotic infections by accelerating fungus growth. Mycotic infection should be suspected in all cases of malignant otitis externa with no signs of improvement after intensive antibiotic therapy and requires multiple deep tissue biopsies and isolation from blood cultures or fistula exudates [1, 12].

doi: 10.2298/SARH1606315D

Imaging studies have an irreplaceable role in determining the extent of the disease and the response to therapy. CT scan and MR imaging are both used in assessment of NOE; CT scan is superior in visualization of bone erosion, while MRI has an advantage in evaluating intracranial and neurological complications. However, their utilization in determining disease resolution after treatment is limited due to inability of differentiation between active inflammation and tissue remodeling. According to a study by Al-Noury and Lotfy [13], all patients had cortical bone changes on CT scan and about two thirds of patients had soft tissue changes on MR imaging, a year after disease resolution. On the other hand, some nuclear medicine studies, such as ^{67}Ga scanning or ^{111}In-labeled leukocyte scintigraphy combined with SPECT/CT are powerful tools that can differentiate between healing tissue and active inflammation and therefore are used to document resolution of infection [14].

Advanced stages of NOE have been associated with multiple cranial nerve neuropathies. Facial nerve paralysis is usually the first neurological disorder in NOE and it is reported in about 25% of cases [15]. It represents a sign of disease progression, but it doesn't seem to be an indicator of adverse prognosis [16]. Decompression is not treatment of choice in such cases since it will not restore neural function due to damaging of nerve integrity and replacing it with granulation tissue. Fortunately, thanks to newer powerful therapy, the great majority of patients experience improvement or total recovery of facial nerve function.

Involvement of cranial nerves IX, X, XI, and XII is typically in that order and represents a sign of infection spreading to jugular foramen, presenting with symptoms such as dysphonia or dysphagia. Further spread of the disease, involving petrous apex, may affect cranial nerves V and VI with signs of Gradenigo's syndrome. Scull base osteomyelitis secondary to malignant otitis externa is associated with multiple cranial nerve palsies in about 40% of patients. Also, the recurrence of necrotizing infection affects the severity of neurological presentation. It is reported that four fifths of patients with recurrent NOE suffered from facial nerve paralysis, which was twice as often as glossopharyngeal and vagus nerves' paralysis concurrently, while all lower cranial nerves were involved in 20% of patients [17]. Lower cranial nerve palsies are significantly more likely to have a good treatment response compared with facial nerve palsy [18]. Our patient suffering from recurrent necrotizing otitis complicated with scull base osteomyelitis had a complete resolution of all neurological deficits. Facial nerve had the longest period of complete functional recovery, in comparison with other cranial nerve involved by infection.

Treatment of NOE comprises control of glycemia level, improvement of general condition, local debridement of granulation tissue, cleansing of external auditory canal and aggressive antimicrobial treatment for at least six weeks. Great effort must be taken to make accurate diagnosis and begin with adequate treatment in early stages of the disease in order to minimize complication rate. Verim et al. [19] found that time period of more than 30 days elapsed between the onset of symptoms for NOE and admission to hospital has a significant impact on patient outcome and affects the duration of recovery.

Accurate timing of treatment suspension represents a difficult challenge for clinicians due to possibility of residual disease in spite of clinical resolution. ^{67}Ga or ^{111}In-labeled leukocyte scintigraphy combined with SPECT/CT should be repeated every two to four weeks during the treatment until normalization [14]. Factors that contribute to recurrent necrotizing otitis are still to be determined, although insufficient duration of treatment may play a significant role.

In fungal NOE, continued treatment for more than 12 weeks with amphotericin B is recommended, but its toxic profile has to be taken into consideration, especially in patients with serious comorbidities [1]. It is reported that voriconazole may be very effective agent for management of NOE caused by *Aspergillus* species but its precise role in therapeutic protocol had not still been documented [20]. Fungal infections are more invasive than bacterial forms of the disease and require surgical intervention four times as often, as reported by Hamzani et al. [2].

Surgical treatment has been ceded a leading role to an aggressive antimicrobial therapy and today it is used for local debridement, bony sequestrum removal, or incision of abscess. In some cases, tumors of temporal bone may have a clinical presentation similar to NOE and histopathological analysis is mandatory to exclude the malignancy [21].

Extensive surgical approach is not usually used due to its inability to prevent the spread of necrotizing infection; on the contrary, it brings the risk of further progression of the disease. On the other hand, there is a standpoint that radical mastoidectomy should be carried out in the majority of cases, especially if the fungi are involved as causative factors [1]. Hyperbaric oxygen is also used as an adjuvant treatment for malignant otitis externa, but there is no clear evidence regarding efficiency of this kind of treatment compared with combination of antibiotics and surgery [22].

Despite the fact that, nowadays, clinicians are provided with very potent antimicrobial agents and powerful diagnostic tools, the recurrence of malignant infection is common and mortality rate remains about 20% [17]. Study by Soudry et al. [23] showed that five-year survival is 44% in patients older than 70 years of age. The factors found to be associated with high mortality are multiple cranial nerve involvement, intracranial or scull base extension, and poor initial treatment response.

In conclusion, necrotizing otitis externa is a dangerous disease with high mortality rate in immunocompromised patients. Successful recovery of patients requires early diagnosis and aggressive antimicrobial treatment. In our case, the disease was persistent and aggressive despite prolonged and extensive treatment. The estimation was based on general condition of the patient, presenting symptoms, blood analysis, imaging findings (CT, MR) and swab analysis. However, our experience with conservative therapy in combination with surgery achieved a positive final outcome for the patient.

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Понављајуће малигно запаљење спољашњег ува са захватањем више кранијалних нерава: приказ болесника

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КРАТАК САДРЖАЈ

Увод Некротизирајуће запаљење спољашњег ува је ретка, мада условно летална инфекција спољашњег слушног ходника са ширењем на дубока мека ткива и кост, која може да доведе до некрозе и остеомијелитиса темпоралне кости и базе лобање. Ово обољење је такође познато као малигни отитис због својих агресивних карактеристика и слабог терапијског одговора. Рана дијагноза представља тежак изазов. Приказујемо интересантан случај некротизирајућег запаљења спољашњег ува и дајемо предлоге како избећи дијагностичке и терапијске замке.

Приказ болесника Седамдесетогодишњи болесник је испољио знакове малигног отитиса, компликованог парализом фацијалног живца. Спровођење адекватног дијагностичког и терапијског протокола довело је до резолуције обољења. Релапс малигне инфекције се јавио три месеца након претходне инфекције, изазивајући функционални испад више кранијалних живаца и показујући знакове тромбозе југуларне вене и сигмоидног синуса. Спроведена је агресивна антибиотска терапија и хируршко лечење, што је довело до изузетног опоравка болесника и комплетног опоравка функције кранијалних нерава.

Закључак Малигни отитис представља озбиљну инфекцију са неизвесном прогнозом. Сумња на ово обољење требало би да постоји у случајевима слабог одговора на топикалну терапију, посебно код пацијената са предиспонирајућим факторима. Смернице за некротизирајући отитис спољашњег ува засноване на доказима и даље не постоје; стога би протокол лечења требало прилагодити сваком пацијенту индивидуално.

Кључне речи: спољашње уво; инфекција псеудомонасом; фацијална парализа; база лобање; остеомијелитис

Примљен • Received: 03/09/2015 **Прихваћен • Accepted:** 21/12/2015

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):320-324
DOI: 10.2298/SARH1606320J
ПРИКАЗ БОЛЕСНИКА / CASE REPORT
UDC: 616.132-089-06

Acute renal failure and hepatocellular damage as presenting symptoms of type II aortic dissection

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SUMMARY

Introduction Pericardial effusion can be a consequence of a number of pathological conditions, and as such it can cause impaired left ventricular filling followed by decreased cardiac output and blood pressure. This kind of hemodynamic compromise and its consequences are extremely uncommon unless pericardial effusion causes tamponade.

Case Outline We describe a very rare case of a 30-year old male patient, with an acute aortic dissection type II causing pericardial effusion without clinical nor echocardiographic signs of tamponade, while presenting with an acute renal and hepatic failure. After initial diagnostic uncertainties, and following final diagnosis of an acute aortic dissection, this patient underwent surgical aortic valve replacement with a satisfactory outcome.

Conclusion It is important to underscore the significance of clinical situation of simultaneously existing acute renal and hepatic failures in the setting of a "non-tamponade" pericardial effusion, following a type II aortic dissection. Although most commonly aortic dissection presents itself with typical clinical symptoms or patient history data, it is not that unusual for it to be hidden in an entirely atypical clinical milieu as the one described in this case.

Keywords: aortic dissection; intimal flap; renal failure; hepatic failure; pericardial effusion

INTRODUCTION

Pericardial effusion is a complication of a number of pathological conditions such as aortic dissection, trauma, malignancies, infections, metabolic disorders, and heart failure [1, 2]. As a consequence of impaired left ventricular diastolic filling, cardiac output and systemic blood pressure are decreased, which in turn leads to reduced hepatic and renal blood flow, thus causing ischemic injury to these organs [1, 3]. These changes are extremely rare if pericardial effusion doesn't cause tamponade.

We describe a very rare case of an acute aortic dissection type II causing pericardial effusion without clinical or echocardiographic signs of tamponade, while presenting with an acute renal failure (ARF) and hepatic insufficiency as well.

CASE REPORT

One day prior to the admission to our hospital, a 30-year-old male patient presented to his primary care clinic complaining of a chest pain spreading to the neck and jaw. He started having these symptoms a couple of days before, and during this period he also became oliguric.

Clinical examination revealed a blood pressure of 120/70 mmHg and 110/65 mmHg (right and left arm, respectively). On auscultation, there was normal breathing, heart sounds were not diminished, while subtle diastolic murmur was heard above the aorta. There was no pulsus paradoxus. Discrete jugular venous distension was visible. The liver wasn't palpable on the right costal margin. There were no signs of skin hemorrhaging.

Electrocardiogram (ECG) showed a sinus rhythm with heart rate of 60 beats per minute and negative T waves in D2, aVF, V5–V6. There were no changes in the ST segment. QRS complex voltage was normal, and electrical alternans was absent.

Initial laboratory test results disclosed raised levels of inflammatory markers, a fall in platelet count, and signs of hepatic and renal failure. Troponin values were also elevated (Table 1). Chest X-ray confirmed widened mediastinum.

There were history data about this patient having prior contact with pesticides, but subsequent toxicology report excluded poisoning as a possible etiology of the liver injury.

Abdominal ultrasound showed that there were no focal hepatic lesions. The kidneys were normal in size. There was no hydronephrosis, and the blood flow pattern, which was measured on the basis of Doppler examination, was also normal. Abdominal aorta dimension was normal and there were no signs of dissection or thrombotic masses in its lumen.

Focused echo examination revealed dilated aortic root and the ascending part of the aorta,

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Table 1. Complete blood cell count and plasma biochemical values on admission and during the critical phase of the illness

Variables	Time (hours)		Reference range
	8:00	20:00	
White blood cell count	18.5	18.1	3.4–9.7 $\times 10^9$/L
Red blood cell count	5.2	3.5	4.34–5.72 $\times 10^{12}$/L
Hemoglobin	154	107	138–175 g/L
Platelet count	81	37	158–424 $\times 10^9$/L
Hematocrit	/	0.311	0.415–0.53 L/L
Mean corpuscular volume	/	89.1	83–97.2 fL
Sedimentation rate	55	/	3–15 mm/hour
Aspartate aminotransferase	3,250	3,630	0–37 U/L
Alanine aminotransferase	3,630	5,195	0–41 U/L
Gamma glutamyltransferase	/	66	0–55 U/L
Total bilirubin	9	10	0–20.5 µmol/L
Lactic acid dehydrogenase	6,710	7,780	220–460 U/L
Potassium	3.9	4.4	3.5–5.1 mmol/L
Blood urea nitrogen	13.6	22.7	2.5–7.5 mmol/L
Creatinine	436	551	59–104 µmol/L
Glomerular filtration rate	15	11	>60 mL/min/1.73 m^2
International normalized ratio	1.53	1.51	0.8–1.2
Prothrombin time	20	19	10–14
Activated partial thromboplastin time	36.5	43.2	22–32 seconds
C-reactive protein	85	/	0–3 mg/L
Creatine kinase	92	311	0–200 U/L
Creatine kinase MB	31	71	0–24 U/L
Troponin I	5.3	7	>0.30 µg/L
D-dimer	/	24.06	>0.5 mg/L
Fibrinogen	/	3.6	2.0–4.0 g/L

accompanied by a moderate to severe aortic regurgitation. There was also evidence of a moderate circular pericardial effusion. Left ventricular walls were hypertrophic.

This led to a decision to perform a contrast-enhanced computed tomography (CT) scan, which disclosed a dilated ascending part of the aorta (Figure 1a), as well as a structure which was highly suspicious of an intimal membrane located in the same part of the aorta (Figure 1b). This poor visualization of an intimal flap and its atypical configuration were interpreted to be a part of the dissection process, since a moderate pericardial effusion (up to 16 mm) of hemorrhagic density was also noted (Figure 1a).

Since these initial diagnostic findings were considered to be highly suggestive of an aortic dissection, this patient

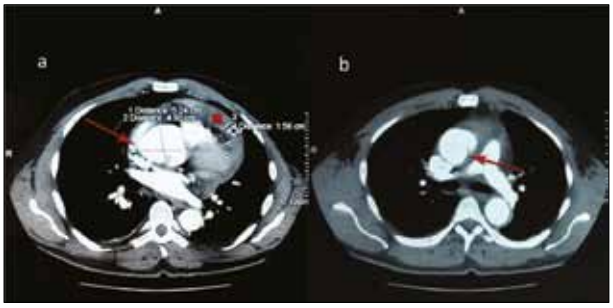

Figure 1. Contrast-enhanced computed tomography: (a) dilated ascending part of the aorta (arrow) with a moderate circular pericardial effusion (arrowhead); (b) poor visualization of an intimal flap (arrow) located in the same segment of the aorta

was referred to the Emergency Care Unit of our tertiary care hospital.

On the admission, physical examination was repeated and it wasn't significantly different from the previous one. Subsequent laboratory tests revealed a further deterioration of the liver and renal function, substantial fall in hemoglobin levels and red blood cell count, as well as additional drop in platelet count. D-dimer value was elevated (Table 1).

Because of the existing ARF, a nephrologist was consulted regarding the safety of repeating a CT scan with intravenous contrast application. Since the patient would most probably need hemodialysis later on, and as time was of the essence, the nephrologist's decision was against performing a CT scan again.

Based on a high clinical suspicion that this was a case of an acute aortic dissection, transthoracic echocardiogram (TTE) followed by a transesophageal echocardiogram (TOE) were performed instead. TTE revealed an intimal membrane located in the ascending part of the aorta. This was most clearly visible only after the patient was positioned in the right lateral decubitus (Figure 2a). Previously noted circular pericardial effusion was also evident. At this point, there were no echocardiographic criteria for tamponade. On TOE, a proximal extension of the dissection was revealed – a very thin intimal flap was identified just above the non-coronary cusp (Figure 2b). The dissection process was traceable up to the origin of the great vessels off the aortic arch. Moderate to severe aortic regurgitation was present (Figure 2c). Also, the aortic wall above the non-coronary cusp appeared to be thinned, so it was

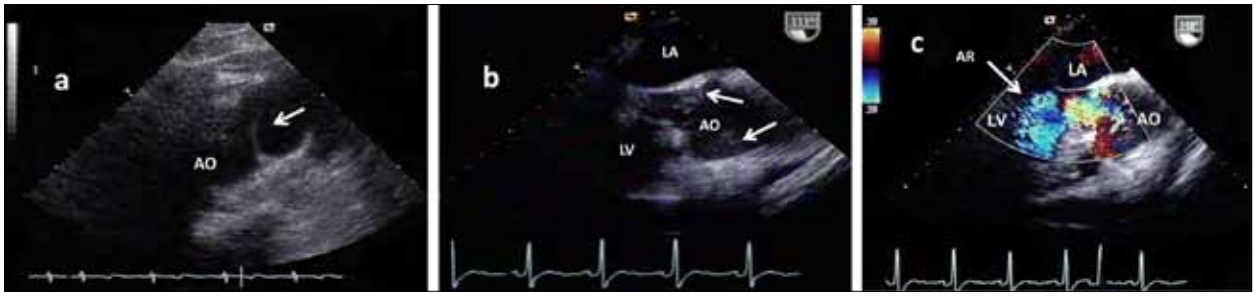

Figure 2. (a) Transthoracic echocardiogram (right lateral decubitus); an intimal membrane located in the ascending part of the aorta (arrow); (b) transesophageal echocardiogram; proximal extension of the dissection – thin intimal flap (arrows) originating just above the non-coronary cusp with a thinned aortic wall in that segment; (c) aortic regurgitation (arrow)
AO – aorta; AR – aortic regurgitation; LA – left atrium; LV – left ventricle

considered to be a possible place of communication with the pericardial sac (Figure 2b).

Urgent aortic root replacement surgery using the Cabrol technique was performed [4]. Within the next couple of hours the patient's urine output started to increase. In the upcoming days, his renal function started to normalize, which was followed by his other laboratory results returning to normal as well.

Postoperative echo examination, performed four weeks after the procedure, showed a properly functional prosthetic aortic valve. Peak velocity and pressure gradient across the valve were 2.68 m/s and 28 mmHg, respectively, while mean pressure gradient was 17 mmHg. Mild transvalvular aortic regurgitation was present. Graft that was placed in the ascending part of the aorta was also visible. There was no pericardial effusion.

DISCUSSION

Current literature is very limited regarding case reports of a type II aortic dissection presenting both with symptoms of an ARF and hepatocellular damage occurring as a consequence of several pathologic mechanisms inducing malperfusion and ischemic injury to these organs.

Laboratory tests were reflective of the setting of an acute aortic dissection: elevated values of systemic inflammatory biomarkers (sedimentation rate, white blood cell count, C-reactive protein, D-dimer), as well as raised troponin levels [5, 6, 7]. Extremely low platelet count, prolonged prothrombin time, and elevated D-dimer value could be explained by an existing acute liver injury and beginning of a consumptive coagulopathy [8].

An aortic dissection process itself is characterized by elevated D-dimer values, independent of consumptive coagulopathy. For this reason, measuring D-dimer values is an integral part of laboratory testing and flowchart for diagnosis and decision-making in acute aortic syndrome [9]. This kind of biochemical testing is most suitable for ruling out an acute aortic dissection in patients with a low likelihood of the disease [9].

The acute hemorrhagic state that this patient was in was to some extent a result of blood loss both into the pericardium and a false lumen. Some reports describe the concept of platelet dysfunction (impaired platelet aggregation) as additional contributing factor for the state of altered hemostasis and bleeding tendency [8]. We think that endothelial and liver injury were both contributive to the impairment of hemostasis in this case.

Decreased glomerular filtration rate and elevated blood urea nitrogen and creatinine levels were a clear consequence of the existing ARF. His rising aspartate aminotransferase and alanine aminotransferase levels were indicative of a shock liver [10].

Malperfusion syndrome occurs in up to 30% of patients with acute aortic dissection [9]. It is known that pericardial effusion can cause impaired left ventricular diastolic filling, which in turn leads to decreased cardiac output and systemic blood pressure. These hemodynamic changes can ultimately result in a reduced renal and hepatic blood flow, thus causing ischemic injury to these organs [1, 3]. It is, however, important to emphasize that pericardial effusion is not the only pathologic factor contributing to the malperfusion of visceral organs. In this particular case, the other key mechanism of renal and hepatic damage was the dynamic compression of the true lumen due to high pressure blood accumulation in the false lumen, which is the result of a large proximal inflow into the false lumen and insufficient outflow into the distal aorta [9]. Moderate to severe aortic regurgitation furthermore lowered the effective stroke volume, thus also contributing to hypoperfusion of visceral organs.

Since this patient developed symptoms a couple of days before being admitted to his primary care clinic, it is possible that fast accumulation of even a small amount of blood in pericardium initially led to substantial hemodynamic instability, which later entered into spontaneous resolution. This could explain the fact why this patient did not develop clinical signs of cardiac tamponade. Still, we hypothesize that even without echocardiographic or clinical signs of tamponade, this moderate pericardial effusion played an important role in creating a hemodynamic compromise of systemic circulation with the occurrence of both hepatic and renal failure.

ARF can additionally be explained with possible hormonal changes. Decreased left ventricular trans-mural pressure causes a decrease in atrial natriuretic peptide secretion [11]. Also, increased secretion of antidiuretic hormone in the setting of pericardial effusion has been noted in animal studies [12, 13]. Its physiologic effect implies to renal vasoconstriction and volume retention. Since this was probably a case of unrecognized primary hypertension, it is plausible that this patient suffered from a subclinical form of hypertensive renal impairment. This could have facilitated exacerbation of renal failure because of altered hemodynamics secondary to the pericardial effusion [1]. Liver damage can also be explained by the existing venous hypertension and congestion, which led to the mechanical stretch of hepatocytes.

Regarding the incomplete result of the CT scan, there are a number of pitfalls that can cause a diagnostic problem. Some of these are related to technical factors like the improper timing of contrast material administration relative to image acquisition, which in turn results in poor visualization of an intimal flap and a false negative diagnosis [14]. In this particular case this could have been one of the explanations, since manual injection of contrast material was used, rather than the automated intravenous contrast injection which has the ability of applying high flow rates. Conventional spiral CT angiography of the aortic root is also prone to cardiac and aortic wall motion artifacts [14]. Here, a non-ECG-gated recording of these structures was done, leaving it liable to mistakes regarding the dynamic changes of aortic root dimensions and its shape throughout the cardiac cycle [15]. It is also possible that the proximal extension of the dissection was overlooked since it was located immediately above the non-coronary cusp and thus disguised as part of the aortic valve apparatus.

doi: 10.2298/SARH1606320J

In this case report we also wanted to emphasize the significance of TTE, in particular its right lateral decubitus as a mandatory part of the ascending aorta examination, since that was the position where the first clear image of an intimal flap was obtained from.

TOE was also a diagnostic method of choice in this case due to the state of ARF that this patient was in, since another contrast application would deteriorate renal function even more.

One of the key learning points of this case report is to underscore the importance and raise awareness about clinical situations of simultaneously existing ARF and hepatic failure, in the setting of a "non-tamponade" pericardial effusion, following a type II aortic dissection.

Although most commonly aortic dissection presents itself with typical clinical symptoms or patient history data, it is not that unusual for it to be hidden in entirely atypical clinical milieu as the one described in this case. Therefore, aortic dissection should be thought of as a "master of disguise" and should always cross the clinician's mind, especially in clinical settings that seem ambiguous.

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Акутна бубрежна и хепатична инсуфицијенција као презентујући симптоми аортне дисекције типа II

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КРАТАК САДРЖАЈ

Увод Перикардни излив може настати као последица низа патолошких стања и довести до смањеног дијастолног пуњења леве коморе, што даље води смањењу минутног волумена и артеријске тензије. Овакав хемодинамски компромис циркулације и његове последице су узузетно ретки уколико перикардни излив не изазове тампонаду срца.

Приказ болесника Акутно настала аортна дисекција типа II код болесника старог 30 година довела је до појаве перикардног излива који није био компликован клиничким ни ехокардиографским знацима тампонаде срца, али се презентовала знацима акутне реналне и хепатичне инсуфицијенције. Након спроведене дијагностике, болесник је подвргнут хитној хируршкој интервенцији замене аортне валвуле са задовољавајућим исходом.

Закључак Важно је истаћи значај клиничке слике у којој истовремено постоји акутна бубрежна и хепатична инсуфицијенција на терену аортне дискеције типа II компликоване перикардним изливом без знакова тампонаде срца. Аортна дисекција се често презентује типичним клиничким симптомима и уобичајеним анамнестичким подацима. Клинички куриозитет представља њена појава и последице настале у околностима приказаним у овом случају.

Кључне речи: аортна дисекција; интимални флап; ренална инсуфицијенција; хепатична инсуфицијенција; перикардни излив

Примљен • Received: 12/11/2015 **Прихваћен • Accepted:** 09/03/2016

Early post-transplant lymphoproliferative disorder – Case of fatal lymphoma after kidney transplantation

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SUMMARY

Introduction Post-transplant lymphoproliferative disorder (PTLD) is a common malignancy following organ transplantation. Risk for PTLD is associated with the use of anti-thymocyte globulin in the prevention and treatment of acute rejection following kidney transplantation.

Case Outline We report a case of fatal PTLD presented with sudden onset of fever. A 33-year-old male patient with primary diagnosis of left kidney agenesia underwent kidney transplantation six years following hemodialysis treatment initiation. Deceased donor was a 66-year-old female whose cause of death was cerebrovascular accident. Immunosuppressive regimen consisted of basiliximab, corticosteroids, tacrolimus, and mycophenolate mofetil. Six months upon transplantation the patient was hospitalized due to fever of unknown origin. All microbiological samples were negative, but abdominal ultrasound revealed round solid mass in the right native kidney. Right nephrectomy was performed showing tumor 35 × 35 × 20 mm in size within the 70 × 40 × 35 mm kidney. Pathohistological analysis confirmed very rare monomorphic B-cell PTLD – B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma.

Conclusion We consider this case of PTLD following kidney transplantation particular because of the tumor mass in native kidney after basiliximab induction and rare pathohistology. In a transplanted patient with fever, PTLD must always be considered, irrespective of immunosuppressive regimen.

Keywords: kidney transplantation; post-transplant lymphoproliferative disorder; immunosuppression; fatal outcome

INTRODUCTION

Post-transplant lymphoproliferative disease (PTLD) is a common cause of cancer-related mortality following solid organ transplantation that occurs in 2–3% of solid organ recipients [1, 2]. Five-year mortality is between 35% in the United States and 60% in Europe [3, 4]. Mortality is higher in recipients with disseminated lymphoma, brain localization, invasion of serous membranes and T-cell PTLD [5]. The outcome of early onset PTLD in adult organ recipients is also shown in Table 1 [6–13].

PTLD is typically aggressive, with a rapid onset. According to the most recent World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues, PTLD is divided into three categories: early lesions, polymorphic PTLD, and monomorphic PTLD [14]. Monomorphic PTLD is mostly of B-cell origin, while T-cell PTLD and Hodgkin lymphoma-like PTLD are uncommon. Overlapping morphological features rarely appear. Almost 90% of B-cell PTLD as well as 15% of T-cell PTLD are associated with Epstein–Barr virus (EBV) infection [15]. Type of organ transplanted, recipient age, and intensity of immunosuppression are some of PTLD risk factors [16]. Incidence is high immediately after transplantation, decreasing subsequently, then rising again four to five years after the transplantation.

Generally, treatment entails reducing immunosuppression or administering anti-cancer agents and rituximab [17].

CASE REPORT

A 33-year-old Caucasian male patient with left kidney agenesia and end stage right kidney disease underwent a deceased donor kidney transplant from a 66-year-old Caucasian female donor. The patient had been treated with hemodialysis for six years prior to the transplant. Hypertension, chronic anemia, appendectomy, and subtotal parathyroidectomy were evident in the patient's medical history.

Viral serology of the donor and recipient prior to the transplant detected both (EBV) and cytomegalovirus (CMV) IgG antibodies.

Recipient human leukocyte antigen (HLA) typing confirmed A26, -; B38, 61; DR 4,11, while the donor had A 11, 31; B 38, 62; DR 4, 15. Immunosuppressive regimen included basiliximab 20 mg on days 0 and 4, followed by corticosteroids, tacrolimus (TAC), and mycophenolate mofetil (MMF). Prophylactic valganciclovir was applied to prevent CMV infection. The recipient was treated with hemodialysis for

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Table 1. Early PTLD case reports with patient outcome in adult organ recipients

Author	Transplanted organ	Outcome
Bennett WM, et al. [6]	Kidney	Fatal
Biller P, et al. [7]	Kidney-pancreas	Fatal
Kitajima K, et al. [8]	Kidney	Remission
Gao C, et al. [9]	Kidney	Remission
Fedrigo M, et al. [10]	Heart	Fatal
Miyagi S, et al. [11]	Kidney-pancreas	Remission
Vishnu P, et al. [12]	Liver	Remission
Garceau P, et al. [13]	Heart	Remission

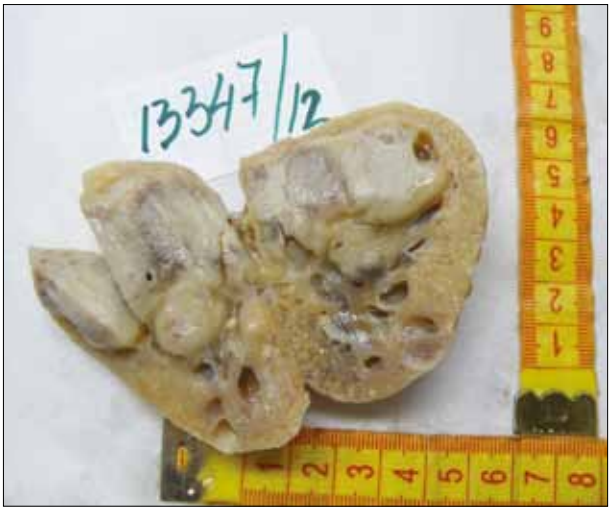

Figure 1. Macroscopic examination of the kidney: white-gray homogenous tumor with homogeneous appearance without necrosis and hemorrhage in small native cystic kidney after long term dialysis

five weeks after the transplantation due to delayed graft function. Six weeks following transplantation the patient developed severe leukopenia that required MMF temporary discontinuation and recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF) administration. Sternal puncture revealed toxic, hypocellular bone marrow. Abdominal computed tomography (CT) confirmed end-stage right kidney without solid mass. Adverse events also included urinary tract infection, diarrhea and worsening anemia. The patient was discharged after almost three months with serum creatinine level of 236 μmol/l, creatinine clearance of 39 ml/min, and proteinuria of 1 g/24 hours.

Six months post-transplantation the patient was hospitalized due to a fever. All microbiological samples were negative, but abdominal ultrasound revealed round solid mass in the right native kidney. Right nephrectomy was performed showing tumor 35 × 35 × 20 mm in size within 70 × 40 × 35 mm kidney (Figure 1).

A few days following the nephrectomy, the patient complained of pain in the lower abdomen. Abdominal CT detected multiple focal lesions in the liver and spleen, enlarged paratracheal, paraaortic, and paracaval lymph nodes, bilateral diffuse infiltrative lung lesions and pleural effusions on both sides. Microscopic examination of the tumor tissue revealed large lymphoid cells with oval or irregular nuclei, scant cytoplasm, of centroblast and immunoblast type, with rare large multinuclear cells, some of

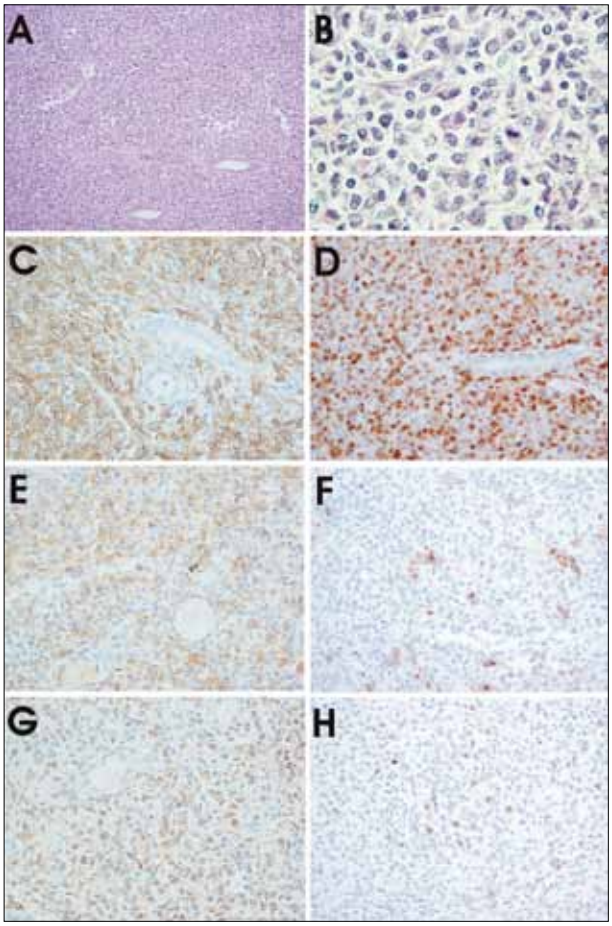

Figure 2. Microscopic examination: B-cell lymphoma, unclassifiable, with features of both diffuse large B-cell lymphoma and classical Hodgkin lymphoma; (A) diffuse proliferation of large lymphoid cells with scattered pleomorphic cells resembling lacunar and Hodgkin cells (arrows) (hematoxylin and eosin staining, ×200); (B) sheets of large cells with scanty cytoplasm and oval to round vesicular nuclei with prominent nucleoli (hematoxylin and eosin staining, ×1,000). Tumor cells were strongly positive for (C) CD20 (×400), (D) MUM1 (×400), (E) CD30 (×400), (F) CD15 (×400), (G) bcl-2 (×400), and (H) Epstein–Barr virus (×400).

which correspond to classical Hodgkin and Reed–Sternberg cells (Figure 2A, B). There were CD3+/CD5+ T lymphocytes and rare histiocytes around the neoplastic cells. Immunohistochemistry confirmed that tumor cells were negative for epithelial membrane antigen (EMA), terminal deoxynucleotidyl transferase (TdT), CD3, CD5, CD10, CD21, cyclin D1, anaplastic lymphoma kinase-1 (ALK-1) and human herpes virus-8 (HHV-8). Notably, positivity was identified for vimentin, leukocyte common antigen (LCA), PAX-5, CD79-alfa, CD43, CD23, and p53. In addition, tumor cells were positive for CD20, Multiple Myeloma Oncogene -1 (MUM-1), CD30, CD15 (focal in HRS cells) and bcl-2 (Figure 2C-G). Proliferative activity was moderate and almost 60% of the tumor cells expressed Ki-67. HRS cells were EBV-latent membrane protein (LMP) positive (Figure 2H).

Unfortunately, the patient died before the initiation of treatment.

doi: 10.2298/SARH1606325L

DISCUSSION

The frequency of PTLD differs in response to many variables, including allograft type, EBV seropositive status, intensity of immunosuppression and recipient age. Our patient had higher age-related risk for early PTLD [15]. The recipient had HLA A26, B38 that are strongly related to higher incidence of EBV-positive PTLD [18]. The donor was without PTLD protective HLA A1, B8, or DR3. The overall number of HLA mismatches was not associated with higher PTLD risk [15].

While EBV and CMV IgG negative patients have higher PTLD risk, our patient was both EBV and CMV IgG positive [19, 20].

Studies suggest that PTLD risk seems to be correlated with cumulative immune suppression [21]. Stronger immunosuppressive properties of TAC comparing to cyclosporine A might be associated with higher PTLD risk [22]. Increased incidence of PTLD was not related to MMF [23]. Monoclonal antibodies, such as basiliximab, do not increase the PTLD risk, while repeated treatment of acute rejection might increase it [24]. In the presented case, the patient was not treated by additional immunosuppression for acute rejection and only TAC treatment might be related to higher PTLD risk.

Although renal graft lymphoma infiltration was reported, this is an unusual case of PTLD lymphoma within native kidney [25]. Previously, native kidney PTLD lymphoma infiltration has been commonly described in recipients of non-renal solid organs [26]. Rare cases of PTLD with tumor mass in native kidney after kidney transplantation might be suspected as renal cell carcinoma [27].

B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma is a new entity in the updated World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues, which introduces provisional borderline categories for lymphoma that demonstrate overlapping between well-established entities [14].

We consider this case of PTLD in renal transplant recipient noteworthy because of (1) tumor mass in native kidney after kidney transplant, (2) PTLD appearance after basiliximab induction, and (3) pathohistological features overlapping between diffuse large B-cell lymphoma and classical Hodgkin lymphoma.

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Рана посттрансплантациона лимфопролиферативна болест – случај лимфома са смртним исходом после трансплантације бубрега

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КРАТАК САДРЖАЈ

Увод Посттрансплантациона лимфопролиферативна болест (ПТЛБ) често је малигно обољење после трансплантације органа. Ризик болести је повећан у случају примене антитимоцитног глобулина за превенцију и лечење акутног одбацивања после трансплантације бубрега.

Приказ болесника Приказан је случај ПТЛБ са смртним исходом, која се манифестовала изненадном појавом повишене температуре. Трансплантација бубрега урађена је после шест година лечења дијализом болеснику мушког пола, старости 33 године, са агенезијом левог бубрега. Донор је била жена старости 66 година преминула од можданог удара. Имуносупресивна терапија се састојала од базилихимаба, кортикостероида, микофенолат мофетила и такролимуса. Шест месеци после трансплантације болесник је хоспитализован због повишене температуре непознатог узрока. Сви микробиолошки узроци су били негативни, али је ултразвучни преглед абдомена указао на кружну солидну промену у десном бубрегу. Урађена је десностранa нефректомија, која је указала на тумор 35 × 35 × 20 *mm* у бубрегу величине 70 × 40 × 35 *mm*. Патохистолошки налаз је потврдио редак облик мономорфне ПТЛБ – Б ћелијски лимфом, некласификован, са особинама дифузног крупноћелијског Б лимфома и класичног Ходгкиновог лимфома.

Закључак Осим ретког патохистолошког облика ПТЛБ после индукције базилихимабом, случај је значајан због ретке локализације у нативном бубрегу код болесника који је претходно лечен хемодијализом. Код примаоца алографта бубрега у случају повишене температуре непознатог узрока увек морамо диференцијално дијагностички размотрити ПТЛБ независно од примењеног имуносупресивног протокола.

Кључне речи: трансплантација бубрега; посттрансплантациона лимфопролиферативна болест; имуносупресија; смртни исход

Примљен • Received: 14/07/2015 **Ревизија • Revision:** 13/10/15 **Прихваћен • Accepted:** 14/12/2015

Metastatic malignant melanoma of the uterus diagnosed by colposcopy

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SUMMARY

Introduction Primary and metastatic malignant melanomas represent a rare diagnosis with a small number of described cases. The aggressive nature of the tumor, non-specific symptoms, difficult diagnosis, and no official protocol about the treatment result in poor disease prognosis.

Case Outline The authors presented a 41-year-old multigravida patient. She had an operation of malignant melanoma in the occipital area of the head. She went to her gynecologist because of increased pale pink vaginal secretion. Gynecological examination didn't show any significant abnormalities apart from a slightly enlarged uterus. Papanicolaou test and vaginal secretion examination were normal. Colposcopically, a significant dark brown hyperpigmented area around 1 cm in size was observed on the posterior lip of the cervix, near the orifice and cervical canal, suspicious of melanoma, which was proven on targeted biopsy of the hyperpigmented change on the cervix, and by magnetic resonance imaging of the lesser pelvis. Classic hysterectomy with adnexectomy and regional pelvic lymphadenectomy were performed.

Conclusion This case report pointed out the significance of applying colposcopy in diagnosing suspected metastatic melanoma of the uterine cervix, along with other diagnostic methods and anamnestic data.

Keywords: malignant melanoma; uterus; colposcopy; metastases

INTRODUCTION

Malignant melanoma (MM) of the skin is a tumor developed by malignant transformation of melanocytes. The term comes from the pigment melanin, which is most frequently present although its forms are clinically different, from hyperpigmented to amyloid [1]. A common characteristic of all MMs is that they are locally aggressive and prone to early lymphogenic and vascular metastases [2]. The percentage of MMs in relation to all carcinomas is 1%, and estimated incidence of MMs in the genital region is in 3–7% range, out of which the vulva is the most common site of melanoma occurrence [3]. Due to such a low incidence, MM of the uterine cervix is a rare entity. Since 1989, only about 81 cases of uterine MMs have been described in the literature [4].

With respect to gender, the frequency of MM in the incidence of all malignant tumors is 1.6% for men and 1.5% for women [5].

CASE REPORT

The patient was a 41-year-old multigravida female. She went to her gynecologist in November 2014 because of increased pale pink vaginal secretion. Anamnesis showed that the patient had no previous serious gynecological problems and that she had been regularly undergoing routine gynecological examinations. Her medical history showed that one year previously she underwent an excision of hyperpigmented skin change in the occipital area of the head. The operation was performed in October 2013. Histopathology after the surgical excision of the lesion showed melanoma malignum nodular cutis exulcerans. The cells were epithelioid and a significant inflammatory infiltration (peritumoral and tumoral) was present, necrotic and ulcerative areas were observed, there was no angioinvasion and the number of mitoses was 10/mm^2. The skin lesion was completely removed (including more than 2 cm of the surrounding healthy tissue). The disease was graded level III according to Breslow (10 mm) and level IV according to Clark. Considering that the lesion was removed along with a region of healthy tissue and that X-ray of the lungs and echo of the liver and soft tissue were normal, the consulting doctors decided that there were no indications for specific oncologic treatment and suggested future monitoring with new analyses and tests to be performed in two months.

Until her visit to the gynecologist, there was no evidence of recurrence of the primary disease.

The patient did not see her gynecologist in the previous year. The last Papanicolaou test and colposcopy were performed when primary MM was diagnosed and surgically removed, and the findings were normal.

General examination of the visible skin did not show any new pigmented skin lesions. Gy-

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necological examination did not show any significant abnormalities. The vulva and vagina were normal with no macroscopically visible changes on the cervix, except for slight pinkish secretion flowing from external orifice of the cervical canal to posterior vaginal fornix. The uterus in anteversoflexion was slightly enlarged, which corresponds to the size of uterus after two deliveries (as is the case with our patient). Adnexa could not be palpated. Parametrium was without changes. During the examination, smears were taken for Papanicolaou test and vaginal secretion was sampled.

Papanicolaou test results were normal (PA: NILM). Vaginal secretion examination was also normal (group II). The patient was referred to the Clinic of Gynecology and Obstetrics, where colposcopy and pelvic ultrasound were performed.

Colposcopy findings were satisfactory. Transition zone was completely visualized (TYPE 1). Exocervical epithelium was squamous and iodine positive. A significant dark brown suspected hyperpigmented area around 1 cm in size was observed at 9 o'clock position, near the orifice and cervical canal. Figure 1 shows the cervix after it was covered with acetic acid. The epithelium of the exocervix is squamous, its pale pink color is normal, and it is covered with irregular, spotty area of dark brown pigmentation at 9 o'clock position. Figure 2 shows the colposcopic image of the same cervix after it was covered with Lugol's solution. It can be observed that there is the area of re-epithelialization around the orifice, partially iodine positive, and part of the cervix around the orifice at 9 o'clock position where previously hyperpigmentation was located, which appeared gray-blue after reacting to Lugol's solution.

The ultrasound examination showed the uterus in anteversoflexion (normal anatomical position), 9 cm in size, with endometrium which was hyperechogenic along the cavum and extended into the cervical canal 25 mm wide in the widest part of the central areas. The adnexa had normal position and size. The pouch of Douglas was empty and without free fluid.

Targeted biopsy of the hyperpigmented change on the cervix was performed, as well as fractional explorative curettage of the cervical canal and uterine cavity.

Histopathology findings of cervix biopsy and cervical canal and cavity curettage also showed malignant melanoma.

In order to check for local spread of the disease and possible presence of other metastases, the patient underwent magnetic resonance imaging of the lesser pelvis, X-ray of the lung, and echo of the liver. Magnetic resonance imaging findings confirmed the presence of tumors in the uterine cavity and cervix, which could correspond to secondary MM deposits, as shown in Figures 3 and 4. The change was described as heterogeneous (predominantly hyper-, but also hypodense), partially entering the stroma but without penetrating the myometrium or spreading to parametrium. The X-ray of the lungs, echo of the liver, and examination of other systems did not point to the presence of other suspicious metastatic deposits.

The patient and all her medical records were presented to consulting oncologists. Considering the localized solitary metastatic process, the consulting doctors decided to perform classic hysterectomy with adnexectomy and regional pelvic lymphadenectomy. After the surgery, the patient's histopathology findings were to be examined again by the consulting doctors in order to decide on further treatment.

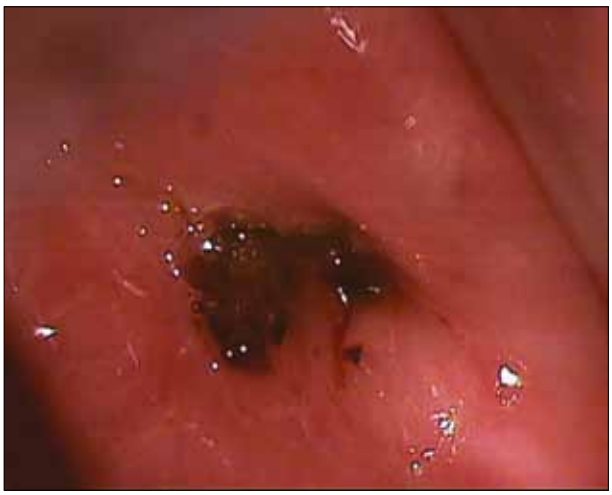

Figure 1. Colposcopic image of malignant melanoma after coating the cervix with acetic acid

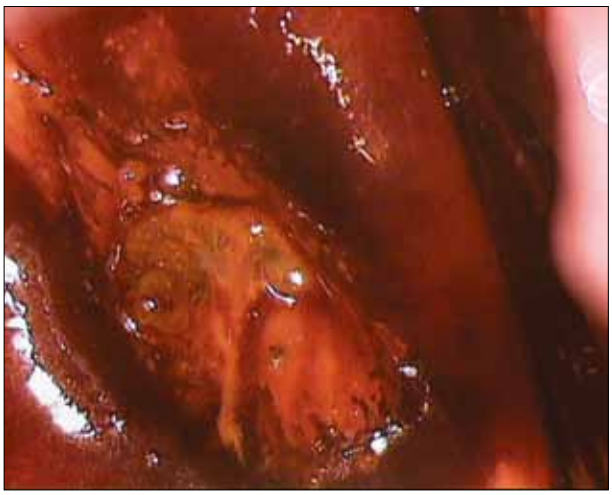

Figure 2. Malignant melanoma of the cervix after coating with Lugol's solution

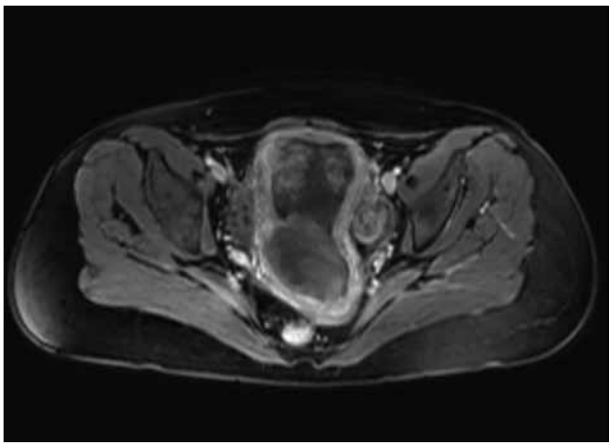

Figure 3. Magnetic resonance imaging of the lesser pelvis – cross section

The surgery was performed on December 19, 2014. The surgery was uneventful. Apart from slightly enlarged and

doi: 10.2298/SARH1606329Z

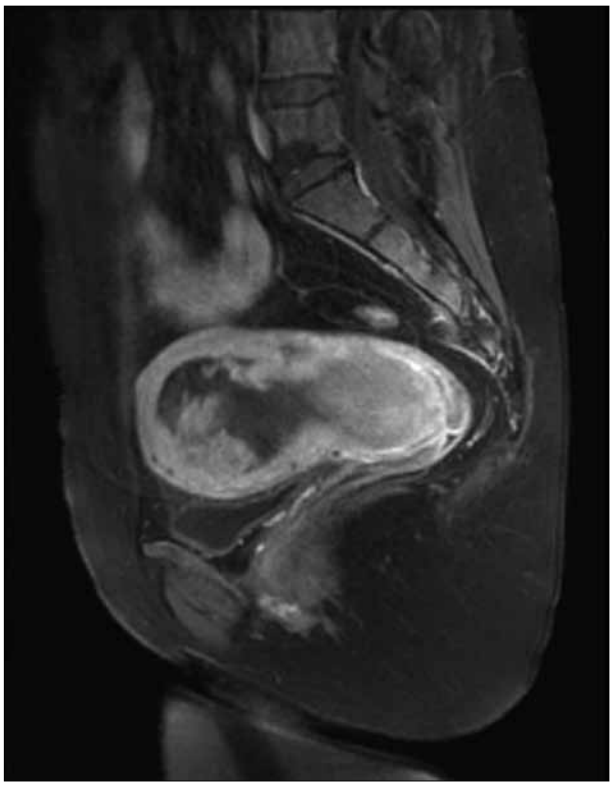

Figure 4. Magnetic resonance imaging of the lesser pelvis – longitudinal section

softened uterus, intraoperative findings of the lesser pelvis did not show any other changes. Palpation and exploration of other organs of the abdominal cavity (intestines, omentum, liver, peritoneum) did not reveal any pathological changes and hyperpigmentation. Figure 5 shows the longitudinal and cross-sectional sample of the surgically removed uterus.

The final postoperative histopathological findings showed presence of malignant melanoma in the tissue of the cervix and corpus with areas of cells of epithelioid and fusiform type, dark granulated pigmentations with number of mitoses around 8 mm^2. The sampled fat tissue and regional iliac lymphatic nodes did not have any metastatic processes.

Immunohistochemical staining confirmed the diagnosis of MM in uterus, as shown in Figures 6 and 7. Tissue samples were fixed in 10% buffered formalin solution, embedded in paraffin wax, divided into 4 μm thick sections and stained with hematoxylin and eosin. Representative material was stained with a panel of antibodies using the labeled streptavidin-biotin-peroxidase complex method according to the manufacturer's instructions (LSAB Kit, Dako, Glostrup, Denmark). The primary antibodies used were mouse monoclonal antibodies for melanosome (clone HMB-45) and rabbit polyclonal antibody for S-100 protein. The chromogen was 3, 3'-diaminobenzidine (DAB), and the slides were lightly counterstained with Meyer's hematoxylin. All reagents were acquired from Dako company.

According to anamnestic data, immunohistochemical parameters and the absence of junctional changes, the malignant melanoma of the uterus was considered to be a metastatic process.

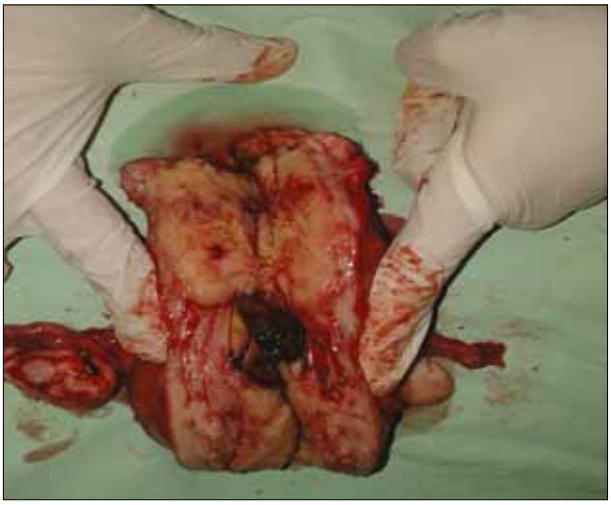

Figure 5. Longitudinal and cross-sectional macroscopic appearance of the surgically removed uterus

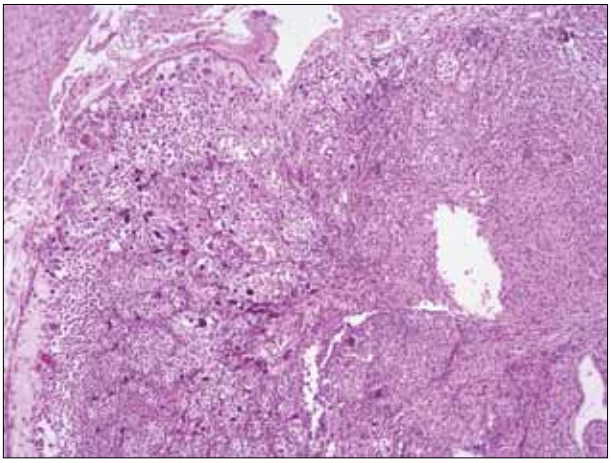

Figure 6. Atypical epithelioid melanocytes with pleomorphic nuclei and abundant cytoplasm with dusty melanin (H&E, ×10)

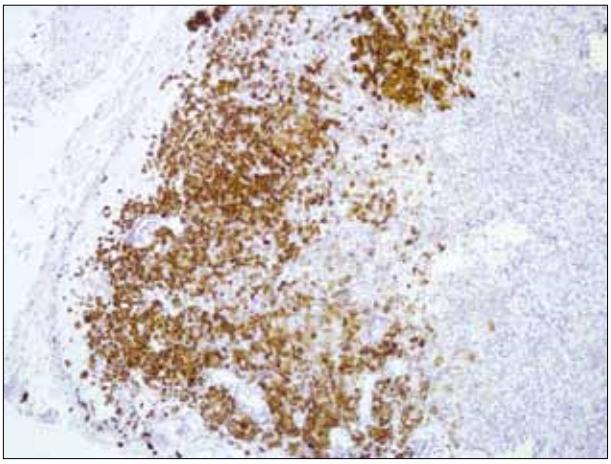

Figure 7. Diffuse immunoreactivity of HMB-45 (Streptavidin-biotin-peroxidase complex method, Meyer's hematoxylin counterstain, ×10)

Postoperative course was uneventful. The patient was generally well, released from gynecology clinic and advised to refer to the consulting oncologists for mixed-type localization (which deals with treatment and monitoring of MM) for further monitoring and continuation of specific oncological treatment of MM.

The consulting panel of doctors referred the patient to positron emission tomography to evaluate the disease spread. The patient has not returned for further check-ups since.

DISCUSSION

The degree of MM invasion can be determined in two ways: measuring the thickness of the infiltrated skin (according to Clark) and measuring the thickness of tumor in millimeters (according to Breslow) [6, 7].

Survival depends on the stage of the disease as well as its location. The prognosis and survival are most favorable when MM is located in the extremities, then the trunk, and the worst prognosis is obtained when MM is in the area of the head and neck [8].

About 20% of patients with skin MM have recurrent disease. The most common location is the place of previous treatment and regional lymphatic glands. Distant visceral metastases are most often found in the lungs, the liver and the brain. With regard to time, metastases are most frequently detected within the first five years after diagnosis [9].

In patients with metastatic disseminated changes, the prognosis is poor and characteristics and classification of the primary tumor (according to both Clark and Breslow) are no longer significant for prognosis and outcome. The treatment in these cases is palliative and its goal is to reduce the tumor mass and prevent complications caused by metastases [10].

The most important prognostic parameters for patients with distant metastatic changes are the number of metastatic places, their location, and length of remission [11].

Patients with one metastatic location have higher survival probability than patients with numerous metastases [12]. Patients with skin metastases have better prognosis than those with visceral metastases [11]. Distant metastases in lymphatic nodes and soft tissues are present in 59% of cases, while visceral metastases are most common in the lungs, with 31–36% of cases [13]. The prognosis depends on the number of lymphatic nodes with metastases, not on their size. The location of metastatic lymphatic nodes is also important because it has been demonstrated that patients with infiltrated armpit lymphatic nodes more often develop visceral metastases than patients with infiltrated inguinal nodes [14].

The concept of primary MM of the cervix is a rare diagnosis, which originated in 1960, when Cid [15] described melanocytic cells in the cervix.

This is an aggressive malignant tumor with late diagnosis because it causes non-specific problems that do not point to this disease. Apart from sparse non-specific symptoms which most often include increased vaginal secretion or irregular bleeding, there are no other specific symptoms. It can clinically manifest itself in the form of exophytic proliferation or polyps of red, blue, dark or dark brown color [16].

Cytological and histopathological picture is also not specific, especially with non-pigmented tumor cells. Pathological preparation shows strings of pleomorphic anaplastic cells with large, round or oval nucleus with protruded nucleolus and sparse cytoplasm in which yellow-brown melanin-positive pigment can be observed [17].

Cytological smear of uterine cervix MM can be normal and false-negative [18].

As an additional support to histological and differential diagnosis, immunohistochemical staining is used, including monoclonal bodies to melanin, anti-S-100 protein, anti-tyrosinase, anti-Mart-1/Melan-A and HMB-4 [19].

A particular problem appears with differential diagnosis of primary and metastatic MM of the cervix. The criteria proposed by Norris and Taylor [20] can help to differentiate primary from secondary MM of the cervix. According to these criteria, primary MMs are characterized by the following: 1) presence of melanin in the cervical epithelium; 2) absence of melanoma in other parts of the body [20, 21], and 3) evidence of junctional changes in the cervix.

The treatment of MM is primarily surgical and in some cases it can include chemotherapy, radiological, and immunological treatment [22]. Surgical treatment of cervical MM implies radical hysterectomy with or without lymphadenectomy, with upper vaginectomy [23]. Radiation therapy in MM has shown limited indications due to the relative radioresistance and is usually reserved as a treatment for palliation of recurrent disease, advanced disease, involved surgical margins, and involved lymph nodes [16]. Immunotherapy means application of high-dose interleukin-2 and is given to a small number of patients with metastatic disease [24]. As for chemotherapy, the most commonly used combination has been cisplatin, bleomycin, and vinblastine, or dacarbazine monotherapy in advanced stages of the disease [25].

Staging of uterine cervix MM is performed by the International Federation of Gynecology and Obstetrics (FIGO) staging, but very often American Joint Committee on Cancer (AJCC) staging is used for indicating the extent of the disease, stages of the disease, involvement of lymph nodes and distant metastases [26].

However, due to a small number of described cases, there are no sufficient statistical data or official recommendations in terms of therapeutic protocol and monitoring of MM located in the uterus. Literature mostly describes the initial treatment which consists of radical hysterectomy, which may or may not be associated with lymphadenectomy. Therefore, having all the described facts in mind, the prognosis is poor and includes early visceral metastases [16].

Primary and metastatic MMs represent a rare diagnosis with a small number of described cases. The aggressive nature of the tumor, non-specific symptoms, difficult diagnosis and no official protocol on the treatment result in poor disease prognosis. This paper points out the significance of colposcopy in identifying and diagnosing malignant melanoma of the uterine cervix in patients who had undergone melanoma surgery.

doi: 10.2298/SARH1606329Z

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Метастатски малигни меланом материце дијагностикован колпоскопијом

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КРАТАК САДРЖАЈ

Увод Примарни и метастатски малигни меланоми представљају ретку дијагнозу са малим бројем описаних случајева. Агресивна природа тумора, неспецифични симптоми, тешка дијагноза и неофицијални терапијски протокол резултују у лошој прогнози болести.

Приказ болесника Аутори су представили 41 годину стару вишеротку као пацијенткињу. Она је имала операцију малигног меланома окципиталне регије поглавине. Отишла је код свог гинеколога због бледе ружичасте вагиналне секреције. Гинеколошки преглед сем лако увећане материце није показао никакве сигнификатне абнормалности. ПАП тест и преглед вагиналног секрета су били нормални. Колпоскопски – присутно је сигнификантно тамно браон хиперпигментовано поље око 1 *cm* у промеру, опсервирано на задњој усни грлића материце, поред орифицијума и цервикалног канала, које је било суспектно на меланом, што је доказано циљаном биопсијом хиперпигментисане промене цервикса и магнетном резонанцом мале карлице. Урађена је класична хистеректомија са аднексектомијом и регионалном пелвичном лимфаденектомијом.

Закључак Овај приказ случаја је показао могућност колпоскопије да укаже на суспектан налаз на грлићу материце, који у склопу осталих дијагностичких и анамнестичких параметара може да помогне у дијагнози метастатског меланома материце.

Кључне речи: малигни меланом; материца; колпоскопија; метастазе

Примљен • Received: 04/03/2015 **Прихваћен • Accepted:** 29/05/2015

Srp Arh Celok Lek. 2016 May-Jun;144(5-6):334-339
ПРИКАЗ БОЛЕСНИКА / CASE REPORT
DOI: 10.2298/SARH1606334V
UDC: 616.5-002-06; 616.35-006.81

Anorectal melanoma and seborrheic dermatitis – A case report

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SUMMARY

Introduction Anorectal melanoma (ARM) is a rare and aggressive neoplasm with predisposition for early infiltration, distant spread, and unfavorable prognosis. It has been speculated that *Malassezia* yeasts could possibly have an impact on skin carcinogenesis and development of melanoma, especially in patients with seborrheic dermatitis (SD), due to production of aryl hydrocarbon receptor (AhR) agonists.
Case Outline A 52-year-old man with intensive SD complained of a four-month-long rectal bleeding, tenesmus, pain, and difficulty during defecation. On examination, a rectal tumor was detected and histopathology of tumor tissue revealed ARM with positive protein S100, melanoma antigen HMB45 and melan-A expression. After the diagnosis was established, abdominoperineal resection of the anus and rectum was performed, since the tumor was large, obstructive, and the anal sphincter was invaded.
Conclusion Because of the possible impact of intensive SD to the cross-link between *Malassezia* yeasts AhR agonists and skin carcinogenesis, we discussed on this matter and reviewed the literature data regarding ARM. In addition to "pathogenic" and "non-pathogenic" *Malassezia* subtypes based on AhR agonist production, future studies on *Malassezia* metabolites, their carcinogenic effect in the skin and development of melanoma are needed. If the cross-link between *Malassezia* AhR agonists and skin carcinogenesis exists, timely prevention of ARM could be done with *Malassezia* eradication, especially in patients with severe SD.
Keywords: anorectal melanoma; cancerogenesis; *Malassezia*; aryl hydrocarbon receptor; seborrheic dermatitis

INTRODUCTION

Anorectal melanoma (ARM) is a rare and aggressive neoplasm with predisposition for early infiltration, distant spread, and unfavorable prognosis, which represents 0.05–4.6% of all anal malignancies [1, 2]. Although being behind cutaneous and retinal, ARM, the third most common melanoma, accounts for 0.4–1.6% of all melanomas [2, 3]. ARM originates from melanocytes, the carcinogenic changed pigmented dendritic-like cells, which are present in all three mucosal zones of the anal canal [4]. Although solar ultraviolet radiation (UVR) is the most important carcinogen for melanocytes, not all melanomas can be attributed to ambient UVR, since some tumors arise on sun protected localizations, such as colon and anal region [5].

The literature data indicate possible link between melanoma, specific products of *Malassezia* yeasts, and aryl hydrocarbon receptors (AhR) [6, 7, 8]. *Malassezia* (*Pityrosporum*) species are lipophilic yeasts that are recognized as skin commensals capable of producing an extensive array of bioactive indolic compounds, such as malassezin, indolo[3,2-b]carbazole (ICZ), and indirubin, especially in pathogenic condition, e.g. seborrheic dermatitis (SD) [7]. Recent data show that the presence of different *Malassezia* species, the yeast density, and different enzyme production, could be important for *Malassezia* role in pathogenesis of different conditions and in different patient groups, especially those mediated by activation of AhR [7–10]. AhR are widely expressed in humans and are involved in various signaling pathways critical to normal cell homeostasis. Dysregulation of these physiological processes is known to contribute to skin tumor induction, by 'initiation,' 'promotion,' and 'progression,' which represent different stages of the carcinogenic process. AhR are expressed in multiple tumor types, suggesting their pro-oncogenic role. It has been proposed that compounds produced by *Malassezia* yeasts are responsible for alterations in melanocytic function [7, 11]. *Malassezia* yeasts may produce enzymes important for melanoma progression making melanoma more sensitive to AhR-activation simultaneously. Therefore, AhR could play a central role in the ecological 'cross-talk' between *Malassezia* yeasts and development of melanoma in the upper part of the epithelium/epidermis.

CASE REPORT

A 52-year-old man with a four-month history of rectal bleeding, bowel obstruction symp-

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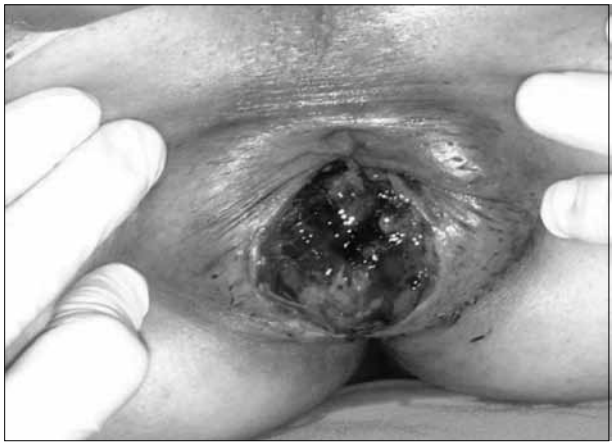

Figure 1. Anorectal melanoma (ARM) located above and below the dentate line with obstruction of the lumen of the rectum

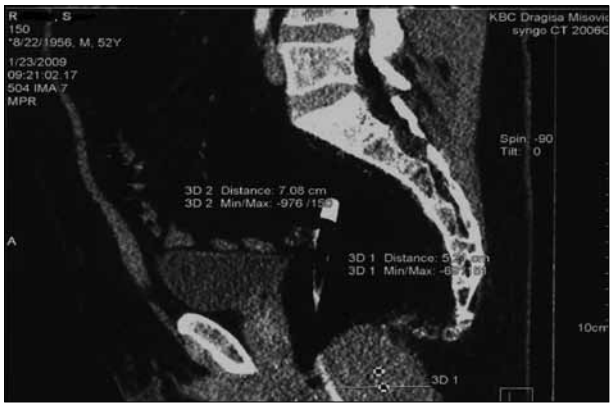

Figure 2. Computed tomography scanning of the small pelvis revealed circumferential rectal thickening (7.2 cm in length and 1.8 cm thickness) located 3.2 cm from the anus, leading to the narrowing of the rectal lumen with several enlarged perirectal nodes

toms, and 10 kg weight loss was referred to our clinic. Anamnestic data revealed untreated extensive long-standing SD. A huge anorectal tumor in the region of the dentate line was detected by clinical inspection (Figure 1). Biopsies demonstrated that it was a malignant melanoma, as protein S100, melanoma antigen HMB-45, and Melan-A expression were found. On computed tomography (CT) scanning, a circumferential anorectal thickening 3.2 cm above the anus, 7.2 cm in length and 1.8 cm in thickness, narrowing the rectal lumen, was detected, associated with several adjacent enlarged perirectal lymph nodes (Figure 2). CT scans of the thorax and abdomen showed no signs of distant metastases. In the inguinal region, no enlarged lymph nodes were detected.

After the diagnosis was established, abdominoperineal resection of the rectum was performed, since the tumor was large, obstructive, and the anal sphincter was invaded (Figure 3). The patient recovered well from the operation and was dismissed on the ninth postoperative day.

Histology analysis confirmed malignant metastatic epithelioid melanoma. The tumor showed invasion into the muscular layer. Only one of 14 removed perirectal lymph nodes was histologically positive on paraffin embedded sections. Melanoma was classified as stage 3. No adjuvant therapy was prescribed by the oncologist.

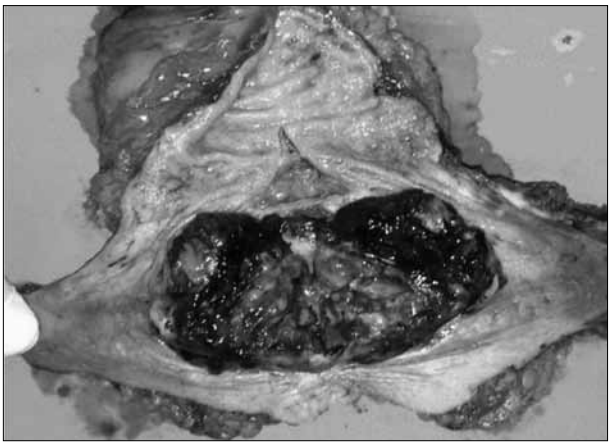

Figure 3. Gross finding of bisected anorectum with present ARM

Eleven months after the initial surgery treatment was performed, the patient began to feel pain in the perineal region. Clinical examination showed palpable tumor mass in the perineal area. The 7.5 × 6.2 cm tumor was confirmed by magnetic resonance imaging and associated with enlarged lymph nodes in the inguinal region. The abdominal magnetic resonance imaging showed no signs of distant metastases. A second surgical procedure was carried out, showing recurrence of ARM in the small pelvis, with no infiltration of the prostate, urinary bladder or pelvic bones. The tumor was removed, the patient recovered well from the operation and was released on the tenth postoperative day and directly forwarded to a dermatologist to initiate treatment of SD and to an oncologist to initiate chemotherapy. Ten months after the second operation, the patient was disease-free, with SD in remission.

DISCUSSION

ARM is a rare disease with exceedingly poor prognosis, worse than cutaneous melanoma, with a median survival of one year and with five-year survival rate of 10% [12]. ARM could be located in the anal canal or at the anal verge (2/3), or in the distal rectum (1/3), and often presents with non-specific local symptoms such as rectal bleeding (55%), rectal masses (34%), anal pain (13%), and change in bowel habits (63%) [13, 14]. However, women are more likely than men to be diagnosed with ARM (male to female ratio is 0.75) [15], and lesions are usually non-specific, amelanotic, and commonly mistaken for benign conditions such as hemorrhoids or rectal polyposis, which prolongs the establishment of the diagnosis [16, 17].

Apart from UVR, the relevant impact of other factors on carcinogenesis in melanoma remains controversial. Exposure to solar UVR is the most important independent carcinogen for melanoma, but additional factors related to the host or local environment may contribute substantially to the pathogenesis of melanoma [18]. The host dependent factors and the polymorphisms of genes encoding for the biosynthesis of detoxifying enzymes are important for melanoma cases in different patient groups [19]. Therefore, UVR-independent anatomical distribution of

melanoma is in line with the current hypothesis that carcinogenic factors acting *in loco* independently or in addition to UVR, may play a distinctive role in the pathogenesis of ARM. In this setting, the important locally modifying factor in human skin carcinogenesis could be lipophilic yeasts of the genus *Malassezia*, previously known as *Pityrosporum*. The genus is characterized by the number of different species production of bioactive enzymes and production of certain metabolites [9, 18]. This is further in line with the observed coincidence of ARM and *Malassezia* colonization areas that are commonly found in some relatively UVR-protected areas, which are also locations of ARM [23]. Epidemiological observations that indirectly link *Malassezia* with melanoma come from the study of patients with Parkinson's disease in whom SD and skin carcinomas appeared in higher rates than expected by chance, despite the fact that the most of other malignancies are found with reduced prevalence in this group of patients [9, 19]. The core mechanism by which *Malassezia* yeasts could probably mediate the carcinogenesis acts through the production of locally acting potent indolic AhR ligands [7]. The AhR belong to orphan receptor group that in adults mediate metabolism of xenobiotics through induction of detoxifying enzymes [20]. AhR are members of the Per-Arnt-Sim superfamily of transcription factors and operate as a key mediators of diverse chemical and physical environmental damages to the cell, including UVR [21, 22]. Recent evidence indicates that AhR ligands are able to modify differentiation of keratinocytes *in vitro* and accelerate terminal skin development [23]. Moreover, AhR have pluripotent functions in adult human skin, e.g. they regulate many aspects of immune response, the cell cycle, and also part of the UVR induced cell alterations [21, 22]. The relationship of rapidly metabolized AhR ligands to carcinogenesis, including those naturally-occurring, like ICZ and indirubin, is more complex because they usually occur as mixtures affecting complex molecular interactions with potentially variable biological outcomes [7].

According to literature data, the presence of AhR ligands from *Malassezia* spp. in the skin could modify epidermal carcinogenesis and induce melanoma by (i) affecting the immune state of the skin; (ii) perturbing the homeostasis between cell cycle progression and apoptosis; (iii) increasing expression of matrix metalloproteinase-1 (MMP-1) in keratinocytes with consecutively enhanced tumor invasion; (iv) affecting survival of the initiated tumor cells; (v) modulating UVR induced carcinogenesis of the epidermis [7, 8]. Within the above framework, a key component of the proposed association to be dissected in future studies will be the behavior of *Malassezia* yeasts under UVR. The increased prevalence of SD among patients with parkinsonism also highlights the impact of the patients' genetic profile, which is important in SD pathogenesis [9, 24].

Although there are various treatment strategies for ARM, surgical treatment remains the main therapeutic modality, with radical abdominoperineal resection (APR) and conservative wide local excision (WLE) as the most common [25]. The necessity of APR in the treatment of ARM beyond WLE remains controversial. Because of the low incidence of this disease, treatment guidelines are based on small retrospective studies. Ross with colleagues showed a 29% recurrence rate in primary ARM after APR, and a 58% recurrence rate after WLE [26]. In a study by Thibault et al. [13], 37 patients had curative resection with no significant survival difference between WLE and APR when comparing disease stage and five-year survival. Before 1997, approximately 70% of all patients underwent APR, while after 1997 almost 90% of patients underwent WLE [14]. The benefits of WLE are obvious, and include fast recovery time from a significantly less invasive procedure, minimal impact on bowel function, and avoidance of permanent colostomy [13]. WLE with or without adjuvant therapy seems to offer good locoregional control without reducing the survival rate, and may be a therapeutic modality of choice for patients with small, superficial ARM [14, 27]. APR should be offered to patients with obstructive bowel symptoms, locally advanced and invasive ARM, or as a salvage therapy following recurrence [27]. Moreover, the role of adjuvant chemotherapy and immunotherapy in the treatment of ARM has not been established, particularly in cases involving metastatic disease. Some patients with metastatic melanomas have been shown to respond to therapy with interferon-α and interleukin-2, suggesting that metastatic melanoma is susceptible to immune assault [28]. However, ARM has always been considered to be a radioresistant tumor [27], as radiation therapy provides no benefit, except occasionally for palliative care in cases with unresectable tumors [28].

In conclusion, besides surgery, the optimal treatment strategy for ARM requires multidisciplinary approach, taking into consideration the possible link between *Malassezia* yeasts and skin carcinogenesis, especially in patients with severe SD. Based on AhR agonist production, future studies are needed to determinate a cross-link between ARM and *Malassezia* yeasts. If a cross-link between agonists (malassezin, ICZ, or indirubin) and carcinogenesis exists in patients with severe SD, timely prevention of melanoma and ARM could possibly be done with *Malassezia* eradication.

ACKNOWLEDGMENT

The study was supported by the Ministry of Education, Science and Technology of the Republic of Serbia, research grant No. OI 175034.

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Аноректални меланом и себореични дерматитис – приказ случаја

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КРАТАК САДРЖАЈ

Увод Аноректални меланом (АРМ) ретка је и агресивна неоплазма са израженом способношћу ране инфилтрације, удаљеног ширења и лоше прогнозе. Познато је да кваснице рода *Malassezia* имају способност продукције протеина, који представљају агонисте за *aryl hydrocarbon* рецепторе (*AhR*), што се доводи у везу са њиховим могућим канцерогеним потенцијалом и способношћу да учествују у развоју меланома коже, посебно код пацијената са себореичним дерматитисом (СД).

Приказ болесника У раду је приказан пацијент стар 52 године, мушкарац са израженим СД и са симптомима аналног крварења, грчева, бола и отежане дефекције током четири месеца. Дијагностикован је тумор ануса, а патохистолошко испитивање је потврдило сумњу да се ради о АРМ са позитивним налазом протеина *S*100, меланома антигена *HMB*45 и меланоцитног антигена *melan-A* (*MART*1). По постављању патохистолошке дијагнозе начињена је абдомино-перинеална ресекција ануса и ректума с обзиром на величину тумора, његову опструктивну природу и инвазију аналног сфинктера.

Закључак Због могуће повезаности СД и продуката гљива рода *Malassezia* са процесом карциногенезе у кожи и последичним развојем АРМ, у раду је дискутован приказани случај уз преглед литературе. Подела на „патогене" и „непатогене" *Malassezia* врсте може да се доведе у везу са њиховим потенцијалом да продукују агонисте *AhR* рецептора, због чега су неопходна даља испитивања. Уколико се потврди веза између ових агониста и канцерогенезе коже код пацијената са израженим СД, могуће је развити стратегију превенције меланома правовременом ерадикацијом гљива рода *Malassezia*.

Кључне речи: аноректални меланома; канцерогенеза; *Malassezia*; *aryl hydrocarbon* рецептор; себореични дерматитис

Примљен • Received: 28/10/2015 **Ревизија • Revision:** 11/01/16 **Прихваћен • Accepted:** 09/03/2016

Laterality in living beings, hand dominance, and cerebral lateralization

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SUMMARY
To date, lateralization in living beings is a phenomenon almost mythologically unexplored. Scientists have proved that lateralization is not exclusively a human feature. Investigations in molecular biology, protein structure, mobility of bacteria, and intracellular lateralization in ciliates, shows important and universal nature of lateralization in living systems. Dominant lateralization implies the appearance of a dominant extremity, or a dominant sense during the performance of complex psychomotor activities. Hand dominance is usually defined as a tendency to use one hand rather than another to perform most activities and this is considered to be the most obvious example of cerebral lateralization and exclusive characteristic of humans. However, there are some exceptions in other species. The dominant hand is able to perform more complex and subtle manual tasks than the non-dominant hand, and this behavioral superiority is the absolute result of additional cerebral support. The asymmetry of brain organization was confirmed in rats, chimpanzees, dogs and birds, some fishes and lizards. The relationships between hand dominance with brain structure and function remain far from clear. For a long time, lateralization was considered unique to humans, but recently it has become clear that lateralization is a fundamental characteristic of the organization of brain and behavior in all vertebrates. It has been questioned to what extent lateralization in humans and other vertebrates may be comparable.
Keywords: laterality; handedness; cerebral dominance

LATERALITY IN LIVING BEINGS

Lateralization may be defined as a localization of function, or activity on one body side which is dominant over the other [1]. To date, lateralization in living environment is a phenomenon not explored satisfactorily. Scientists have proved that lateralization is not exclusively a human feature. Investigations in molecular biology, protein structure, mobility of bacteria, and intracellular lateralization in ciliates, show important and universal nature of lateralization in living systems [2]. Morphological asymmetry is a common feature of animal body plans, from shell coiling in snails to organ placement in humans [3]. Left–right asymmetries of brain and behavior are now known to be widespread among both vertebrates and invertebrates and can arise through a number of genetic, epigenetic, or neural mechanisms. A right-hemisphere dominance for emotion seems to be present in all primates so far investigated, suggesting an evolutionary continuity going back at least 30 to 40 million years [4]. Based on the current body of literature, the general perception remains that while other animals may demonstrate some lateralized behaviors, no other animal shows this trait to an equal level of significance as population-level right-handedness in humans [5].

Dominant lateralization implies the appearance of a dominant extremity, or a dominant sense during the performance of complex psychomotor activities. The lateralization appears as right and left at the same time, equal by function, symmetrical in the performance of activities [1]. Dominant lateralization appears as right, which is most often, or left, which is much rarer. Lateralization in humans is determined at the level of upper extremities, at the level of the senses of sight and hearing and at the level of lower extremities [1].

A fundamental question which as yet remains unanswered is why laterality evolved at all. One of the explanations proposes that lateralization may serve a double purpose. It maximizes the skill and strength level and reduces duration of dependence of children. Secondly, it expedites or facilitates the "fight and flight" reflex response for dexterous individuals [6]. No satisfactory explanation has been offered as to why and how laterality in living beings evolved [6]. Laterality is one of the central topics of the development of neuropsychology and it is an inexhaustible inspiration for researchers.

HAND DOMINANCE

Hand dominance is usually defined as a tendency to use one hand rather than the other to perform most activities, and this is considered to be the most obvious example of cerebral lateralization and exclusive characteristic of humans [7]. As always, every rule has its exceptions – parrots that live in Australia, on the Indonesian archipelago, and on the Pacific islands, as well as one species of frogs. Both

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species show a surprising dominance of the right extremity, which can only be represented by "blind street" in evolution [8]. Around two thirds of chimpanzees are right-handed, especially in gesturing and throwing [6]. In essence, primates generally exhibit mixed handedness. The reason that primates do not exhibit consistent handedness is due to the fact that they use their hands and arms for locomotion. It is also observed that when the supporting limb tires, chimpanzees change hands. Sometimes the fishing-dominant hand becomes the supporting hand and vice versa. In this context, having a dominant handedness would be dangerous and would impair food-gathering [6]. Human/animal partition is no longer tenable, and scientists are reviewing the following four (nonexclusive) possible drivers for the origin of population-level right-handedness: skilled manipulative activity, as in tool use; communicative gestures; organizational complexity of action, in particular hierarchical structure; and the role of intentionality in goal-directed action. Fully testing these hypotheses will require developmental and evolutionary evidence, as well as modern neuroimaging data [9].

The question emerges on what is the main discriminating agent between *Homo sapiens* and other mammals, which made the human species predominantly right-handed during the course of evolution [10]. Handedness, particularly right-handedness prevalence of up to 93%, has been demonstrated in the 35,000-year-old remains of a Neanderthal and 500,000-year-old tools of hominids [6].

In any case, the dominant hand is able to perform more complex and subtle manual tasks than the non-dominant hand, and this behavioral superiority is the absolute result of additional cerebral support [2]. One meta-analysis, examining the incidence of sinistrality involving 81 studies from Africa, America, Asia, and Australia, showed that the proportion of left-handedness ranged from 5% to 30% [6]. One study of a large sample (n = 2,546) of primary schoolchildren in Belgrade, Serbia, reports a prevalence of left-handedness as 7.6% [11]. Another study among Belgrade high school students (n = 1,189) found a slightly lower prevalence of 6.8% [2]. The proportion of strongly left-handed persons was only 3.3% among university students in Belgrade (n = 1,113) [12]. Our fourth study was performed on adult residents of the Stari Grad municipality in Belgrade (n = 1,202), which reported the proportion of left-handedness of 5% [13].

Some people do not consistently use the dominant hand, i.e. they prefer one hand for one manual activity, and the other one for another activity. This situation is described as "mixed-handedness" [1]. In contrast to this, "ambiguous handedness" is inconsistent use of the dominant hand for the same manual activity [1]. There is also an inconsistency between the hand dominance and feet or eye dominance [1]. Intuitively mixed-handedness may seem advantageous in almost every context, as if a person had two equally dexterous hands. No satisfactory explanation has been offered as to why and how single-handedness evolved. Mixed-handedness and ambidexterity are exceedingly rare. Some studies report that 3.7% of the population is mixed-handed; ambidexterity is very rare with no reported prevalence in the literature [6]. To study handedness variations in humans, it is important to choose typical tasks among human populations from different cultures. Thus, some tasks commonly used in Western societies to measure handedness, such as writing or teeth brushing, are meaningless in other cultures [14]. Despite strong neuropsychological correlates for handedness, methods of assessment are not uniform or consistent across development [5]. What is currently lacking is a way to clearly identify the left-hander categories in order to better estimate fitness costs and benefits associated with each category [14].

Certain studies have shown that social and cultural factors may alter the "natural" dominant hand in three ways: a) changing hand dominance for one activity without changes in hand dominance for other activities that are carried out with one hand; b) reducing the degree of hand dominance; c) completely changing hand dominance, which results in reducing the prevalence of left-handedness in the population [15]. For example in China, there is a strong social pressure for right-handed writing and eating, which has drastically decreased the proportion of left-handers for these tasks compared with other tasks [14]. Studies show that there are 13% of left-handers in their late twenties, and less than 1% of left-handers among persons in their eighties [2]. This finding could be explained by the fact that the socio-cultural pressure against left-handed people was much more pronounced in the past than it is today, when this attitude is much more liberal. Another possible explanation is based on the assumption of a shortened lifespan of left-handed people [16]. The first study that tested the possibility of social modulation affecting behavioral lateralization in animals was the study on chicks [17]. An ancient debate between Plato and Aristotle on handedness is well-known. Plato, a right-handed Greek philosopher, argued that the hand dominance was a learned trait, whereas Aristotle, his left-handed student, claimed in his *Metaphysics* that people are either right-handed or left-handed by nature [18].

CEREBRAL LATERALIZATION

Initially, it was thought that the entire left hemisphere was dominant for most neurological functions and that the right hemisphere was subdominant [19]. This standpoint was based on the first series of studies revealing that lesions in the left hemisphere were responsible for the impairment of such an important and visible task such as speech [1]. In the following phases of the research it was observed that the right hemisphere could be dominant in left-handers. Speech and laterality movement are noted as a whole and are determined by each other. In addition, where a predominance of hand and speech appears, the dominance for all other functions of the brain is also expected [2]. The asymmetry of brain organization was confirmed in rats [20], chimpanzees [21], dogs [22], and birds [23, 24]. It was even found among some fishes [17] and lizards [25]. Inconsistent asymmetry of brain orga-

nization was found in cats [26]. Chimpanzees also show left-sided enlargement in two cortical areas homologous to the main language areas in humans, i.e. in Broca's and in Wernicke's area. Even chimpanzees and dogs, as well as many other species can learn to respond to simple spoken instructions, but cannot produce anything resembling human speech [4]. After Broca's investigations, interest flagged for a while, but was revived a century later, in the 1960s, with the study of a patients who had undergone split-brain surgery, in which the main commissures connecting the two hemispheres were cut as a means of controlling intractable epilepsy [4]. Testing of each disconnected hemisphere again revealed that the left hemisphere is specialized for language and the right hemisphere for emotional and nonverbal functions [4]. This work won Roger W. Sperry the Nobel Prize for Physiology and Medicine in 1981. Evidence that the right hemisphere was more specialized for perception and emotion also led to speculation, some of it far-fetched, about the complementary roles of the two sides of the brain in maintaining psychological equilibrium [4].

Until the 1960s it was believed that the functional asymmetry is an exclusive characteristic of the human species. However, studies have shown that canaries stop singing as a consequence of lesions only in the left hemisphere [23]. Left-hemisphere dominance for vocalization has been shown in mice and frogs, and may well relate to the leftward dominance for speech – although language itself is unique to humans and is not necessarily vocal, as sign languages remind us [4]. Sherman and collaborators have shown the asymmetry in the rat cerebral hemispheres, or other functional asymmetry, such as the position of their tail and their activities in the open air [27]. In the house mouse, the ultrasonic calls emitted by young mice to evoke maternal caring behavior are preferentially recognized by the left hemisphere [28].

Some progress in the study of cerebral dominance was achieved in the second half of the 20th century by the introduction of non-invasive techniques that are now commonly applied, such as the functional transcranial Doppler sonography and functional magnetic resonance imaging. Functional transcranial Doppler sonography measures cerebral blood flow in accordance with the neuronal activity in the anterior, middle, and posterior cerebral arteries [2]. This method continuously registers blood flow in accordance with brain activity, provides an excellent temporal resolution compared to other neuroimaging techniques, and is simple for use. It is applicable for patients with whom good cooperation cannot be established, as well as for children. This method has shed light on the organization of the hemisphere itself, within the cognitive, motor and sensory function in adults and children [29]. Postmortem studies were carried out and have shown an initial substrate for functional asymmetry [30]. It is interesting to mention that several of the gross anatomic asymmetries in both fetal and mature human brain were demonstrated in the fossil sculls of our ancestors [19]. Scientists have shown that *planum temporale*, a triangular area situated on the superior temporal gyrus, represents a structural marker for asymmetry of the cerebral hemisphere, and believe that their study will contribute to a better understanding of the origin of lateralization [31]. Neurochemicals as well as structural asymmetry must be taken into account if one is to understand lateral differences in function [31].

ORIGINS AND ASSOCIATION BETWEEN HAND DOMINANCE AND CEREBRAL LATERALIZATION

The simplest theory of lateralization, which is widely accepted, is that patterns of asymmetry are strongly determined genetically. It is well-known that the left hemisphere of the brain is typically dominant for speech and motor activity, while the right hemisphere is responsible for the artistic aptitude, spatial orientation, attention, and many aspects of emotional life [19]. According to this, the patterns of anatomical, biochemical, and functional asymmetry of brain organization in humans, as in the animal kingdom, are strictly genetically determined [19]. Two left-handed parents produce the highest proportion of left-handed children, i.e. approximately 30–40% [32]. The incidence of left-handedness is higher when the mother is left-handed and the father is not, than when the father is left-handed and the mother is not [17]. One of the most influential and widely cited genetic models was called the Right Shift Theory or Theory Shift to the Right by Marian Annett [33]. One of the key settings of Annett's theory is the distribution of the manual dexterity between the left and the right hand. In the world of other primates and lower animal species, the distribution is in the form of a normal curve. Simply put, chimpanzees, cats, birds, etc. are equally skilled with both left and right extremities, which are equally frequently used. The distribution is normal and identical to *Homo sapiens*, except that it is "moved" to the right (right shift). That distribution shift to the right is genetically coded, and the specific gene responsible (the so-called Right Shift Gene) was not destined to produce the dominance of the right hand, but to induce a specific cerebral asymmetry, i.e. to induce a neurological speech center in the left cerebral hemisphere during the earliest stages of development. As a result, the right hand appears as dominant in relation to the left [33]. If left-handedness were purely genetically caused, both identical twins would have identical dominant handedness. Studies have reported that left-handedness occurs in only 76% of twins [34]. Therefore, large studies with better genome coverage are needed to clearly identify the genes involved in the relative hand skills and hand preferences [14]. This may either suggest that the specific deviations, such as the twin, sex, and maternal effects, may be best explained by the environmental factors, as suggested in the literature [17]. The associations between left-handedness and various health problems have often led to a distinction between the pathological left-handedness, which would arise from developmental stresses, and the familial left-handedness which would be due to genotype [35]. This hypothesis considered that some people are left-handed because they have suffered different types of pathology. The in-

creased proportion of lefthanders in clinical populations with central nervous system disorders (e.g. schizophrenia, epilepsy, mental retardation or learning disabilities) can be explained by the claim that early brain insult may cause the individual to switch to the opposite hand for unimanual activities [36].

The modification of dominance by intrauterine environmental changes increases diversity to a far greater extent than a rigid genetic mechanism would allow [19]. Geschwind and Galaburda [19] pointed out that "the lateralization is a central topic in biology and medicine, not simply a secret interest in a small number of researchers." If living beings were genetically predestined to be right-handed, then left-handedness, as some believe, may be considered the failure to be right-handed. Scientists have tried to explain what type of development mistakes have been made. They suggested the possible factors that lead to developmental abnormalities and irregular, or "anomalous" brain dominance; the anomaly is considered to be everything that does not fit the majority and different from the usual standards [19]. Particular attention was paid to the intrauterine environment and factors acting during intrauterine development. In the womb, male and female fetuses share the same maternal and placental hormones. It has been found that sex hormones, such as testosterone, may affect the proliferation and migration of neurons in the brain of the fetus in the critical periods of the development, by acting on the appropriate hormone receptors and enzymes [19]. Testosterone receptors have been identified in the nerves and other tissues in the body. In experimental studies, plasma testosterone in male rat fetuses increases rapidly when mother is exposed to stress [19]. A sudden increase in the level of testosterone in the womb under the influence of stress, combined with an additional testosterone from the testicles, can cause a slow and irregular development particularly of the left hemisphere, because it develops more slowly than the right [19]. This may be one of the explanations why left-handed people are more frequent in men. It is proposed that left-handedness in females can be associated with increased sensitivity of testosterone receptors, but with elevated levels of this hormone in utero [19]. This model has also undergone numerous criticisms [37].

The birth-stress model is the most controversial explanation for the origin of left-handedness [38]. This model radicalized previous interpretation of pathological left-handedness, noting that each case of non-right-handedness is pathological. According to this, non-right-handedness originates from stress at birth, primarily in the firstborn child and in fourth and subsequent births; according to this model, all types of non-right-handedness can be considered pathological side-effect of this stress [38]. This finding is supported by the higher incidence of left-handedness in twins compared with singletons, simply because twins may be more exposed to birth stress [17].

Some studies have shown that the energy that the brain of the male fetus receives during exposure to ultrasound can affect the migration of neurons and consequently produces anomalous cerebral dominance [39]. One study also confirmed the association between exposure to ultrasound in pregnant mothers and later left-handedness in boys [40].

It could also be interesting and easy to study the effects of pregnancy during different seasons and under differing conditions of light, temperatures, and other variables [2]. There are studies that have examined the effect of light on bird eggs and later development of behavioral lateralization [17]. Research in Serbia has shown that maternal cigarette smoking during pregnancy and APGAR score less than 7 at birth were significant risk factors for left-handedness [41].

Handedness and cerebral asymmetries are detectable even in the fetus. In photographs of the brains of 16-week-old fetuses one can observe the typical asymmetry similar to the adult brains [4]. A new study shows a leftward asymmetry of the choroid plexus in two thirds of first-trimester human fetuses. This is the earliest brain asymmetry so far identified and may be a precursor to other asymmetries, including that of the *temporal planum*, which is evident from the 31st week of gestation [4]. Ultrasound recordings have shown that by the 10th week of gestation, the majority of fetuses move the right arm more than the left, and from the 15th week, most suck the right thumb rather than the left – an asymmetry strongly predictive of later handedness [4]. Although these data suggest that predispositions for handedness are already present early in ontogeny, they do not exclude a role for environmental factors affecting lateralization later in life [17]. Since both hand preference and language asymmetries are expressed very early, even before birth, a systematic comparison of the very early development of both behavioral traits is needed to understand this relationship. At present, such data are clearly lacking. Fagard [42] concludes that this evidence favors the view that the two asymmetries develop relatively independently.

It has long been thought that speech and language in a broader sense are inseparably linked to the dominance of the hand, but this relationship was never simple and it is not easily explained [1]. Just to illustrate the complexity of these relations it should be said that while almost all (98%) right-handed persons have speech center located in the left hemisphere, which is a fact established in the second half of the 19th century, the vast majority (70%) of left-handed persons also have speech center located in the same hemisphere [43]. Nevertheless, there are also cases with bilaterally positioned speech center. The latter are almost exclusively left-handed individuals, and very rarely right-handed [43]. Some authors believe that about 30% of left-handers have the speech center in the right hemisphere, or that it extends through both brain hemispheres [44]. Recent studies suggest that neuropsychological substrate for lateralization of speech and hand dominance may not be the same and that there is an independent influence on the development of each of these dominations, which confirms several new genetic and neurological studies [45, 46]. Unfortunately, in animals even less is known about typical development and to what extent early manipulations still exert their effect in adulthood. Such long-term

studies take time, but are very relevant for further progress in the field [46].

CONCLUSION

In general, the relationships between hand dominance and brain structure and function remain far from clear. For a long time, lateralization was considered unique to humans, but recently it has become clear that lateralization is a fundamental characteristic of the organization of brain and behavior in all vertebrates. Animal models open new and exciting insight into the function and evolution and provide the opportunity to experimentally study the causes and consequences of lateralization. It has been questioned to what extent lateralization in humans and other vertebrates may be comparable. It is likely that humans may have species-specific adaptations in their lateralized behavior. This may explain the strong human lateralization in handedness, but lateralization of brain and behavior, being such a fundamental aspect of the organization in vertebrates, must share common principles for humans and other vertebrates. There is evidence for both genes and environment to affect the development of behavioral lateralization, but evidence for both, and especially their interaction, is surprisingly incomplete. With the identification of the human genome, and the use of animal models, we believe that substantial progress can be made in the near future.

ACKNOWLEDGEMENT

The study was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, projects No. 41020 and No. 175078.

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Латерализованост живих бића, доминантност руке и церебрална латерализација

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КРАТАК САДРЖАЈ

Латерализованост живих бића до данас је феномен који је готово митолошки неистражен. Научници су доказали да латерализованост није искључиво карактеристика људске врсте. Истраживања у молекуларној биологији, структури протеина, мобилности бактерија и интрацелуларној организацији код цилијата показују значајну и универзалну природу латерализованости у живим системима. Доминантна латерализованост подразумева појаву водећег екстремитета, или водећег чула током обављања сложених психомоторних активности. Доминантност руке се обично дефинише као тежња да се користи једна рука радије него друга за обављање већине активности и ово се сматра најочигледнијим примером церебралне доминације и искључивом карактеристиком људске врсте. Међутим, постоје изузеци и код других врста. Доминантна рука је у стању да обавља сложеније и суптилније мануелне активности од недоминантне руке, а ова бихевијорална супериорност је апсолутни резултат додатне церебралне подршке. Асиметрија мождане организације потврђена је код пацова, шимпанза, паса, птица, неких риба и гуштера. Односи између доминантности руке са структурама и функцијама мозга још увек су нејасне. Латерализованост се дуго сматрала искључивом карактеристиком човека, али не тако давно постало је јасно да је латерализованост основна карактеристика организације мозга и понашања код свих кичмењака. Остаје питање у којој мери се латерализација код људи и других кичмењака може упоређивати.

Кључне речи: латерализованост; доминантност руке; церебрална латерализација

Примљен • Received: 09/11/2015 **Ревизија • Revision:** 02/02/16 **Прихваћен • Accepted:** 24/03/2016

Current issues on sublingual allergen-specific immunotherapy in children with asthma and allergic rhinitis

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SUMMARY
In 1993 the European Academy of Allergy and Clinical Immunology was the first official organization to recognize that sublingual administration could be "promising route" for allergic desensitization. A few years later, the World Health Organization recommended this therapy as "a viable alternative to the injection route in adults." The first meta-analysis showed sublingual allergen specific immunotherapy (SLIT) effectiveness for allergic rhinitis and another study showed SLIT can actually help prevent the development of asthma both in adults and in children. The main goal of this review article is to present insight into the most up-to-date understanding of the clinical efficacy and safety of immunotherapy in the treatment of pediatric patients with allergic rhinitis and asthma. A literature review was performed on PubMed from 1990 to 2015 using the terms "asthma," "allergic rhinitis," "children," "allergen specific immune therapy." Evaluating data from double-blind placebo-controlled randomized clinical trials (DB-PC-RCTs), the clinical efficacy (assessed as the reduction of symptom score and the need of rescue medicament) of SLIT for allergic rhinitis and allergic asthma, has been confirmed in various meta-analysis Outcomes such as rhinoconjunctivitis score and medication scores, combined scores, quality of life, days with severe symptoms, immunological endpoints, and safety parameters were all improved in the SLIT-tablet compared with placebo group. SLIT safety has been already proven in many DB-PC-RCTs and real-life settings. In accordance with all of the above mentioned, the goals for future trials and studies are the development of comprehensive guidelines for clinical practice on immunotherapy, embracing all the different potential participants. The importance of allergen immunotherapy is of special relevance in the pediatric age, when the plasticity and modulability of the immune system are maximal, and when preventative effects can be reasonably expected.
Keywords: allergen immunotherapy; children; asthma; allergic rhinitis

INTRODUCTION AND HISTORICAL BACKGROUND TO SUBLINGUAL IMMUNOTHERAPY

The first data concerning allergen immunotherapy (AIT) dates back to the beginning of the 20th century when Freemen and Noon were the first to use allergen extracts for desensitizing patients [1, 2]. Although the first clinical trials of allergen-specific sublingual immunotherapy to pollen date back to the 1920s [3], it was not used until the 1970s when interest in the mucosal route was re-examined by a group of German investigators who showed the clinical efficacy of sublingual allergen specific immunotherapy (SLIT) in comparison with subcutaneous allergen specific immunotherapy (SCIT) [4]. The next step in SLIT evolution was revealed by Scadding's and Brostoff's [5] double-blind placebo-controlled trial (DB-PCT) on SLIT efficacy [5]. At the same time, a group of Italian allergists investigated the sublingual manner of allergen administration in order to make this kind of therapy more available [6]. Early papers with sublingual allergen immunotherapy demonstrated positive results, and in 1993 the European Academy of Allergy and Clinical Immunology was the first official organization to recognize that sublingual administration could be a "promising route" for allergic desensitization [7, 8]. A few years later, SLIT safety in adults and children (age >5) was shown [9, 10]. Based on eight DB-PCTs on clinical efficacy of SLIT drops, the World Health Organization recommended in 1998 that this therapy can be considered to be "a viable alternative to the injection route in adults" [11]. A recent Cochrane review analyzing symptoms and/or medication scores proved the efficacy of SLIT in 49 randomized control trials (RCTs) with 4,589 children and adults affected by allergic rhinitis (AR) (with or without asthma or conjunctivitis), compared with placebo [12].

The main goal of this review article is to present insight into the most up-to-date understanding of the clinical efficacy and safety of immunotherapy in the treatment of pediatric patients with allergic rhinitis and asthma. A literature review was performed on PubMed up until 2015 using MeSH terms "asthma," "allergic

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rhinitis," "children," "immune therapy." Additional articles were identified by a manual search of the list of references in the initial search.

EPIDEMIOLOGY OF ASTHMA AND RHINITIS IN SERBIA

The list of articles analyzing the prevalence and epidemiology of childhood atopic diseases, mainly asthma, dermatitis, and rhinitis, is extremely long and extensive. The numbers varied from too low to too high mainly due to a great heterogeneity in the methodological and statistical background. However, the International Study of Asthma and Allergies Phase Three has valuable influence, involving 98 countries worldwide and 236 Phase Three Centers – in other words, it encompasses around 1,059,053 children of two age groups from 236 centers in 98 countries [13, 14]. The prevalence rate of allergic rhinitis, asthma, and eczema in Serbia has been investigated as a part of the International Study of Asthma and Allergies Phase Three. The survey was conducted in five regional centers with different geographical and urban characteristics. Around 14,000 children were enrolled, aged six to seven years and 13–14 years. Prevalence rate of asthma has been 6.59% in the six to seven years age group, and 5.36% in the 13–14 years age group. Prevalence of allergic rhinitis has been 7.17% in six to seven years age group, and 14.89% in the 13–14 years age group. In total, we found asthma in 5.91%, rhinitis in 11.46%, and eczema in 14.27% of the children [15].

CLINICAL EFFICACY

Evaluating data from DB-PC-RCTs, the clinical efficacy (assessed as the reduction of symptom score and the need of rescue medicament) of SLIT for allergic rhinitis and allergic asthma has been confirmed in various meta-analyses (Tables 1 and 2) [12, 16–19].

However, significant clinical and methodological heterogeneity was shown among studies and some issues are still a matter of debate. Recently, a study by Nelson et al. [20], in addition to confirming clinical efficacy of SLIT in reducing nasal and ocular symptoms and the use of rescue medications, also observed no differences in clinical efficacy in mono- and poly-sensitized patients. The effect of disease modification of an SQ-standardized grass SLIT-tablet two years after three years of treatment has been shown in a randomized trial in patients with moderate-to-severe grass pollen induced rhinoconjunctivitis. Outcomes such as rhinoconjunctivitis score and medication scores, combined scores, quality of life, days with severe symptoms, immunological endpoints, and safety parameters were all improved in the SLIT-tablet, compared with placebo group [21]. Results from a 15-year-long prospective study by Marogna et al. [22] show that long-lasting effects of SLIT are in direct correlation with the treatment's duration. Analyzing 59 adult patients on SLIT, the authors concluded that four years of SLIT treatment is optimal to achieve long lasting effects. Duration of five years or more adds only non-additional benefits [22]. The SLIT approach in very young children has raised skepticism concerning the use of soluble allergen drops in an age group that cannot sufficiently hold sublingual allergen long enough under the tongue to deliver allergens to mucosal immune cells [23]. The current study might provide evidence that preventive SLIT over a treatment period of one to two years would deliver enough allergen as to mediate immunologic changes [24]. Nevertheless, optimizing allergen dosing and treatment duration for the use of preventive immunotherapy (sublingual or subcutaneous) in very young children is one of the main questions to be solved. Overall, the current pilot study underlines that the approach of immunomodulation by preventive allergen-specific immunotherapy in early infancy is feasible, and larger studies should delineate more details of optimal application modalities [25]. Literature data showed that SLIT can prevent the development of new sensitization, comparing with the standard pharmacotherapy, even six years after cessation of the treatment [26]. A study of sublingual immunotherapy done by Malling et al. [27] showed that the allergen used for immunotherapy is historically the predominant cause of symptoms, the beneficial effect of SLIT in polysensitized

Table 1. Comparison between SLIT studies

Symptom scores	Reference	Study population	Studies (N)	Active participants (n)	Placebo (n)	Heterogeneity (I^2)
Rhinitis	Wilson D, 2003	Adults and children	21	484	475	73%
Rhinitis	Penagos M, 2006	Children	10	245	239	81%
Rhinitis	Radulović S, 2011	Adults and children	49	2,333	2,256	81%
Asthma	Calamita Z, 2006	Adults and children	9	150	153	64%
Asthma	Penagos M, 2008	Children	9	232	209	94%

Heterogeneity (I^2) = 0–40%: might not be significant; 30–60%: may represent moderate heterogeneity; 50–90%: may represent substantial heterogeneity; 75–100%: considerable heterogeneity

Table 2. Medication score

Medication scores	Reference	Study population	Studies (N)	Active participants (n)	Placebo (n)	Heterogeneity (I^2)
Rhinitis	Wilson D, 2003	Adults and children	17	405	398	44%
Rhinitis	Penagos M, 2006	Children	7	141	138	86%
Rhinitis	Radulović S, 2011	Adults and children	38	1,737	1,642	50%
Asthma	Calamita Z, 2006	Adults and children	6	132	122	92%
Asthma	Penagos M, 2008	Children	7	192	174	95%

doi: 10.2298/SARH1606345Z

participants is similar to that observed for monosensitized patients. This is of importance particularly in patients with respiratory allergies [27].

In the pediatric age range, allergic diseases represent a special problem, with specific aspects, that include their possible evolution (allergic march) [28–30], the problems related to the long-term pharmacotherapy [31], the compliance (which is in charge of caregivers), and the objective difficulties in correctly deliver inhaled drugs. In addition, the quality of life of the children themselves and of their parents (drug treatment, emergency unit visits, impaired school performance and absenteeism), is usually affected [32, 33].

On the other side, quality of life in children with asthma revealed poor score only for physical activities in children, while very poor score for parents and very high level of anxiety related to their children's asthma [34].

Many clinical trials and meta-analyses have convincingly demonstrated that AIT is effective in reducing symptoms and drug consumption, with a consequent improvement of the overall quality of life [35].

CLINICAL INDICATIONS

Recommendations from various allergy organizations to minimize risk and improve efficacy suggest the following considerations for initiating immunotherapy: 1) demonstrating the presence of IgE-mediated disease – atopic constitution in early childhood; 2) documentation that specific sensitivity is involved in symptoms – positive *in vivo* tests on aeroallergens and *in vitro* tests minimum class three on aeroallergens; 3) documentation of the severity and duration of symptoms of allergic rhinitis and asthma [7]. The first dose should be taken in the presence of a doctor (observation period of 30–60 minutes). In this way, patients are reassured about the safety of SLIT, their follow-up can be organized, and compliance structured. SLIT drops or tablets are recommended to be taken usually for three years as continuous treatment during the year or pre- and co-seasonal treatment.

SAFETY OF SLIT IN ALLERGIC CHILDREN

SLIT safety has been already proven in many DB-PC-RCTs and real-life settings [36].

Life-threatening and non-life-threatening severe systemic adverse events (SAEs) related to SLIT for allergic asthma, allergic rhinitis, or allergic rhino-conjunctivitis involving pediatric population are very rare even in DB-PC-RCTs. The prevalence of SAE was lower than 20%, whereas the prevalence of severe SAEs was between 1% and 2%, with only one study reported epinephrine use [37–39]. In real-life settings, most of the systemic reactions reported by post-marketing surveys were mild and resolved spontaneously without any treatment [40–45]. Potential risk factors for systemic adverse reactions are still confusing. Concerning the vaccine-related risk factors, the most relevant are the use of non-standardized extracts, administration of products containing a mixture of many allergens, and overdosing [46]. On the other side, cardiovascular diseases and long-term therapy with non-cardio selective beta-blockers, as well as uncontrolled asthma, oral lesions, and infections can be marked as potential triggers of SAEs related to the patient [47, 48].

The most common SLIT-related local adverse events include oropharyngeal signs and symptoms and gastrointestinal reactions with great variability of their prevalence, rated between 50% and 85% [49, 50, 51].

ORAL TOLERANCE

The immunological effects of SCIT have already been described in a great number of studies. Nevertheless, the questions on SLIT immune modulatory effects are strongly debated. It is well known that SLIT affects both humoral and cell-mediated immune responses. Concerning the allergen specific IgG4, it is proven that SLIT increases its level, although this effect is less intensive comparing with SCIT. The most important immunological effects of SLIT are reduction in mucosal infiltration by effector cells (neutrophils and eosinophils), increase in allergen-specific IL-10 production, and modulation in Th1 and Treg activity in the oral mucosa. The oral mucosa is described as a site of natural immune tolerance induction. Antigen presenting cells (APCs) in the oral mucosa were suggested to be the main actors for the modulation of IL-10 and TGF-β-secreting Tregs observed following sublingual immunotherapy. After allergen uptake by specialized APCs in the oral mucosa, they migrate to regional lymph nodes. These professional APCs are characterized by the expression of high levels of MHCclass I and II, costimulatory molecules such as CD40, CD80, CD86, and high levels of the IgE receptor FcεRI. As mentioned before, these cells are able to release IL-10 in a TLR4-dependent manner and induce T-cells with a regulatory phenotype *in vitro*, after contact with allergen [52, 53].

FUTURE PERSPECTIVES

Despite a great amount of DB-PC-RCTs and meta-analyses, there are no official guidelines on clinical practice on SLIT. In real life, detailed investigations (that have to be performed before the decision on immunotherapy) in children of preschool age are difficult due to poor cooperation of a child and its parents. Secondly, the lack of information and standardized protocols on allergen-specific immunotherapy among pediatricians, allergologists, and all relevant subspecialties make this situation even more complicated. As there is great interest for this kind of treatment, European Academy of Allergy and Clinical Immunology has launched a new project on Guidelines for Clinical Practice on Allergen-Specific Immunotherapy. The main goals for future trials and studies are the development of comprehensive guidelines for clinical practice on immunotherapy, embracing all the different potential

participants (i.e. clinicians, immunologists, GPs, allergen technologists, industry research department representatives, regulatory bodies, allied health representatives, and patient organizations) [54].

CONCLUSION

Subcutaneous immunotherapy (SCIT), which remained the only administration route for several decades, is effective and safe when properly prescribed and administered, but remote risk of severe side effects is present. For this reason, the international guidelines consider the age below five years a relative contraindication to SCIT. In contrast, SLIT seems to be safe and is presently considered as a viable alternative option for traditional subcutaneous route. In addition to the safety profile, SLIT is also convenient because no injection is needed and it can be managed at home. Recently, a number of studies proved the efficacy of simplified schedule (short build-up and once daily dosing) which have been introduced in order to make the SLIT even acceptable.

All of the above mentioned – proven clinical efficacy in a great number of studies in pediatric population, better safety profile comparing with SCIT (with the possibility of using it in children below the age of five years), disease modifying capability with the possibility of having the long-term effects – implicate that if we want to consider modern approach to allergic diseases, we have to take AIT into consideration. These facts take on special relevance in the pediatric age, when the plasticity and modulability of the immune system are maximal, and when preventative effects can be reasonably expected.

ACKNOWLEDGEMENT

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant No. III41004.

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Актуелни проблеми у сублингвалној алерген-специфичној имунотерапији код деце са астмом и алергијским ринитисом

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КРАТАК САДРЖАЈ

Године 1993. Европско удружење алерголога и клиничких имунолога препознало је сублингвални пут примене алрегена као „обећавајући" у процесу десензибилизације на алергене. Неколико година касније Светска здравствена организација (СЗО) препоручује овај вид терапије као „алтернативу" инјекционом путу примене алергена код адултних пацијената. Прва метаанализа показује ефикасност сублингвалне алерген-специфичне имунотерапије (СЛИТ) код пацијената са алергијским ринитисом, док је једна друга студија потврдила да СЛИТ може успешно да превенира развој астме код деце и одраслих са алергијским ринитисом. Главни циљ овог ревијалног рада је да презентује најновија знања и ставове на тему клиничке ефикасности и сигурности имунотерапије у лечењу педијатријских пацијената са алергијским ринитисом и астмом. Истраживање је извршено претрагом радова у базама *MEDLINE* и *PubMed* у периоду од 1990. до 2015. године користећи кључне речи астма, алергијски ринитис, деца и алерген-специфична имунотерапија. Већ од педијатријског узраста АИТ има посебно место у дечјем узрасту када су пластицитет и способност модулације имунског система максимални и када је оправдано очекивати значајан превентивни ефекат. Анализирајући резулате двоструко слепо плацебо контролисаних рандомизираних клиничких студија (на основу смањења симптом скора и употребе спасоносних лекова), ефикасност СЛИТ-а је показана у великом броју метаанализа. Резулати као што су риноконјунктивитис скор, лек скор, симптом-лек скор, квалитет живота, број дана са тешким симптомима, имонолочки параметри, сигурносни профил били су значајно бољи у групи пацијената који су били на сублингвалној алерген-специфичној имунотерапији у односу на пацијенте који су били на плацебу. Сигурност СЛИТ-а је показана у бројним двоструко слепо плацебо контролисаним студијама и *real life* студијама. У складу са свим претходно поменутим, главни циљеви будућих истраживања треба да се фокусирају на развој јасних водича за клиничку примену сублингвалне алерген-специфичне имунотерпије, укључујући знања и потенцијале различитих специјалиста. Алерген-специфична имунотерапија у педијатријском узрасту је нарочито важна зато што је пластицитет и модулабитет имунског система тада највећи, као и очекивани превентивни ефекти ове терапије.

Кључне речи: алерген-специфична имунотерапија; деца; астма; алергијски ринитис

Примљен • Received: 19/06/2015 **Ревизија • Revision:** 30/11/2015 **Прихваћен • Accepted:** 07/12/2015

Препоруке за гастропротекцију у превенцији крварења из дигестивног тракта

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КРАТАК САДРЖАЈ

Инциденца крварења из гастроинтестиналног тракта у већини популације износи око 1 на 1.000 становника годишње. Преко 65% свих епизода гастроинтестиналног крварења повезано је са употребом лекова. Најчешћи узрочници крварења су нестероидни антиинфламаторни лекови и мале дозе ацетил-салицилне киселине. Морталитет унутар првих месец дана након епизоде крварења износи око 10–12% и није се значајно мењао у последњих десет година. Због тога превенција крварења има несумњив значај. Правилна селекција пацијената, одговарајући избор лека, примена најмањих ефикасних доза потенцијално улцерогених лекова и примена лекова који инхибирају лучење желудачне киселине представљају главне превентивне мере крварења из гастроинтестиналног тракта.

Кључне речи: гастроинтестинално крварење; нестероидни антиинфламаторни лекови; желудачна киселина

УВОД

Инциденца крварења из горњег дела дигестивног тракта креће се од 35 до 172 (око 100 најчешће) на 100.000 становника годишње [1]. Инциденца највећим делом зависи од старости, тако да је код особа старијих од 80 година преко десет пута већа него код оних који су стари 40 до 60 година [1]. Морталитет у првих 30 дана након крварења је 10–12% и у последњих десет година је непромењен упркос значајном побољшању интензивне неге критично оболелих и успеха примарне ендоскопске хемостазе [2]. Највећи број пацијената умире због погоршања основне болести или услед кардиореспиратоних и цереброваскуларних компликација, а не од искрварења.

У последње три деценије лекови су постали најчешћи узрок крварења из ГИТ-а и узрокују преко 65% свих крварећих епизода [3, 4, 5]. Око 30% епизода крварења последица је употребе малих доза (75–100 *mg*) ацетил-салицилне киселине (*ASA*). Нестероидни антиинфламаторни лекови (НСАИЛ) на другом су месту и одговорни су за 18% свих епизода крварења. Са по 5% у етиологији крварења из ГИТ-а учествују орални антикоагуланси, глукортикоиди и селективни инхибитори преузимања серотонина (*SSRI*) [1–4] (Табела 1). Истовремено прописивање више потенцијално улцерогених лекова делује синергистички на појаву крварења. Најчешће се при ендоскопији код пацијената са крварењем дијагностикују дуоденални и гастрични улкус, а потом ерозивни гастритис, дуоденитис и езофагитис [6]. Лезије су углавном мултипле и захватају више органа дигестивног тубуса, што отежава ендоскопско или хируршко лечење. НСАИЛ и *ASA* су снажни аналгетици, тако да је крварење узроковано овим лековима подмукло, јер се јавља без претходног бола.

Прецизни епидемиолошки подаци о дигестивном крварењу у Србији нису доступни, али се може сматрати да наши пацијенти деле судбину становника Западне Европе. Ако се узме у обзир да у Србији живи око 7,5 милиона становника, годишње крвари око 8.000 људи, а 750 људи годишње умре због крварења узрокованих лековима! От-

Табела 1. Лекови најчешћи узрочници крварења из горњих партија дигестивног тракта
Table 1. Drugs involved in upper gastrointestinal bleeding

Лекови Drugs	Учесталост (%) Frequency (%)
АСК ASA	30.8
НСАИЛ NSAIDs	18.1
SSRI	6.0
ОАК OAC	5.2
Кортикостероиди Corticosteroids	4.2
Клопидогрел Clopidogrel	3.3
ASA + Клопидогрел ASA + Clopidogrel	1.6

АСК – ацетилсалицилна киселина; НСАИЛ – нестероидни антиинфламаторни лекови; *SSRI* – селективни инхибитори поновног преузимања серотонина; ОАК – орални антикоагуланси

ASA – acetylsalicylic acid; NSAIDs – nonsteroidal anti-inflammatory drugs; SSRI – selective serotonin reuptake inhibitors; OAC – oral anticoagulants

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прилике исти број људи умре годишње у саобраћајним несрећама у Србији. Зато је неопходна хитна акција професионалаца у здравственом систему и државе. Значај овако великог, општег проблема је потцењен у стручној јавности и целом друштву.

Последњих деценија су у циљу гастропротекције осмишљене стратегије које су смањиле, али нису потпуно елиминисале ризик од крварења. Постоје веома јасни научни докази да привржености гастропротекцији двоструко умањује ризик од крварења у реалном животу [7]. Гастропротекција мора да се примењује онолико дуго колико се узима улцерогени лек, јер прекид гастропротекције повећава ризик за крварење за 45% [4].

Постоји велики јаз између обиља научних доказа и реалног живота [1–4, 7]. Само 22% пацијената старијих од 65 година користи гастропротекцију. Неадекватна заштита пацијената последица је чињенице да ни лекари, а ни пацијенти нису довољно едуковани у овој области.

АЦЕТИЛ-САЛИЦИЛНА КИСЕЛИНА (*ASA*) У МАЛИМ ДОЗАМА (75–100 *mg*)

Број пацијената које је потребно лечити малом дозом *ASA* (*NNH*) да би се изазавало једно крварење варира од 270 до 833 [8]. Огроман број људи свакодневно узима *ASA*, који је најчешћи узрок крварења из ГИТ-а, пре свега због великог апсолутног броја пацијената који узимају лек [8]. Пуферизовани облици *ASA* и облици који се ослобађају у танком цреву не смањују значајно учесталост крварења, а много су скупљи [9].

Фактори ризика за крварење код пацијената који су на хроничној терапији са *ASA* су [9]:

- анамнеза улкусне болести
- анамнеза претходног крварења из дигестивног тракта
- истовремено узимање других антиагрегационих лекова, оралних антикоагуланаса (ОАК), глукокортикоида, НСАИЛ или *SSRI*

Ако су присутна најмање два од три доленаведена фактора:

а. старост преко 60 година
б. одраније дијагностикована диспепсија
в. одраније дијагностикована рефлуксна болест једњака

За сада се не препоручује рутинско тестирање и лечење *H. pylori* инфекције код свих будућих корисника *ASA*, већ само код оних који имају одраније позитивну анамнезу улкусне болести [10]. Тестирање треба да буде само издисајним тестом или ендоскопијом.

МИНИМАЛНА И ОПТИМАЛНА ПРЕПОРУКА ЗА ГАСТРОПРОТЕКЦИЈУ У ТОКУ *ASA* ТЕРАПИЈЕ

Гастропротекцију треба да добију пацијенти:

Оптимална препорука
- Сви са бар једним фактором ризика

Минимална препорука
- Сви који су имали улкус или крварење из ГИТ-а
- Сви са два фактора ризика

За гастропротекцију се користе искључиво лекови из групе *PPI* (блокатори протонске пумпе): омепразол, пантопразол, лансопразол, есомепразол и рабепразол. Они се дају у једној јутарњој дози пола сата пре доручка (20 *mg* омепразола, пантопразола и есомепразола, односно 15 *mg* лансопразола). У овој индикацији су *H*2 блокатори (ранитидин, фамотидин) инфериорни [11].

Гастропротекција се узима онолико дуго колико се узима *ASA*.

Са гастропротекцијом се не сме прекидати, јер њено укидање драматично повећава ризик од крварења. Безбедоносни профил дуготрајне примене *PPI* је повољан и корист далеко надмашује потенцијални ризик од њихове дуготрајне употребе [11]. *PPI* значајно смањују и кардиоваскуларни морталитет код корисника *ASA*, с обзиром на то да скоро 20% пацијената одбија терапију са *ASA* због диспепсије, а додатак *PPI* повећава адхеренцу за терапију *ASA*-ом за више од 5%, што доприноси смањењу кардиоваскуларног морталитета.

Лечење некрварећег улкуса код пацијената који су на терапији *ASA*-ом не захтева прекид узимања *ASA*-е. Треба ординирати *PPI* у јутарњој и у вечерној дози 14 дана и даље трајно наставити само са јутарњом дозом.

У току терапије крварећег улкуса изазваног *ASA*-ом, терапија *ASA*-ом се не сме прекидати дуже од пет дана, јер то значајно повећава морталитет. Тестирање на *H. pylori* и ерадикација инфекције су обавезни. Код *H. pylori* негативних потребан је *PPI* трајно, јер без гастропротекције ови пацијенти имају осмоструко већи ризик за поновно крварење [12].

ДВОЈНА АНТИАГРЕГАЦИОНА И КОМБИНОВАНА ТЕРАПИЈА

Кардиоваскуларне болести (КВБ) водећи су узрок смрти у Србији и у свету. Највећи део морталитета последица је акутног коронарног синдрома (*ACS*) и цереброваскуларног инсулта (ЦВИ). Најефикаснији начин лечења *ACS* јесте уградња стентова у инфарктну коронарну артерију. Двојна антиагрегациона терапија (*DAPT*), тј. комбинација *ASA* са клопидогрелом, обавезан је део терапијских протокола у циљу превенције тромбозе стента. Ризик од крварења је већи што је јачи антитромботични ефекат лека. Појава крварења је важан, независни предиктор морталитета (3,5 пута већи у првих годину дана у случају појаве крварења) и других компликација *ACS* [13]. Око 20–30% свих крварења су у ГИТ-у, јављају се код 0,5–2,3% пацијената и то су најчешћа крварења која нису повезана са процедуром уградње стента. Најчешће се јављају током хоспитализације или унутар првих 30 дана.

Фактори ризика за појаву ГИ крварења су:
- анамнеза о постојању ранијих ГИ крварења или горушице
- старост болесника преко 60 година

doi: 10.2298/SARH1606351D

- примена ОАК, НСАИЛ или гликопротеинских II*b*/III*a* антагониста
- стање циркулаторног шока
- потреба за инотропном подршком циркулације
- присуство коморбидитета (бубрежне инсуфицијенције и шећерне болести)
- пушење
- присуство *H. pylori* инфекције

На основу познавања ових предиктора конструисан је калкулатор [14] за израчунавање ризика за појаву ГИ крварења код срчаних болесника, бесплатно доступан на интернет-адреси http://www.asariskcalculator.com. Поред ризика од појаве крварења, калкулатор прорачунава и корист (ефикасност у односу на безбедност) од коришћења малих доза *ASA* у превенцији атерокслеротичних догађаја.

Крварења су још већи проблем код болесника на *DAPT* са атријалном фибрилацијом (АФ) због обавезе да се у превенцији емболијских компликација узимају и ОАК. Индикација за ОАК се поставља на основу скорова ризика: најпознатији су *CHADS2* и *CHA2DS2-VASC*. Коришћење калкулатора [14] овде има још већи значај.

Код болесника са високим ризиком за појаву крварења важно је да се правовремено поправе коректибилни фактори ризика: циркулаторни шок мерама реанимације, тешка анемија трансфузијама еритроцита, а код болесника на елективном програму перкутане коронарне интервенције (*PCI*) ерадикација *H. pylori* инфекције пре започињања *DAPT*. У планирању *PCI* процедуре предност се даје стентовима без лека (*BMS*) због краће примене *DAPT*.

МИНИМАЛНА И ОПТИМАЛНА ПРЕПОРУКА ЗА ПРЕВЕНЦИЈУ ГИ КРВАРЕЊА КОД ПАЦИЈЕНАТА НА *DAPT* ТЕРАПИЈИ

- Проценити ризик крварења свим болесницима којима се планира или се даје *DAPT*, коришћењем одговарајућих скорова или калкулатора.
  - Гастропротекцију треба да добију болесници са *ACS* и повећаним ризиком од ГИ крварења (животна доб ≥ 60 год. и било који од горе побројаних фактора ризика). Ако је једини фактор ризика старост, није потребно давати *PPI*.
  - Код болесника са АФ и елективном уградњом стента, примену стентова са леком (*DES*) ограничити само на јасно дефинисане индикације (дугачке лезије у проксималним сегментима коронарних артерија, дијабетичари).
  - Код болесника са АФ и елективном *PCI* процедуром са металним стентом без лека, *DAPT* са ОАК давати током месец дана, а потом наставити ОАК и *clopidogrel*. Гастропротекцију давати свима током првих месец дана, а касније само онима са високим ризиком за крварење.
  - Код болесника са АФ и *ACS*, или болесницима са АФ и елективном *PCI* процедуром који добију стент са леком (*DES*), тројну терапију (аспирин, клопидогрел и ОАК) давати у краћем временском периоду (током 3–6 месеци), а дуже (годину дана) селекционисаној групи болесника са малим ризиком од крварења. Гастропротекцију обавезно давати свима док су на тројној терапији, а касније само онима са високим ризиком.

Лекови избора у превенцији ГИ крварења су инхибитори протонске пумпе (*PPI*). Доста се дискутује о безбедности истовремене примене *PPI* са клопидогрелом [7]. Наиме, клопидогрел је пролек који се активира у јетри под дејством различитих ензимских система од којих је *CYP*2*C*19. Већина *PPI* се такође разграђује преко *CYP*2*C*19 ензимског система, па постоји могућност компетиције за овај ензимски систем са клопидогрелом. Афинитет различитих *PPI* за *CYP2C*19 није једнак и највећи је за омепразол, а нешто мањи за есомепразол. Афинитет пантопразола је веома мали, јер се доминантно разграђује преко ензима *CYP*3*A*4, па се не очекује инхибиција са антиагрегационом активношћу клопидогрела. Проблем интерреакције *PPI* са новим антитромбоцитним лековима (прасугрелом и тикагрелором) изгледа да не постоји. Данас се као *PPI* избора код болесника на *DAPT* препоручује пантопразол [15]. Лечење болесника на *DAPT* са ГИ тегобама или крварењем спада у домен заједничког рада гастроентеролога и кардиолога. Ендоскопија се сматра безбедном методом код особа које су хемодинамски стабилне.

НЕСТЕРОИДНИ АНТИИНФЛАМАТОРНИ ЛЕКОВИ

НСАИЛ су група лекова који смирују бол, нормализују повишену температуру и смирују запаљенску реакцију. Делују смањујући активност ензима циклооксигеназа (*COX*), које су важне за синтезу простагландина, а они су неопходни за настајање и одржавање запаљенске реакције. Активношћу ензима циклооксигеназе 1 (*COX*-1) стварају се простагландини битни за регулацију физиолошких функција организма (повећање протока крви кроз зид дигестивног тракта и лучење слузи која штити слузницу од нагризајућег деловања киселине у желуцу). Ензим циклооксигеназа 2 (*COX*-2) катализује настанак простагландина, који учествују у запаљенским реакцијама и изазивају бол. Већина НСАИЛ смањује активност и *COX*-2 и *COX*-1 ензима (неспецифични НСАИЛ). На тај начин НСАИЛ могу повећати ризик од крварења из ГИТ-а за два до четири пута [16, 17]. *COX*-2 селективни НСАИЛ (*sCOX*-2) подједнако добро смањују запаљење и бол као и неселективни НСАИЛ, али мање ремете заштиту слузнице ГИТ-а и тиме смањују учесталост крварења из ГИТ-а [18]. Ризик од ГИТ крварења се повећава уколико је полуживот лека дужи и уколико лек делује јаче антиагрегационо [19].

Фактори ризика за крварење из ГИТ-а су:

- животна доба (старије особе имају већу склоност ка крварењу из ГИТ-а)
- подаци о ранијем крварењу из ГИТ-а, пептичком улкусу и/или диспепсија

- употреба високих доза НСАИЛ
- истовремено узимање два НСАИЛ, или НСАИЛ и *ASA*, ОАК и кортикостероида
- *H. pylori* инфекција [20, 21].

Учесталост појединих фактора ризика за крварење из ГИТ-а у популацији особа које узимају НСАИЛ није подједнака за све факторе ризика. Пошто је остеоартритис (ОА) једна од најчешћих болести у популацији, а оболели веома често узимају НСАИЛ, подаци о учесталости појединих фактора ризика за крварење из ГИТ-а у популацији ових болесника су значајни. У узорку од 17.105 болесника са ОА [22], процентуална учесталост појединих фактора ризика за крварење из ГИТ-а је била: старост >60 година – 76,1%, диспепсија – 36,4%, НСАИЛ + аспирин – 21,4%, висока доза НСАИЛ – 20,3%, НСАИЛ + антикоагуланс – 12,6%, некомпликовани улкус – 12,4%, НСАИЛ + кортикостероид – 7,9%, компликовани улкус – 3,3%. У истој групи болесника са ОА, један фактор ризика је имало 27,5% болесника, два фактора ризика истовремено 37,4%, а три фактора ризика истовремено 20,2% болесника. Само 7,5% болесника из ове групе није имало ниједан фактор ризика за крварење из ГИТ-а, а 7,5% је имало четири или више фактора ризика истовремено.

На основу учесталости и врсте факора ризика за крварење из ГИТ-а, болесници се деле у три групе: болесници са малим ризиком, болесници са умереним и великим ризиком од крварења.

Мали ризик од крварења имају болесници без горепоменутих фактора ризика за крварење из ГИТ-а.

Умерени ризик за крварење имају болесници са бар једним од следећих фактора ризика:

- старији од 60 година
- уз НСАИЛ узимају и малу дозу *ASA*
- уз НСАИЛ узимају и кортикостероиде
- имају податке о клинички испољеном пептичком улкусу
- имају податке о диспепсији
- узимају велику дозу НСАИЛ
- узимају два НСАИЛ

Високи ризик за крварење из ГИТ-а имају болесници који су раније крварили из ГИТ-а, или истовремено узимају НСАИЛ и *OAT*, или имају истовремено три фактора за умерени ризик од крварења [20].

Очекивана учесталост крварења за болеснике са ниским ризиком је 1,5 крварења на 100 болесник/година. Код болесника са умереним ризиком, очекивана учесталост крварења је 1,5–10 крварења на 100 болесник/година, а за оне са великим ризиком очекује се >10 крварења на 100 болесник/година.

Особе млађе и средње животне доби, без факора ризика за крварење из ГИТ-а су једине које имају мали ризик од крварења из ГИТ-а током узимања НСАИЛ.

МИНИМУМ И ОПТИМУМ ЗАШТИТЕ ПАЦИЈЕНАТА КОЈИ УЗИМАЈУ НСАИЛ

Крварење из активног улкуса

Ако је *Helicobacter pylori* (*Hp*) инфекција доказана, онда је ерадикација инфекције први корак, и то чим почне унос хране на уста. После ерадикације у складу са прихваћеном водичима добре праксе (*Maastricht* IV), наставља се и доза одржавања *PPI* у случају улкусне нише желуца, до ендоскопски потврђеног излечења.

Ако нема *Hp* инфекције, а постоји НСАИЛ као узрок, онда се саветује прекид НСАИЛ или замена неселективног лека селективним *COX*-2 инхибитором уз додавање *IPP* [23, 24].

Превенција развоја улкуса у току терапије НСАИЛ

Први корак је стратификација кардиоваскуларног (КВ) – велики и мали, и гастроинтестиналног (ГИ) ризика – мали, средњи и велики ризик (Табела 2).

Минимум заштите слузнице (Табела 3) подразумева две групе пацијената: ако је процењени ГИ ризик велики, онда у превенцији улкуса треба тестирати и лечити *Hp* инфекцију ако је има, елиминисати друге потенцијално штетне факторе и додати намању дозу *IPP* уз најмању потребну дозу НСАИЛ.

Ако је реч о крварећем улкусу, минимум заштите био би ерадикација *Hp* инфекције ако је има, забрана НСАИЛ ако је могуће, ако није, онда трајно *IPP* (ако је могуће неселективни НСАИЛ заменити селективним *COX*-2 инхибитором).

Оптимална заштита слузнице (Табела 4) подразумева код малог КВ и ГИ ризика најмању дозу најмање улцерогеног лека, а код свих осталих категорија, и средњег и великог ризика, трајно узимање најмање дозе *PPI*.

Табела 2. Превенција улкуса изазваног нестероидним антиинфламаторним лековима
Table 2. Prevention of ulcus induced by nonsteroidal anti-inflammatory drugs

Кардиоваскуларни ризик Cardiovascular risk	Гастроинтестинални ризик Gastrointestinal risk		
	Мали Low	Средњи Medium	Велики High
Мали Low	Најмања доза најмање улцерогеног лека Lowest dose of the safest drug	НСАИЛ + ИПП NSAIDs + PPI	*Cox-2* + ИПП Cox-2 + PPI
Велики High	НСАИЛ + ИПП NSAIDs + PPI	НСАИЛ + ИПП NSAIDs + PPI	Забрана НСАИЛ и *Cox*-2 NSAIDs and Cox-2 prohibited

ИПП – инхибитори протонске пумпе; *Cox-2* – селективни инхибитори циклооксигеназе 2

PPI – proton pump inhibitors; Cox-2 – selective inhibitors of cyclooxygenase-2

doi: 10.2298/SARH1606351D

Табела 3. Минимум заштите слузнице током коришћења нестероидних антиинфламаторних лекова
Table 3. Minimal gastroprotection during nonsteroidal anti-inflammatory drug use

Превенција ако је велики ГИТ ризик Gastroprotection with high risk	Ерадиковати *Helicobacter pylori* инфекцију, ако је има *Helicobacter pylori* eradication if bacteria is present
	Елиминисати друге штетне факторе Eliminate other adverse factors
	ИПП уз најмању довољну дозу НСАИЛ PPI with the smallest effective NSAID dose
Крварећи улкус Bleeding ulcer	Ерадикација *Helicobacter pylori* ако има инфекције *Helicobacter pylori* eradication if bacteria is present
	Елиминисати друге штетне факторе Eliminate other adverse factors
	Прекид НСАИЛ, а ако мора да се узима, онда ИПП стално (ако може *COX*-2 уместо неселективних НСАИЛ) Discontinue NSAID, but if necessary then PPI continuously (if feasible Cox-2 instead of non-selective NSAIDs)

Табела 4. Оптимум заштите слузнице током коришћења нестероидних антиинфламаторних лекова
Table 4. Optimal gastroprotection during nonsteroidal anti-inflammatory drug use

Мали ризик Low risk	Најмања доза најмање улцерогеног лека Lowest dose of the safest drug
Средњи и велики ГИТ ризик Medium and high risk	Ерадикација *Helicobacter pylori* инфекције, ако је има Helicobacter pylori eradication if infection is present
	Елиминисати друге штетне факторе Eliminate other adverse factors
	ППИ уз најмању довољну дозу НСАИЛ PPI with the smallest effective NSAID dose
Крварећи улкус Bleeding ulcer	Ерадикација *Helicobacter pylori* инфекције, ако је има *Helicobacter pylori* eradication if infection is present
	Елиминисати друге штетне факторе Eliminate other adverse factors
	Прекид НСАИЛ, а ако је неопходно да се узима, онда ИПП стално (ако може *COX*-2 уместо неселективних НСАИЛ) Discontinue NSAID, but if necessary then PPI continuously (if feasible Cox-2 instead of non-selective NSAIDs)

КОРТИКОСТЕРОИДИ

Кортикостероиди (КС) су група лекова са широком применом у различитим гранама медицине. Иако се ови лекови користе деценијама, још увек постоје недоумице да ли су и у којој мери кортикостероиди улцерогени.

Резултати метаанализа које су имале за циљ да процене улцерогеност лекова из групе кортикостероида су контроверзни [25, 26]. Месер и сарадници су показали да терапија КС повећава ризик за настанак улкуса 2,3 пута и 1,5 пута ризик за крварење из улкуса [25]. Кон и сарадници су показали да инциденца улкуса није битно повећана у току терапије КС [26]. Различит резултат ових метаанализа вероватно је последица разлика у критеријумима за укључивање пацијената у студије на основу којих је рађена метаанализа.

Најчешће индикације за КС терапију у групи пацијената са ГИТ крварењем су артритис у 25%, хронична опструктивна болест плућа (ХОБП) у 17% и бронхијална астма у 14% случајева.

Инциденца улкуса и ризик за крварење директно зависе од дозе кортикостероида [26]. У току терапије ниским и средњим дозама КС ризик за крварење из ГИТ повећан је 1,6, а у току терапије високим дозама КС 3,3 пута. Истовремена употреба КС и НСАИЛ повећава ризик за крварење из ГИТ четири пута у току лечења ниским, а чак 12 пута у току лечења високим дозама КС. Уколико пацијенти уз ниске дозе КС користе и мале дозе *ASA*, не повећава се ризик за крварење из ГИТ, али високе дозе КС уз ниску дозу *ASA* носе 4,43 пута већи ризик за крварење из ГИТ-а [4].

Фактори ризика за крварење код пацијената на терапији кортикостероидима су:

- високе терапијске дозе кортикостероида
- истовремена употреба кортикостероида са једним или више лекова из групе нестероидних антиинфламаторних лекова (НСАИЛ), аспирина (*ASA*), селективних инхибитора преузимања серотонина (*SSRI*) и/или оралних антикоагуланаса (*OAT*)
- анамнестички податак о ранијем улкусу гастродуоденума и/или крварењу из ГИТ-а

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Гастропротекцију треба да добију пацијенти:

- који су крварили или имали улкус
- на високим дозама КС
- који уз КС користе још један или више лекова из групе: НСАИЛ, ОАК, *SSRI*, као и *ASA* или клопидогрел

Гастропротекција се даје онолико дуго колико се узима КС терапија, а за гастропротекцију се користи искључиво лек из групе *PPI*.

Проблем ерадикације *Hp* инфекције пре започињања кортикостероидне терапије није обухваћен Мастрихт консензусом [10]. Вероватно се треба водити чињеницом да код пацијената са ранијим улкусом и крварењем

из улкуса гастродуоденума, независно од употребе кортикостероида, постоји индикација за ерадикацију инфекције. *Hp* је независан фактор ризика за настанак улкуса гастродуоденума, те као такав највероватније може само потенцирати улцерогеност кортикостероида. Са друге стране, дуготрајна терапија лековима из групе *IPP* повезана је са настанком атрофичног гастритиса и код тих пацијената индикована је ерадикација инфекције по Мастрихт IV консензусу [10].

ОРАЛНИ АНТИКОАГУЛАНСИ

Крварења у току терапије ОАК лековима најчешће су порекла ГИТ-а (41%) и уринарног тракта (14%) [27].

У половини случајева крварења у току *OAT* су тзв. *велика крварења* (енгл. *major bleed*). Просечна дужина хоспитализације ових пацијената је чак 23 дана, а морталитет 10% у току епизоде *великог крварења*. По поновном увођењу *OAT*, један од 12 пацијената ће поново крварити [27]. У 58% случајева крварења лезија је пептички улкус, док су у 42% случајева крварења другог порекла [27]. Употреба већег броја улцерогених лекова повећава ризик за крварење из ГИТ-а.

Показано је да је ризик за поновну епизоду крварења могуће смањити тако што ће *INR* бити редовно и стриктно контролисан тако да буде у терапијском опсегу. Такође је неопходно пре поновног увођења *OAT* ендоскопски верификовати зарастање улкуса [28].

Фактори ризика за крварење код пацијената који започињу или су на хроничној терапији оралним антикоагулансима су:

- претходно крварење из ГИТ-а
- старост (особе старости изнад 65–70 година имају пет пута већи ризик за крварење)
- Истовремена употреба антиагрегационе терапије или лекова из група КС, НСАИЛ, *SSRI*
- *Hp* инфекција
- женски пол
- *CYP2C9* и/или *VKORC1* генски полиморфизам
- високе дозе *OAT* (*INR* > 4.5)
- нестабилан *INR* (*TTR* < 60%, *TTR – time in therapreutic range*)
- коморбидитет (*HTA*, болести јетре, бубрега, *CMP*, малигнитет)

Ризик је кумулативно већи што је присутно више фактора ризика [29].

ОПТИМАЛНА ПРЕПОРУКА ЗА ГАСТРОПРОТЕКЦИЈУ У ТОКУ ОАК

Гастропротекцију треба да добију пацијенти:

- са макар једним фактором ризика
- старији од 65 година
- са нестабилним *INR*-ом
- у току првих 90 дана *OAT*

МИНИМАЛНА ПРЕПОРУКА ЗА ГАСТРОПРОТЕКЦИЈУ У ТОКУ ОАК

Гастропротекцију треба да добију пацијенти:

- који су крварили или имали улкус
- који уз *OAT* користе и још један или више лекова из група НСАИЛ, КС, *SSRI* или антиагрегационих лекова

За профилаксу се користе искључиво лекови из групе *PPI*, а лекови се дају све време док се узима ОАК. Мастрихт консензус за дијагностику и лечење *Hp* инфекције не доноси јасне препоруке за пацијенте на оралној антикоагулантној терапији. Мастрихт консензус [10], са друге стране, јасно дефинише да дуготрајна терапија инхибитором протонске пумпе представља индикацију за ерадикациону терапију.

СЕЛЕКТИВНИ ИНХИБИТОРИ ПРЕУЗИМАЊА СЕРОТОНИНА

SSRI користе се у лечењу депресије, булимије и опсесивно-компулзивних поремећаја. Механизам којим *SSRI* повећавају ризик за крварење из ГИТ-а укључује пораст киселе желудачне секреције у току употребе *SSRI* и антагонистички утицај на агрегацију тромбоцита. Сматра се да пацијенти који узимају лекове из групе *SSRI* имају низак апсолутни ризик за крварење из ГИТ-а [30]. Истовремена употреба НСАИЛ уз *SSRI* повећава ризик за крварење из ГИТ-а осам пута, а уколико пацијент поред НСАИЛ узима и салицилате уз *SSRI*, ризик за крварење из ГИТ-а повећава се 28 пута [31]. Уколико пацијент узима инхибитор протонске пумпе у циљу гастропротекције, ризик за крварење из ГИТ-а у току терапије *SSRI* смањује на ниво ризика у здравој популацији. Истовремена употреба оралне антикоагулантне терапије и *SSRI* повећава ризик од *велике епизоде* крварења за 4,4 пута [32].

Табела 5. Фактори ризика за крварење из гастроинтестиналног тракта (ГИТ) код пацијената на терапији селективним инхибиторима преузимања серотонина
Table 5. Risk factors for upper gastrointestinal bleeding in patients taking SSRI drugs

Фактор ризика Risk factor	Релативни ризик у односу на пацијенте који не узимају *SSRI* Relative risk compared with patients not taking SSRI
Helicobacter pylori	2.73
Мушки пол Males	1.15
Старост Advanced age	1.26
НСАИЛ NSAIDs	3.91
АСК ASA	3.00
Раније дијагностикован улкус Previous ulcer	7.31
Претходна епизода крварења из ГИТ Previous gastrointestinal bleeding	3.01

doi: 10.2298/SARH1606351D

Примена *SSRI* и антиагрегационе терапије, у случају монотерапије клопидогрелом повећава ризик за крварење из ГИТ-а за 1,54 пута, док двојна антиагрегациона терапија (аспирин и клопидогрел) уз *SSRI* носи ризик од 1,57 пута. [33]

Фактори ризика за крварење код пацијената који започињу или су на хроничној терапији *SSRI* [34] приказани су у табели 5.

МИНИМАЛНА ПРЕПОРУКА ЗА ГАСТРОПРОТЕКЦИЈУ У ТОКУ ТЕРАПИЈЕ ЛЕКОВИМА ИЗ ГРУПЕ *SSRI*

Гастропротекцију треба да добију пацијенти:
- који су крварили или имају улкус
- који уз *SSRI* користе још и један или више лекова из група НСАИЛ, ОАК, КС, антиагрегационих лекова

ОПТИМАЛНА ПРЕПОРУКА ЗА ГАСТРОПРОТЕКЦИЈУ У ТОКУ ТЕРАПИЈЕ ЛЕКОВИМА ИЗ ГРУПЕ *SSRI*

Гастропротекцију треба да добију пацијенти:
- сви са макар једним фактором ризика
- сви са поремећајима микроциркулације (дијабетес, хипертензија)

За гастропротекцију се искључиво користе лекови из групе *PPI*, а примена траје све док се узимају *SSRI*.

Мастрихт консензус за дијагностику и лечење *Helicobacter pylori* инфекције не доноси јасне препоруке за пацијенте на терапији *SSRI* [10]. У одлуци о ерадикацији инфекције треба се водити принципима који су објашњени у поглављу о оралним антикоагулансима и кортикостероидима.

ЗАКЉУЧАК

Неки од данас најчешће примењиваних лекова: антиреуматици, антиагрегациони лекови, орални антикоагулантни лекови и антидепресиви повећавају ризик од крварења у гастроинтестиналном тракту и веома су значајан разлог јатрогеног морталитета и морбидитета. Присуство симптома (диспепсија, рефлукс) није предиктор гастроинтестиналних компликација, па се превентивне мере морају спроводити код свих пацијената који имају одговарајуће факторе ризика. У већини индикација превенција крварења се спроводи леком из групе инхибитора протонске пумпе, а приликом избора лека треба обратити пажњу на конкомитантну терапију због потенцијалних интеракција.

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Recommendation for gastroprotection in gastrointestinal bleeding prevention

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SUMMARY

Incidence of gastrointestinal bleeding in most populations is about 1 per 1,000 inhabitants. More than 65% of all bleeding episodes are associated with drug use. The most often involved are non-steroidal antiinflammatory drugs and low doses of acetyl-salicylic acid. The mortality within the first month after the bleeding episode is about 10–12%, and has not significantly changed in the last decade. Therefore, bleeding prevention is of major importance. Appropriate selection of patients, proper drug choice, application of lowest efficient doses of potentially ulcerogenic drugs, and use of drugs that inhibit gastric acid secretion remain cornerstone preventive measures of gastrointestinal bleeding.

Keywords: gastrointestinal bleeding; nonsteroidal anti-inflammatory drugs; gastric acid

Примљен • Received: 03/06/2015 **Ревизија • Revision:** 13/10/15 **Прихваћен • Accepted:** 01/04/2016

Sudden cardiac death and guidelines for pre-participation examination in athletes

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SUMMARY
Incidence of sudden cardiac death (SCD) in athletes under 35 years of age is between 0.4 and 4.4 in 100,000. The highest mortality is seen in older athletes (≥35 years) who engage in running, mostly because of complications of atherosclerotic coronary ischemic disease. Majority of European countries are guided by European Society of Cardiology's (ESC) pre-participation screening (PPS) recommendations that include electrocardiography (ECG), while in the United States of America the ECG is not a routine part of the PPS examination. In Serbia, the ESC guidelines are being used, but there are no references prescribed by the Ministry of Health. The authors of this study believe that the national strategy for sport improvement should be accompanied with clear and well defined PPS recommendations that could be tenable in our health system.
Keywords: athlete screening; cardiac death; competition

INTRODUCTION

Sudden cardiac death (SCD) in athletes usually happens unexpectedly, to someone who seemed "perfectly healthy", but it can also happen to those with a diagnosed health condition who did not discontinue sports engagement contrary to physician's advices. Practicing sports can potentiate the development of cardiovascular diseases in genetically susceptible individuals or accelerate phenotypic expression. Intensity and extent of a sports activity can influence the manifestation and progression of the pathology; therefore, professional athletes represent a high risk group [1].

The incidence of SCDs in athletes under 35 years of age is between 0.4 in 100,000 in Italy, and 4.4 in 100,000 in the United States of America (USA), but it can be as high as 6 in 100,000 [2–7]. SCD death rate among athletes who are members of the National Collegiate Athletic Association (NCAA) was found to be 2.3 in 100,000 [8]. Harmon et al. [8] concluded that basketball players at USA's Division I universities (rank I indicates the biggest engagement in sports and the highest premiums) were dominantly exposed to sudden deaths of cardiac origin with the incidence of 8.3 in 100,000 athletes yearly. The authors report that, concerning all three ranks, the risk of SCD is three times higher in black race basketball players. The literature data show marked incidence of SCDs among black race athletes [2, 6, 8, 9]. Other sports with high incidence of SCDs are football, swimming, lacrosse, and cross-country running/skiing [2, 8, 9]. Actually, the greatest incidence of SCDs (1 in 7,620) is seen among athletes above 35 years of age, more precisely in the fifth decade of life, who dominantly engage in running and it is most often the result of complications of atherosclerotic coronary ischemic disease [6, 10]. More than 70% of leisure athletes aged 18 to 70 years exercise in intensities above recommended for their individual risk level [11]. Among American military recruits, the incidence of SCDs is 1 in 9,000, and those are mostly young people between 18 and 35 years of life, exposed to everyday strenuous exercises [6]. In relation to sex, there is a dominance of SCDs in males. In the above mentioned study of Harmon et al., male to female ratio was 2.3, although that number is much lower than the ones reported by other authors [5, 8, 10, 12]. The prevalence of cardiovascular diseases that can lead to SCD is 0.3% in a population of young athletes [13].

By comparison, the causes of death in individuals aged 1–18 years it was determined that SCDs happened in 5.8% of all deaths, 14% of which happened during moderate to extreme workout [14]. Out-of-hospital cardiac arrest in children and young adults within King County (WA, USA) was related to physical activity in 25% of cases. In Texas, research conducted on a cohort of children with out-of-hospital cardiac arrest, aged 0–18 years, has shown that arrest appeared in 40% of older children during physical exercise [3].

In our country, according to Sports Association of Serbia's data for 2009, there were 18,263 registered athletes actively competing in all branches of sport. Data of the Alliance for Leisure Sport in Serbia from 2010 state that there were 73,600 members at the time. University

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Sports Federation of Serbia claimed 10,000 members in the same year, and Serbian School Sports Federation regarded all students of elementary and high schools (close to one million children) as active athletes [15]. Health statistics related to SCDs in athletes does not exist. According to Health and Statistics Yearbook for 2014, there were 1,503 deaths in individuals under 35 years of age, and 25 of them had a diagnosis of cardiac arrest, with predominance in male sex [16].

The US National Registry of Sudden Death in Athletes was instituted in 1992 under the Minneapolis Heart Institute Foundation and it encompasses information about young athletes who engage in competitive sports [12]. Additionally, there are several other ways for gathering information about SCDs in athletes [8].

The Minnesota High School League insists that every athlete has insurance in case of a catastrophic event. Such insurance policy includes compensation for deaths related to sports activity that happened during games or practice [17].

PRE-PARTICIPATION CARDIOVASCULAR SCREENING PROTOCOLS

American Heart Association (AHA) asserts that a competitive athlete is one who participates in organized group or individual sports that require systematic training and regular competitions in goal of achieving impeccable results. Leisure athlete is a middle-aged person (≥ 35 years) who participates in various, informal sport activities of recreational type, in permanent regime or with pauses, which do not require systematic training and are not necessarily directed towards achieving impeccable results [1, 6].

According to the Italian law from 1982, pre-participation screening (PPS) of athletes is mandatory and it contains a precise description and extent of examinations that physicians are obliged to follow, for in the contrary they may be found criminally negligent. In USA there are no legal rules for screening. AHA adopted the 36th Bethesda conference consensus and established PPS guidelines on its basis, which were published in 2007. The screening protocol comprises two parts – there are seven questions from personal and three questions from family history in the first part, while the second part covers four examination points [18]. A recent American study showed that out of 257 Division I universities, PPS was mandatory in 100% before any sports activity, of whom 4% required electrocardiography (ECG) and in 0.4% echocardiography was performed on every new-coming athlete [19]. In the majority (63%) of these athletes, with the beginning of a new season, those that had been previously examined were only required to go through personal history questionnaire, usually with their trainer, without the need for a new examination. In addition, in 40% of the cases the questionnaires lacked crucial questions from the AHA recommendations. In conclusion, 92% did not satisfy given PPS standards [19].

Most European countries follow guidelines of the European Society of Cardiology (ESC) that originated in 2005. In 2010, the same group had published guidelines for pre-participation analysis of 12-lead ECG [20].

In general, the biggest difference between European and American beliefs regarding the subject is that the USA does not promote inclusion of ECG in PPS protocol underlining that routine ECG is not cost-beneficial for screening a large number of athletes due to its low specificity and high cost [1, 18, 21]. Interestingly, the ESC guidelines for ECG interpretation in athletes were twice revised in order to give them better specificity, and both times the conferences were organized in the USA – "the Stanford criteria" from 2011 and "the Seattle criteria" from 2012 [22, 23]. By using the Seattle criteria, the number of "abnormal" ECGs appears to be the smallest and there is less need for further diagnostic evaluation [6, 24]. When the 2010 ESC guidelines were used, there were no differences in sensitivity and specificity in detection of abnormal ECG patterns between cardiologists and primary care specialists; however, cardiologists did, with significantly greater sensitivity, correctly diagnose the abnormal patterns [4, 13].

The authors from Bethesda applied a statistical model – the Markov model – in order to compare economical reflections of the following modes of screening: 1) history and examination with referral to cardiologist if a suspicion of an abnormality occurred; 2) history and examination, followed by ECG and referral to cardiologist if anything pointed to an abnormality; and 3) only ECG, with referral to cardiologist if an abnormality was suspected. As a result, a screening based only on ECG had the most favorable profitability [7].

The Survival of Myocardial Infarction Long-term Evaluation (SMILE) study comprises the results of the still active ESC's project – the SMILE project. The project was started in 2006. It was designed to interconnect sports centers in Greece, Germany, France, and Algeria with a center in Italy and with expert members of the ESC sports cardiology study group in case of need for second and, possibly, third opinion. ECG recordings are being digitalized for standardization and history and examinations are being conducted according to 36th Bethesda guidelines. The authors believe that this way of collaboration increases the efficacy of screening without increasing the costs [25, 26].

In November 2013 at an AHA yearly meeting in Dallas, four experts from USA and Europe defended their views regarding PPS in front of a live audience. After their presentation the audience was asked to vote, and 60% of live audience voted for ECG in screening protocols. Of those that voted through the internet, 58% were in favor of PPS with history, examination, and ECG. In the USA there was a slight predominance of those agreeing that ECG should be done (45%) in relation to those who consider history and examination sufficient (35%), while in Italy the majority voted for screening according to ESC guidelines [27].

The target group for implementation of these guidelines is high school children and young adolescents, since this is the age range in which sport participation culminates. However, the guidelines are equally applicable to those under 12 and those above 35 years of age [1, 5].

doi: 10.2298/SARH1606359G

RECOMMENDATIONS IN SERBIA

In our country, according to the current Law on Sports and Regulation on Determining Medical Fitness in Athletes Regarding Engagement in Sports and Participation in Sports Competitions on the territory of the Republic Of Serbia, general and exceptional sports ability of a competitive athlete is determined in competent health institutions, i.e. institutes for sports and sports medicine. The above mentioned regulation also contains a questionnaire with detailed set of personal and family history questions and a medical examination form. This is in fair accordance with global recommendations [28].

Serbia follows the ESC guidelines. The Republic of Serbia's Ministry of Health website does not display the national guideline regarding PPS. Subjects of screening and SCDs are less represented in scientific literature in comparison to other countries. When using keywords *screening*, *athletes,* and *sudden cardiac death*, a small number of, mostly, review articles originating from Serbia occur as the result of index databases search. References in these articles are predominantly from foreign authors and auto- or hetero-citations [29–32].

CONCLUSION

The national strategy for health improvement foresees that by the year 2018 there will be 70% of children engaged in organized sports activities minimally three hours per week and 40% of adults will participate at least once a week in sports and recreation. The strategy also predicts better results of our athletes at international competitions of the highest rank.

The authors believe that this strategy should be accompanied with clearly defined recommendations for pre-participation cardiovascular screening that will be tenable in the existing health system. Also, it is expected that trends in Serbia will follow the world's trend of increase in number of recreational athletes. Having this in mind, our viewpoint is that primary care physicians should be introduced to the policy of cardiovascular examinations in sportsmen through educative courses, as it is not unreasonable to think that a health certificate with health risk assessment will very soon be needed before engagement in any organized athletic recreation.

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Напрасна срчана смрт и препоруке за препартиципациони преглед спортиста

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КРАТАК САДРЖАЈ

Инциденција напрасних срчаних смрти код спортиста млађих од 35 година креће се између 0,4 и 4,4 на 100.000. Највећи морталитет среће се код спортиста старијих од 35 година који се баве трчањем и најчешће је резултат атеросклерозом посредоване коронарне исхемијске болести. Већина европских земаља руководи се препорукама за препартиципациони скрининг (ППС), које обухватају и електрокардиографију (ЕКГ), објављених од стране Европског удружења кардиолога (*European Society of Cardiology, ESC*), док се ЕКГ рутински не ради приликом ППС унутар Сједињених Америчких Држава. У Србији су у употреби *ESC* препоруке, али не постоје водичи препоручени од стране Министарства здравља. Аутори рада сматрају да је у оквиру националне стратегије за развој спорта потребно наћи јасне и добро дефинисане ППС препоруке које би биле примењиве у постојећем здравственом систему.

Кључне речи: скрининг спортиста; срчана смрт; такмичење

Примљен • Received: 14/04/2015 **Ревизија • Revision:** 29/09/15 **Прихваћен • Accepted:** 13/11/2015

Корисни линкови

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Пре подношења рукописа Уредништву часописа „Српски архив за целокупно лекарство“ сви аутори треба да прочитају Упутство за ауторе (*Instructions for Authors*), где ће пронаћи све потребне информације о писању и припреми рада у складу са стандардима часописа. Веома је важно да аутори припреме рад према датим пропозицијама, јер уколико рукопис не буде усклађен с овим захтевима, Уредништво може одложити објављивање рада или чак одбити његово публиковање. Стога се ауторима и потенцијалним сарадницима саветује да пажљиво прочитају ово упутство пре него што приступе припреми рукописа за штампу у „Српском архиву за целокупно лекарство“.

ОПШТА УПУТСТВА. Оригинални и стручни радови се у целини достављају на енглеском језику са сажетком на енглеском и српском. Текст рада куцати у програму за обраду текста *Word*, фонтом *Times New Roman* и величином слова 12 тачака (12 *pt*). Све четири маргине подесити на 25 *mm*, величину странице на формат А4, а текст куцати с двоструким проредом, левим поравнањем и увлачењем сваког пасуса за 10 *mm*, без дељења речи (хифенације). Не користити табулаторе и узастопне празне карактере (спејсове) ради поравнања текста, већ алатке за контролу поравнања на лењиру и *Toolbars*. За прелазак на нову страну документа не користити низ ентера, већ искључиво опцију *Page Break*. После сваког знака интерпункције ставити само један празан карактер. Ако се у тексту користе специјални знаци (симболи), користити фонт *Symbol*. Подаци о коришћеној литератури у тексту означавају се арапским бројевима у угластим заградама – нпр. [1, 2], и то редоследом којим се појављују у тексту. Странице нумерисати редом у доњем десном углу, почев од насловне стране.

При писању текста на енглеском језику треба се придржавати језичког стандарда *American English* и користити кратке и јасне реченице. За називе лекова користити искључиво генеричка имена. Уређаји (апарати) се означавају фабричким називима, а име и место произвођача треба навести у облим заградама. Уколико се у тексту користе ознаке које су спој слова и бројева, прецизно написати број који се јавља у суперскрипту или супскрипту (нпр. ^{99}Tc, IL-6, O_2, $Б_{12}$, CD8).

Уколико је рад део магистарске тезе, односно докторске дисертације, или је урађен у оквиру научног пројекта, то треба посебно назначити у Напомени на крају текста. Такође, уколико је рад претходно саопштен на неком стручном састанку, навести званичан назив скупа, место и време одржавања.

КЛИНИЧКА ИСТРАЖИВАЊА. Клиничка истраживања се дефинишу као истраживања која се односе на једну здравствену интервенцију или на више њих, а ради испитивања утицаја на здравствени исход. Регистарски број истраживања треба да се наведе у последњем реду Кратког садржаја.

ЕТИЧКА САГЛАСНОСТ. Рукописи о хуманим медицинским истраживањима или историјама болести пацијената треба да садрже изјаву у виду писаног пристанка испитиваних особа у складу с Хелсиншком декларацијом и одобрење надлежног етичког одбора да се истраживање може извести и да је оно у складу с правним стандардима. Експериментална истраживања на хуманом материјалу и испитивања вршена на животињама треба да садрже изјаву етичког одбора установе и треба да су у сагласности с локалним правним стандардима.

ИЗЈАВА О СУКОБУ ИНТЕРЕСА. Уз рукопис се прилаже потписана изјава у оквиру обрасца *Submission Letter* којом се аутори изјашњавају о сваком могућем сукобу интереса или његовом одсуству. За додатне информације о различитим врстама сукоба интереса посетити интернет-страницу Светског удружења уредника медицинских часописа (*World Association of Medical Editors – WAME; http://www.wame.org*) под називом „Политика изјаве о сукобу интереса“.

АУТОРСТВО. Све особе које су наведене као аутори рада треба да се квалификују за ауторство. Сваки аутор треба да је учествовао довољно у раду на рукопису како би могао да преузме одговорност за целокупан текст и резултате изнесене у раду. Ауторство се заснива само на: битном доприносу концепцији рада, добијању резултата или анализи и тумачењу резултата; планирању рукописа или његовој критичкој ревизији од знатног интелектуалног значаја; завршном дотеривању верзије рукописа који се припрема за штампање.

Аутори треба да приложе опис доприноса појединачно за сваког коаутора у оквиру обрасца *Submission Letter*. Финансирање, сакупљање података или генерално надгледање истраживачке групе сами по себи не могу оправдати ауторство. Сви други који су допринели изради рада, а који нису аутори рукописа, требало би да буду наведени у Захвалници с описом њиховог рада, наравно, уз писани пристанак.

НАСЛОВНА СТРАНА. На првој страници рукописа треба навести следеће: наслов рада без скраћеница; пуна имена и презимена аутора (без титула) индексирана бројевима; званичан назив установа у којима аутори раде, место и државу (редоследом који одговара индексираним бројевима аутора); на дну странице навести име и презиме, адресу за контакт, број телефона, факса и имејл адресу аутора задуженог за кореспонденцију.

КРАТАК САДРЖАЈ. Уз оригинални рад, саопштење, преглед литературе, приказ болесника, рад из историје медицине, рад за рубрику „Језик медицине“ и рад за праксу, на другој по реду страници документа треба приложити кратак садржај рада обима 100–250 речи. За оригиналне радове кратак садржај треба да има следећу структуру: Увод, Циљ рада, Методе рада, Резултати, Закључак; сваки од наведених сегмената писати као посебан пасус који почиње болдованом речи. Навести најважније резултате (нумеричке вредности) статистичке анализе и ниво значајности. За приказе болесника кратак садржај треба да има следеће делове: Увод, Приказ болесника, Закључак; сегменте такође писати као посебан пасус који почиње болдованом речи. За остале типове радова сажетак нема посебну структуру.

КЉУЧНЕ РЕЧИ. Испод Кратког садржаја навести од три до шест кључних речи или израза. Не треба да се понављају речи из наслова, а кључне речи треба да буду релевантне или описне. У избору кључних речи користити *Medical Subject Headings – MeSH (http://www.nlm.nih.gov/mesh).*

ПРЕВОД НА СРПСКИ ЈЕЗИК. На трећој по реду страници документа приложити наслов рада на српском језику, пуна имена и презимена аутора (без титула) индексирана бројевима, званичан назив установа у којима аутори раде, место и државу. На следећој – четвртој по реду – страници документа приложити кратак садржај (100–250 речи) с кључним речима (3–6), и то за радове у којима је обавезан кратак садржај на енглеском језику. Превод појмова из стране литературе треба

да буде у духу српског језика. Све стране речи или синтагме за које постоји одговарајуће име у нашем језику заменити тим називом.

Уколико је рад у целости на српском језику (нпр. рад из историје медицине, језика медицине и др.), потребно је превести називе прилога (табела, графикона, слика, схема) уколико их има, целокупни текст у њима и легенду на енглески језик.

СТРУКТУРА РАДА. Сви поднаслови се пишу великим масним словима (болд). Оригинални рад обавезно треба да има следеће поднаслове: Увод, Циљ рада, Методе рада, Резултати, Дискусија, Закључак, Литература. Преглед литературе чине: Увод, одговарајући поднаслови, Закључак, Литература. Првоименовани аутор прегледног рада мора да наведе бар пет аутоцитата (референце у којима је био први аутор или коаутор рада) радова публикованих у часописима с рецензијом. Коаутори, уколико их има, морају да наведу бар један аутоцитат радова такође публикованих у часописима с рецензијом. Приказ болесника чине: Увод, Приказ болесника, Дискусија, Литература. Не треба користити имена болесника, иницијале, нити бројеве историја болести, нарочито у илустрацијама. Прикази болесника не смеју имати више од седам аутора.

Прилоге (табеле, графиконе, слике итд.) сместити на крај рукописа, а у самом телу текста назначити место које се односи на дати прилог. Крајња позиција прилога пре штампања рада зависиће од прелома текста.

СКРАЋЕНИЦЕ. Користити само када је неопходно, и то за веома дугачке називе хемијских једињења, односно називе који су као скраћенице већ препознатљиви (стандардне скраћенице, као нпр. ДНК, сида, ХИВ, АТП). За сваку скраћеницу пун термин треба навести при првом навођењу у тексту, сем ако није стандардна јединица мере. Не користити скраћенице у наслову. Избегавати коришћење скраћеница у кратком садржају, али ако су неопходне, сваку скраћеницу поново објаснити при првом навођењу у тексту.

ДЕЦИМАЛНИ БРОЈЕВИ. У тексту рада на енглеском језику, у табелама, на графиконима и другим прилозима децималне бројеве писати са тачком (нпр. 12.5±3.8), а у тексту на српском језику са зарезом (нпр. 12,5±3,8). Кад год је то могуће, број заокружити на једну децималу.

ЈЕДИНИЦЕ МЕРА. Дужину, висину, тежину и запремину изражавати у метричким јединицама (метар – *m*, килограм – *kg*, литар – *l*) или њиховим деловима. Температуру изражавати у степенима Целзијуса (°C), количину супстанце у молима (*mol*), а притисак крви у милиметрима живиног стуба (*mm Hg*). Све резултате хематолошких, клиничких и биохемијских мерења наводити у метричком систему према Међународном систему јединица (*SI*).

ОБИМ РУКОПИСА. Целокупни рукопис рада – који чине насловна страна, кратак садржај, текст рада, списак литературе, сви прилози, односно потписи за њих и легенда (табеле, слике, графикони, схеме, цртежи), насловна страна и сажетак на српском језику – мора износити за оригинални рад, саопштење, рад из историје медицине и преглед литературе до 5.000 речи, а за приказ болесника, рад за праксу, едукативни чланак и рад за рубрику „Језик медицине“ до 3.000 речи; радови за остале рубрике могу имати највише 1.500 речи.

Провера броја речи у документу може се извршити у програму *Word* кроз подмени *Tools–Word Count* или *File–Properties–Statistics*.

ТАБЕЛЕ. Свака табела треба да буде сама по себи лако разумљива. Наслов треба откуцати изнад табеле, а објашњења испод ње. Табеле се означавају арапским бројевима према редоследу навођења у тексту. Табеле цртати искључиво у програму *Word*, кроз мени *Table–Insert–Table*, уз дефинисање тачног броја колона и редова који ће чинити мрежу табеле. Десним кликом на мишу – помоћу опција *Merge Cells* и *Split Cells* – спајати, односно делити ћелије. Куцати фонтом *Times New Roman*, величином слова 12 *pt*, с једноструким проредом и без увлачења текста. Коришћене скраћенице у табели треба објаснити у легенди испод табеле.

Уколико је рукопис на српском језику, приложити називе табела и легенду на оба језика. Такође, у једну табелу, у оквиру исте ћелије, унети и текст на српском и текст на енглеском језику (никако не правити две табеле са два језика!).

СЛИКЕ. Слике се означавају арапским бројевима према редоследу навођења у тексту. Примају се искључиво дигиталне фотографије (црно-беле или у боји) резолуције 300 *dpi* и формата записа *tiff* или *jpg* (мале, мутне и слике лошег квалитета неће се прихватати за штампање!). Уколико аутори не поседују или нису у могућности да доставе дигиталне фотографије, онда оригиналне слике треба скенирати као *Grayscale* (или у боји) у резолуцији 300 *dpi* и снимити их у оригиналној величини.

Уколико је рукопис на српском језику, приложити називе слика и легенду на оба језика.

Слике се у свесци могу штампати у боји, али додатне трошкове штампе сносе аутори.

ГРАФИКОНИ. Графикони треба да буду урађени и достављени у програму *Excel*, да би се виделе пратеће вредности распоређене по ћелијама. Исте графиконе прекопирати и у *Word*-ов документ, где се графикони означавају арапским бројевима према редоследу навођења у тексту. Сви подаци на графикону куцају се у фонту *Times New Roman*. Коришћене скраћенице на графикону треба објаснити у легенди испод графикона.

Уколико је рукопис на српском језику, приложити називе графикона и легенду на оба језика.

СХЕМЕ (ЦРТЕЖИ). Схеме цртати у програму *CorelDraw* или *Adobe Illustrator* (програми за рад са векторима, кривама). Сви подаци на схеми куцају се у фонту *Times New Roman*, величина слова 10 *pt*. Коришћене скраћенице на схеми треба објаснити у легенди испод схеме.

Уколико је рукопис на српском језику, приложити називе схема и легенду на оба језика.

ЗАХВАЛНИЦА. Навести све сараднике који су допринели стварању рада а не испуњавају мерила за ауторство, као што су особе које обезбеђују техничку помоћ, помоћ у писању рада или руководе одељењем које обезбеђује општу подршку. Финансијска и материјална помоћ, у облику спонзорства, стипендија, поклона, опреме, лекова и друго, треба такође да буде наведена.

ЛИТЕРАТУРА. Списак референци је одговорност аутора, а цитирани чланци треба да буду лако приступачни читаоцима часописа. Стога уз сваку референцу обавезно треба навести *DOI* број чланка (јединствену ниску карактера која му је додељена) и *PMID* број уколико је чланак индексиран у бази *PubMed/MEDLINE.*

Референце нумерисати редним арапским бројевима према редоследу навођења у тексту. Број референци не би требало да буде већи од 30, осим у прегледу литературе, у којем је дозвољено да их буде до 50. Број цитираних оригиналних радова мора бити најмање 80% од укупног броја референци, односно број цитираних књига, поглавља у књигама и прегледних чланака мањи од 20%. Уколико се домаће монографске публикације и чланци могу уврстити у референце, аутори су дужни да их цитирају. Већина цитираних научних чланака не треба да буде старија од пет година. Избегавати коришћење апстракта као референце, а апстракте старије од две године не цитирати. Референце чланака који су прихваћени за штампу, али још нису објављени, треба означити са *in press* и приложити доказ о прихватању рада за објављивање.

Референце се цитирају према Ванкуверском стилу (униформисаним захтевима за рукописе који се предају биомедицинским часописима), који је успоставио Међународни комитет уредника медицинских часописа (*http://www.icmje.org*), чији формат користе *U.S. National Library of Medicine* и базе научних публикација. Примере навођења публикација (чланака, књига и других монографија, електронског, необјављеног и другог објављеног материјала) могу се пронаћи на интернет-страници *http://www.nlm.nih.gov/bsd/uniform_requirements.html.* Приликом навођења литературе веома је важно придржавати се поменутог стандарда, јер је то један од најбитнијих фактора за индексирање приликом класификације научних часописа.

ПРОПРАТНО ПИСМО (*SUBMISSION LETTER*). Уз рукопис обавезно приложити образац који су потписали сви аутори, а који садржи: 1) изјаву да рад претходно није публикован и да није истовремено поднет за објављивање у неком другом часопису, 2) изјаву да су рукопис прочитали и одобрили сви аутори који испуњавају мерила ауторства, и 3) контакт податке свих аутора у раду (адресе, имејл адресе, телефоне итд.). Бланко образац треба преузети са интернет-странице часописа (*http://www.srpskiarhiv.rs*).

Такође је потребно доставити копије свих дозвола за: репродуковање претходно објављеног материјала, употребу илустрација и објављивање информација о познатим људима или именовање људи који су допринели изради рада.

ЧЛАНАРИНА И ПРЕТПЛАТА. Да би рад био објављен у часопису *Српски архив за целокупно лекарство*, сви аутори морају бити чланови Српског лекарског друштва (у складу са чланом 6. Статута Друштва), док први аутор мора бити и претплатник на часопис, за годину у којој се рад предаје Уредништву. Установе (правна лица) не могу преко своје претплате да испуне овај услов аутора (физичког лица). Уз рукопис рада треба доставити копије уплатница за чланарину и претплату, као доказ о уплатама. Аутори из иностранства нису дужни да буду чланови Српског лекарског друштва, нити претплатници на часопис за текућу годину. Додатне информације о чланарини и претплати могу се добити на телефоне 011/3245-149 и 011/3346-963, односно имејлом (*srparhiv@bvcom.net*) и на интернет-страници часописа (*http://www.srpskiarhiv.rs*).

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ISSN 0370-8179
ISSN Online 2406-0895

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ISSN 0370-8179
ISSN Online 2406-0895

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61(497.1)861

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