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Ervin G. Erdös [October 16, 1922 (Budapest, Hungary) – November 17, 2019 (Chicago, IL, USA)], a famous American scientist who spent 60 years of research on the discovery, activity and function of several peptidases, enzymes included in the activation and degradation of angiotensins, kinins, oxytocin and substance P. His research has provided a foundation for the development of inhibitors of these enzymes that are widely used today in the treatment of cardiovascular diseases.

In this issue of the Vojnosanitetski pregled, professor Rajko Igić shared with us the memories of Ervin Erdös during his visit to the former Yugoslavia in 1976 (see pp. 762–4).

Ervin G. Erdos [16. oktobar, 1922. (Budimpešta, Mađarska) – 17. novembar, 2019. (Čikago, SAD)], čuveni američki naučnik koji je 60 godina radio na otkrivanju i proučavanju aktivnosti i funkcije nekoliko peptidaza, enzima uključenih u aktivaciju i degradaciju angiotenzina, kinina, oksitocina i supstance P. Njegova istraživanja obezbedila su temelje za razvoj inhibitora tih enzima koji se danas široko koriste u lečenju kardiovaskularnih bolesti.

U ovom broju „Vojnosanitetskog pregleda", profesor Rajko Igić podelio je sa nama uspomene na Ervina Erdosa tokom njegove posete bivšoj Jugoslaviji 1976. godine (vidi str. 762–4).

Lower limb perfusion scintigraphy with 99mTc-MIBI scintigraphy and determination of endothelin in diabetic and nondiabetic patients

Perfuziona scintigrafija donjih ekstremiteta sa 99mTc-MIBI i određivanje endotelina kod bolesnika sa dijabetesom melitusom i kod zdravih ispitanika

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Abstract

Background/Aim. Peripheral artery disease (PAD) is a common macrovascular complication in patients with diabetes mellitus (DM) as a result of impairment of homeostatic mechanisms of the endothelium, thus initiating the process of atherosclerosis. The imbalance between endothelium-derived vasodilators and vasoconstrictors plays an important role in the pathogenesis of diabetic microangiopathy, as well as in other vascular complications in diabetes. Perfusion scintigraphy using technetium-99m-methoxyisobutyl isonitrile (^{99m}Tc-MIBI) can be very useful method for evaluation of the lower limbs muscle perfusion. The aim of this study was: to compare the results of dynamic and static studies of lower limbs tissue muscle perfusion scintigraphy with ^{99m}Tc-MIBI (one-day rest-stress protocol) in patients with and without DM and to determine the perfusion reserve for diagnostic evaluation of PAD in patients with DM type 2, as well as to assess the endothelin-1 (ET-1) levels as a vasoconstrictor agent in patients with and without diabetes. **Methods.** Prospective study was performed in 90 pa-

tients, divided into two groups according to the presence of DM – patients with DM type 2 (DP), 60/90 (67%), and patients without DM (NDP), 30/90 (33%). Lower limbs tissue muscle perfusion scintigraphy was done with ^{99m}Tc-MIBI including two studies ("rest" and "stress"). **Results.** In the DP group significantly lower pick of radioactivity was detected in comparison with the NDP group, in both phases (rest and stress), for both calves. Lower counts from the static phase were registered in the region of both calves. Lower inter-extremity indexes as well as perfusion reserve were found in the DP group. There was a significant difference in concentrations of ET-1 between groups (higher concentrations were found in tzhe DP group). **Conclusion.** This one-day protocol (rest-stress with ^{99m}Tc-MIBI) of perfusion scintigraphy of lower limbs is considered a useful procedure in PAD assessment in patients with DM type 2, especially the asymptomatic form.

Key words:
diabetes mellitus, type 2; periferal arterial disease; perfusion imaging; lower extremity; endothelin-1.

Apstrakt

Uvod/Cilj. Bolest perifernih arterija predstavlja makrovaskularnu komplikaciju dijabetesa melitusa (DM) koja nastaje kao zbog poremećaja homeostatskih mehanizama endotelijuma i kojom započinje proces arterioskleroze. Poremećaj ravnoteže vazodilatatora endotelnog porekla i vazokonstriktora ima veliku ulogu u patogenezi dijabetičke mikroangiopatije, kao i ostalih vaskularnih komplikacija dijabetesa. Cilj rada je bio da se uporede rezultati dinamičkih i statičkih studija perfizione scintigrafije donjih ekstremiteta sa tehnecijum-99ᵐ-metoksiizobutil izonitrilom (99ᵐ-Tc–MIBI) (jednodnevni stres/oporavak test) kod osoba sa DM i kod zdravih ispitanika u cilju određivanja perfuzine rezerve u okviru dijagnostičke evaluacije bolesti perifernih arterija kod

osoba sa DM tip 2, kao i da se odredi nivo endotelina-1 (ET-1) kao vazokonstriktora kod osoba sa i bez DM. **Metode.** Prospektivnom studijom obuhvaćeno je 90 ispitanika podeljenih u dve grupe prema prisustvu (DP)/odsustvu DM tip 2 (NDP). DP grupu sačinjavalo je 60/90 (67%) bolesnika sa DM tip 2, dok je u grupi NDP bilo 30/90 (33%) ispitanika bez DM tip 2. Perfuziona scintigrafija sa ^{99m}Tc-MIBI mišićnog tkiva donjih ekstremiteta sprovedena je u fazi odmora i fazi stresa. **Rezultati.** U DP grupi ustanovljen je značajno niži pik radioaktivnosti u odnosu na NDP grupu ispitanika obostrano i u obe faze sa nižim *inter-extremity* indeksima i sniženom perfuzionom rezervom. Utvrđena je značajna razlika u koncentraciji ET-1 između grupa (veća koncentracija je zabeležena u DP grupi). **Zaključak.** Prikazani jednodnevni protokol perfizione scintigrafije donjih ek-

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stremiteta u fazi odmora i napora je korisna procedura u proceni bolesti perifernih arterija bolesnika sa DM tip 2, naročito u asimptomatskoj formi bolesti.

Ključne reči:
dijabetes melitus, insulin nezavisni; bolest perifernih arterija; perfuziono snimanje; noga; endotelin-1.

Introduction

Peripheral artery disease (PAD) is a common macrovascular complication in patients with diabetes mellitus (DM) as a result of impairment of homeostatic mechanisms of the endothelium, thus initiating the process of atherosclerosis. The normal, healthy endothelium regulates vascular tone and structure and exerts anticoagulant, antiplatelet, and fibrinolytic properties. The maintenance of vascular tone is accomplished by the release of numerous dilator and constrictor substances. A major vasodilative substance released by the endothelium is nitric oxide (NO), originally identified as endothelium-derived relaxing factor (EDRF). The endothelium also produces vasoconstrictor substances, such as endothelin-1 (ET-1) (the most potent endogenous vasoconstrictor identified to date) and angiotensin II. Angiotensin II not only acts as a vasoconstrictor but also as pro-oxidant, and stimulates production of ET-1. ET-1 and angiotensin II promote proliferation of smooth muscle cells and thereby contribute to the formation of atherosclerotic plaque. Activated macrophages and vascular smooth muscle cells, characteristic cellular components of atherosclerotic plaque, produce large amounts of ET-1 [1].

The imbalance between endothelium-derived vasodilators and vasoconstrictors initiates a number of events/processes that promote or exacerbate atherosclerosis. They include increased endothelial permeability, platelet aggregation, leukocyte adhesion, and generation of cytokines. Decreased production or activity of NO, manifested as impaired vasodilation, and increased production of ET, may be one of the earliest signs of atherosclerosis. All these processes play an important role in the pathogenesis of diabetic microangiopathy, as well as in other vascular complications in diabetes [2, 3]. Development of endothelial dysfunction involves several biological mediators including increased expression of ET-1 and altered expression of ET receptors [4]. Increased endothelial ET-1 expression enhances lipid biosynthesis and accelerates the progression of atherosclerosis.

There are a number of diagnostic procedures that, according to the accepted protocols for this vasculopathy, are successively involved in different levels of diagnosis. Despite good anatomic information for the large arteries provided by computed angiography, it is insufficient for the small vessels perfusion [5]. Perfusion scintigraphy using technetium-^{99m}-methoxyisobutyl isonitrile (^{99m}Tc-MIBI) can be very useful for evaluation of the lower limbs muscle perfusion. After intravenous application, ^{99m}Tc-MIBI is rapidly cleared from the circulation and preferentially is accumulated in muscular tissues (including heart) proportionally to regional blood flow [6, 7]. These characteristics of ^{99m}Tc-MIBI make it very suitable for examining regional blood flow, visualization with gamma camera, as well as getting quantitative parameters for regional blood flow changes, including quantitative assessment of tissue perfusion in basal conditions (rest study) and after workload (stress study).

The aim of this work was to compare the results of dynamic and static studies of lower limbs tissue muscle perfusion scintigraphy (TMPS) with ^{99m}Tc-MIBI (one-day rest-stress protocol) in patients with and without DM type 2 and to determine the perfusion reserve for diagnostic evaluation of PAD in patients with DM type 2. Also, the aim was to assess differences of ET-1 levels between two groups of patients (with and without diabetes).

Methods

TMPS was performed through one-day rest-stress protocol with ^{99m}Tc-MIBI. The study was approved by the Ethics Committee and all subjects signed double informed consent form. This was a prospective study performed in 90 patients, divided into two groups according to the presence of DM type 2 – patients with DM (DP) 60/90 (67%), and patients without DM (NDP) 30/90 (33%). In the NDP group, 10 (33.33%) patients had hypertension (HTA), 8 (26.67%) were obese, 7 (23.33%) had hyperlipidemia (HLP) and 6 (20%) were smokers. Analyzing the symptoms, 18 (60%) had calf pain, 11 (36.67%) complained of numbness, and 7 (23.33%) had cold lower extremities. In the DP group 44 (73.33%) had HTA, 26 (43.33%) HLP, 20 (33.33%) were smokers, 50% were obese, 48 (80%) had calf pain, 34 (56.67%) had numbness and 24 (40%) complained of cold legs.

^{99m}Tc-MIBI scintigraphy

Lower limbs TMPS with ^{99m}Tc-MIBI is a non-invasive, functional method that evaluates tissue perfusion in resting condition (rest study) and after workload (stress study), as visually as well as through several quantitative parameters.

Tissue muscle perfusion studies were done with planar technique, with two-headed gama camera (DHV MEDISO Nucline SPIRIT), low energy high resolution collimator (LEHR). Before the initiation of the rest study the patient was positioned in resting mode for 20–30 minutes (separate isolated room was used to avoid external influence and the patients were instructed to remain in a horizontal position during this period of resting mode). The rest study was started with a dynamic phase of tissue-muscle vascularization of both calves after iv. application of 300 MBq of ^{99m}Tc-MIBI, (the rest study time interval was 7 minutes, consisted of 28 frames, with time interval 15s per frame) (Figure 1), followed with a whole body scan (WBS) for tissue perfusion of the whole body in posterior-anterior (PA) position, matrix size $512 \times 1024 \times 16$, speed 15 cm/min.
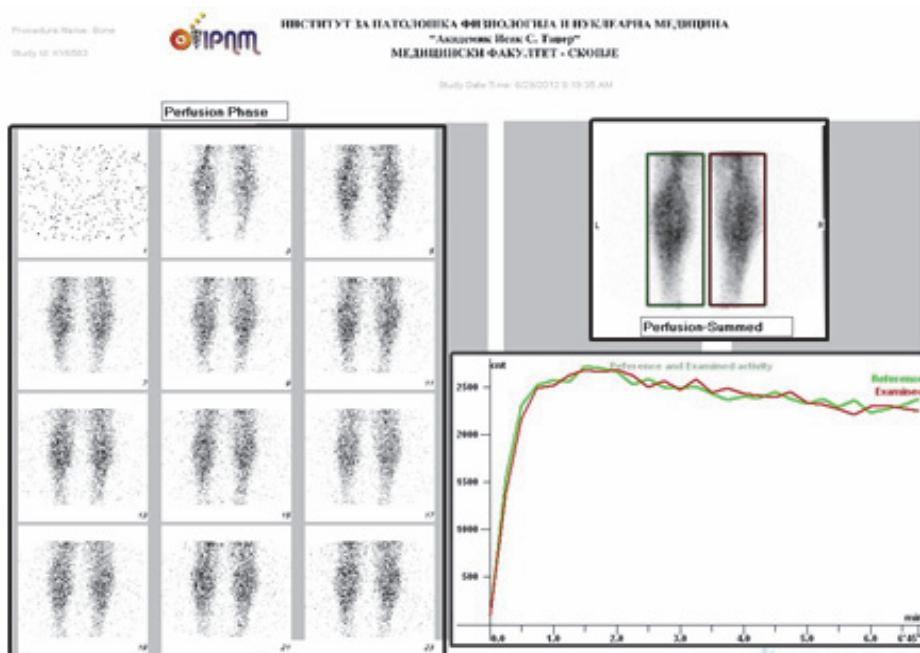
Fig. 1 – Dynamic phase of both calves in the rest study.
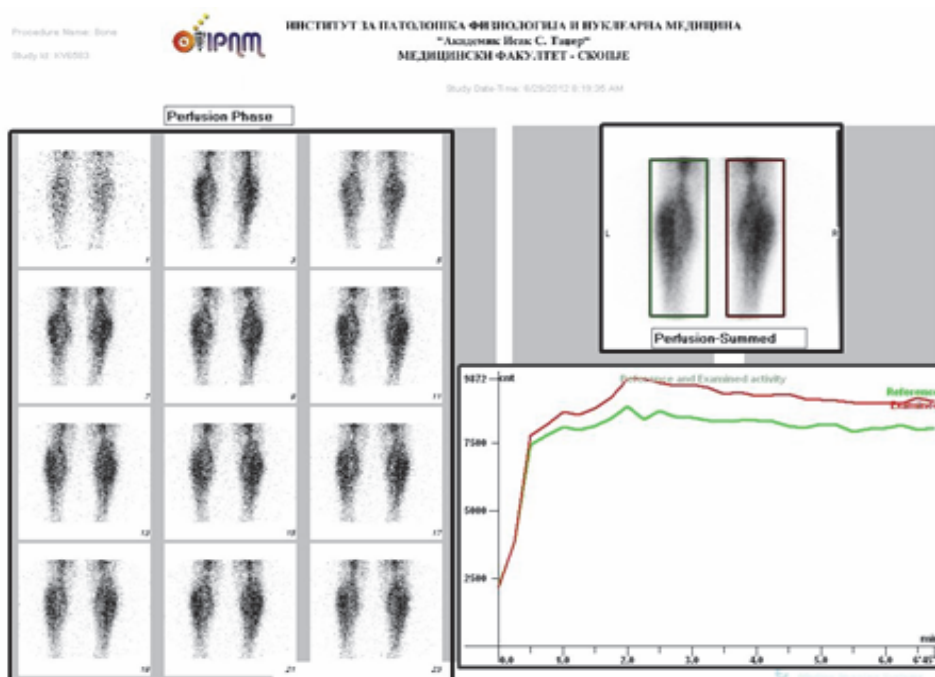
Fig. 2 – Dynamic phase of both calves in the stress study.

The stress study was carried out afterwards and the patient was instructed to perform 30 flexion/extensions of both feet, followed by iv. application of 600 MBq^{99m}Tc-MIBI, when the dynamic phase was started with the same acquisition protocol as in the rest study (Figure 2). After application of the radiopharmaceutical, the patient performed another 30 flexion/extension of the feet. WBS was performed afterwards (with the same aquistion as in the rest study) (Figure 3).

With quantitative analyses of the dynamic phase, radioactive curves were constructed in a time manner (time activity curve – TAC) above the region of interest (Figure 2), positioned above both calves and these parameters were investigated: T maximum (Тмах) – time of maximal uptake of the tracer in each calf and impulses collected in Tmax; radioactivity in 1st minute in calves – (radioactivity above calf in 1st minute) × 100 / maximal radioactivity above calf.

Fig. 3 – Whole body scan in the stress study.

With quantitative analyses of WBS, with registered impulses in the ROI, positioned over calves and the whole body these indices were evaluated: radioactivity in calves – accumulated impulses in both calves in both studies, after drawing symmetrical ROI (Figure 3); intra-extremity index – (for both studies) left calf/left ankle (LC/LA) and right calf/right ankle (RC/RA); index calf/whole body (for both studies) – left calf/whole body and right calf/whole body; perfusion reserve (PR %) for both calves – as a percent of grow of the tissue blood flow in stress study, in comparison with the rest study, was calculated with the formula:

$$PR\ (\%) = \frac{\text{(radioactivity in calf in stress} - \text{radioactivity in calf in rest)}}{\text{radioactivity in calf in rest}} \times 100\%$$

Endothelin-1 measurements

For the determination of ET-1 in our study we used a commercial RIA by the manufacturer Phoenix Pharmaceuticals, Inc. After blood withdrawal, the samples were centrifuged and the serum was stored in a refrigerator at -20°C until analyses performed simultaneously for all samples. ET-1 measurements were taken by a competitive radioimmunoas-

say. The method is based on a competitive reaction of the analyte (ET-1 in the test sample) and the radiolabelled endothelin (^{125}I-endothelin) in the kit, for the limited amount of antibody-specific antibodies in each of the test tubes. According to the competitive conditions, there is an inverse correlation of the bound radioactivity in the formed immune complex and the concentration of the analyte ET-1. The procedure for the determination of ET-1 was carried out in accordance with the conditions and protocol prescribed by manufacturer.

Results

The DP group had significantly lower pick of radioactivity detected in the dynamic phase in comparison with the control group, in both studies (rest and stress) for both calves (Table 1). The number of impulses in the 1st minute for both calves was also significantly lower in the DP group in both studies, as well (Table 2).

Table 1

Number of impulses accumulated at the peak of radioactivity for both calves

Peak of radioactivity	Group	Rest	Stress
		mean ± SD	mean ± SD
Tmax RC	DP	2,158.75 ± 410.6	7,223.62 ± 1,383.4
	NDP	2,427.40 ± 278.8	8,019.47 ± 946.3
p-value		0.0018**	0.0057**
Tmax LC	DP	2,234.75 ± 423.7	7,240.07 ± 1,673.8
	NDP	2,445.43 ± 384.1	7,995.53 ± 1,098.3
p-value		0.024*	0.028*

RC – right calf; LC – left calf; DP – diabetic patients; NDP – nondiabetic patients; SD – standard deviation.
*$p < 0.05$, **$p < 0.01$ (Student's *t*-test for independent samples).

Table 2

Number of counts accumulated in the 1st minute of dynamic phase

T1min	Group	Rest	Stress
		mean ± SD	mean ± SD
RC	DP	1,949.32 ± 404.9	6,752.88 ± 1,248.6
	NDP	2,230.87 ± 284.4	7,671.73 ± 978.1
	p-value	0.001**	0.00068**
LC	DP	2,048.45 ± 435.1	6,924.87 ± 1,314.9
	NDP	2,248.6 ± 442.1	7,646.87 ± 1,080.5
	p-value	0.044*	0.011*

RC – right calf; LC – left calf; DP – diabetic patients; NDP – nondiabetic patients; SD – standard deviation.
*$p < 0.05$, **$p < 0.01$ (Student's *t*-test for independent samples).

The accumulated counts in the region of both calves was insignificantly lower in the DP group compared to the NDP group in the rest study and significantly lower in the stress study ($p = 0.018$). The counts accumulated in the rest study were for LC 16,967.78 ± 3,520.9 in the DP group vs. 17,726.83 ± 3,285.3 in the NDP group, while for RC they were 17,228.07 ± 4,287.5 in diabetic patients vs. 17,772.87 ± 3,242.2 in nondiabetic ones.

Table 3
Intra-extremity index for both calves in both studies (rest and stress)

Variable	Group	Rest		Stress	
		mean ± SD	median	mean ± SD	median
LC/LA	DP	82.17 ± 23.72	74.47	368.16 ± 110.6	356.5
	NDP	82.79 ± 23.31	88.09	389.06 ± 110.1	399.8
	p-value	0.7 (ns)		0.2 (ns)	
RC/RA	DP	84.48 ± 29.09	79.86	368.91 ± 111.9	346.6
	NDP	81.65 ± 19.08	83.23	385.46 ± 104.8	374.6
	p-value	0.86 (ns)		0.43 (ns)	

LC/LA – left calf/left ankle; RC/RA – right calf/right ankle; DP – diabetic patients; NDP – nondiabetic patients.
ns – non-significant (Mann-Whitney test).

In the stress study total counts for the LC these values were 75,546.95 ± 15,864.5 in the DP group, 84,098.9 ± 19,954.7 in the NDP group and for the RC values were 75,059.9 ± 14,851.9 in the DP group and 83,972.8 ± 19,489.8 in the NDP group.

Median for intra-extremity index of the left and right calf was lower in diabetic patients vs. nondiabetic ones, without significance (Table 3). Non-significant differences in indices of calf/whole body were registered in both studies for both calves (Table 4).

Table 4
Index calf/whole body

Variable	Group	Rest	Stress
		mean ± SD	mean ± SD
LC/WB	DM	1.98 ± 0.4	3 ± 0.5
	NDP	1.78 ± 0.3	3.05 ± 0.6
	p-value	0.23 (ns)	0.69 (ns)
RC/WB	DM	2 ± 0.4	2.98 ± 0.5
	NDP	1.8 ± 0.3	3.05 ± 0.6
	p-value	0.24 (ns)	0.55 (ns)

LC/WB – left calf/whole body; RC/WB – right calf/whole body; DP – diabetic patients; NDP – nondiabetic patients; SD – standard deviation.
ns – non-significant (Mann-Whitney test).

Perfusion reserve (PR) of calves (LC, RC) was calculated with the formula: (ROI stress-ROI rest) × 100% / ROI rest. The results showed insignificantly lower PR of LC in diabetic patients compared to nondiabetic ones (40.25 ± 14.7 vs. 44.77 ± 10.3, respectively; $p = 0.32$). Significant difference in PR of RC was registered in diabetic patients in relation to nondibaetic ones (40.02 ± 11.2 vs. 44.53 ± 10.5 in nondiabetic ones, respectively; $p = 0.045$).

There was a significant difference in concentrations of ET-1 between groups (higher concentrations were found in diabetic patients) (Table 5).

Diabetes mellitus is a chronic disease caused by impaired insulin secretion or insulin resistance. Peripheral arterial disease in diabetes is a consequence of an atherogenic process in the lower limb arteries accelerated by multifactorial pathophysiologic mechanisms underlying DM. This process is accompanied also with atherotrombosis in vasculature of other organs including coronary and cerebrovascular system. Having in mind all complications arising from this pathological condition it is of great clinical significance to recognize the early abnormalities in the peripheral circulation. The precise assessment of the prevalence of PAD in diabetic patients is aggravated by the high prevalence of asymptomatic forms, peripheral neuropathy, and the absence/impared function of pain perception, as well as the present limitation of screening methods for its diagnosis. Therefore, in the resolution of asymptomatic and subclinical forms of PAD in these patients, both preventive and diagnostic and curative medical procedures should always be included.

Table 5
Endotelin-1 concentration (pg/mL)

DP	NDP	*p*
mean ± SD	mean ± SD	
105.22 ± 8.8	98.58 ± 8.6	0.042*

DP – diabetic patients; NDP – nondiabetic patient; SD – standard deviation.
*p < 0.05 (Student's *t*-test for independent samples).

Discussion

For this purpose in nuclear medicine ^{99m}Tc-labelled perfusion tracers are used to provide better image quality as well as quantitative processing of the scans. Radiopharmaceutical that was used in our study, ^{99m}Tc-MIBI, is a lipophilic cationic component that injected into animals is distributed into the tissues proportionally to blood flow and is retained in the mitochondria. Given the negative plasma membrane potential and even more negative mitochondrial membrane potential, both potentials contribute to a strong driving force for ^{99m}Tc-MIBI accumulation and sequestration in the mitochondrial matrix. Studies showing that cultured myocardial cells accumulate ^{99m}Tc-MIBI 1,000 times more in mitochondria than in the cytosol, have contributed to its wide application in the field of nuclear cardiology [8]. Biodistribution and kinetics of the ^{99m}Tc-detected components allowed combining myocardial perfusion with perfusion of the lower limbs.

The results from our study clearly pointed to abnormal microvascular perfusion in the affected regions of lower limbs, while the quantification of the tested parameters indicated the extent of perfusion insufficiency. Lower number of

accumulated counts was detected in both calves for both phases in the diabetic patients. In the rest phase of the left calf, total count number was 75,546.95 ± 15,864.5 in the DP group, and 84,098.9 ± 19,954.7 in the NDP group. For the right calf the total count number was 75,059.9 ± 14,851.9, and 83,972.8 ± 19,489.8, respectively for both groups. Still significant decrease of the counts was registered in the stress phase only, due to reactive hyperemia. This is a state when under resting conditions, the limb uses all possible resources for blood supply and self-protection from ischemic consequences, such as collateral circulation and vasodilator response under the action of stimuli that are excreted in response to hypoxia or steel phenomenon. However, under loading conditions it is unable to raise the blood flow to a higher level in order to provide an appropriate metabolic response to the effort.

Perfusion of the lower extremities was also performed in the study of Taillefer [9] in 35 patients using method of post-occlusive reactive hyperemia and resting state. Regions of interest over both thighs and calves were drawn in PA position of imaging, and afterwards inter- and intra-extremity index were calculated. Paradoxically, larger uptake showed muscle blood supply from significantly stenosed blood vessels, which resulted in false positive and false negative results.

In 2001, Cosson et al. [10] investigated by thallium-201 scanning circulation in the muscles of the lower limb in diabetic patients without clinical peripheral vascular disease but with a high cardiovascular risk profile and suggested that scanning of the lower limbs coupled with myocardial scintigraphy is a convenient method of investigating peripheral muscle circulation. They found muscle perfusion defects in 42% of the patients, mainly in the calves.

Significantly lower PR of diabetic patients (without peripheral artery disease) versus the control group (without DM), 70.2 ± 10.7% and 98.6 ± 9.4%, respectively were registered in 2004 by Lin et al. [11]. They used method of 60 plantar and dorsal flexions of the right foot and calculated the perfusion reserve by the formula PR = (ROI right foot – left foot)/ROI (right foot) ×100%.

Lower extremity ischemic disease assessed by thallium-201 was also used by Cizmic et al. [12] in evaluation of diabetic angiopathy. Their results of lower extremities perfusion scintigraphy showed reliable indices of muscle microcirculatory perfusion, with statistically significant correlation between the Doppler hemodynamic indices and thallium-201 perfusion scintigraphy.

Younes et al. [13], in 2017, performed 30–40 dorso-plantar flexions and extensions of the right foot in sitting position and afterwards ROI were drawn over both calves. Using the formula: PR = Stress (right foot) – Rest (left foot)/ROI (left foot) × 100%, significantly lower PR was detected in patients with PAD vs. the control group (28.4 ± 20.3% vs. 65.0 ± 11.4%, respectively; $p < 0.001$).

Perfusion muscle scintigraphy of lower limbs can help in the algorithm for starting using more invasive diagnostic methods such as angiography. In 2007, Soyer and Uslu [14]

published a case of a patient with intermittent claudication in one leg, a preserved circulation evaluated by the Doppler technique, a striking reduction in perfusion in the stress phase recorded with ^{99m}Tc-MIBI muscular scintigraphy and the detection of multiple stenosis with peripheral arterial angiography. Additionally, through the visual analysis of the scans it is possible to locate regions with impaired microvascular circulation, which would contributed to the appropriate therapeutic modalities.

In our case report of diabetic patient in 2016 we performed TMPS and confirmed diabetic angiopathy in both calves, with a borderline value for perfusion reserve of the left calf – 57%, and a lower perfusion reserve of the right calf – 42% (reference values 50–80%) [15].

Tan et al. [16] used two-day protocol of ^{99m}Tc-MIBI TMPS in patients with Behcet disease, using pharmacologic stress plus adding 30 plantar flexions and extensions of the feet. PR was calculated with the formula: PR % = (ROI stress-ROI rest)/ ROI rest) × 100%. They got significantly lower PR in the control group -3.34 ± 8.7%, vs. 8.6 ± 8.5%.

The detection of PR with the method of TMPS was used in patients with rheumatoid arthritis, as a screening tool in the evaluation of the atherosclerotic process by Amin et al. [17] in 2012. Higher PR were noticed in the control group vs. patients with RA (48.3 ± 27.2% vs. 30.7 ± 22.6%, respectively; $p = 0.015$).

The concentrations of ET-1 showed significant higher mean values in the group of diabetic patients vs. the control group, which is consistent with pathogenetic mechanisms of the ET-1 involvement in the onset of microangiopathy. In that context, in several studies it was found that vascular endothelial dysfunction may precede DM type 2, implying that elevated levels of ET-1 can partly be included in development of the metabolic syndrome, mainly through reduction of insulin sensitivity. Considering conducted studies, it was found that ET-1 increases the production of reactive oxygen species (mainly superoxide anions) and thus contributes to the endothelial activation and consecutive endothelial dysfunction in vascular endothelial cells as the main place of ET-1 production. Also, increased circulating levels of ET-1 may promote the initiation and progression of atherosclerosis by inhibiting endogenous NO production in vascular smooth muscle cells (VSMCs), through its inhibitory effect on endothelial nitric oxide synthase (eNOS), and additionally contribute to the development of microcirculatory disorders [18–20].

Conclusion

This one-day protocol (rest-stress with ^{99m}Tc-MIBI) of perfusion scintigraphy of lower limbs is considered a useful procedure in PAD assessment, especially the asymptomatic form, in patients with DM type 2. The investigation of the functional haemodynamic parameters are important for relevant guidance, treatment and risk stratification of these patients with PAD.

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Association of severity of depression, paroxetine use and markers of liver damage with QT interval duration in patients with alcohol dependence

Udruženost težine depresije, upotrebe paroksetina i markera oštećenja jetre sa dužinom QT intervala kod zavisnika od alkohola

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Abstract

Background/Aim. Patients suffered from chronic alcoholic disease very often have depression and cardiomyopathy. Treatment with several antidepressants is associated with prolonged QT interval, ventricular arrhythmias and sudden death. The aim of this study was to investigate the relation between the severity of depression, serum levels of gamma-glutamyl transferase (GGT), as a marker of liver damage, and the possible influence of paroxetine use on duration of QT interval in patient who started treatment of chronic alcoholic dependence. **Methods.** The study included 147 male patients (older than 18 years of age) suffering from alcohol addiction, who were also diagnosed with depressive disorder on the basis of DSM-IV criterion and positive Hamilton Rating Scale for Depression (HRSD) at the beginning of hospitalization. Out of total number of patients, 49 were randomly selected to be treated with antidepressant paroxetine at a dose of 20 mg once daily during 20 days. The global QTc interval was automatically determined. **Results.** By applying the generalised linear model, the statistically significant positive correlation between the length of QTc interval and serum values of GGT, that is, intensity of alcoholism ($p = 0.002$) and values of the HRSD score, that is, intensity of depression ($p = 0.021$) was established in the sample of 147 depressed alcoholic patients before the application of paroxetine. In spite of the vulnerability of patients due to the heart damage and the liver dysfunction arising from alcohol consumption, as well as altered patients' drugs metabolism, no elongation of QTc interval resulting from the application of paroxetine was established. The length of QTc interval 20 days after paroxetine administration was 401.43 ms and before paroxetine administration it was 403.31 ms. The difference in QTc interval length (after and before paroxetine administration) was $\Delta QTc = -1.88$ ms ($p = 0.524$). **Conclusion.** The results indicated that the severity of depression and GGT serum levels positively correlated with the length of QT interval. On the other hand, paroxetine after 20 days of usage did not prolong QT interval.

Key words:
alcoholism; alcohol-induced disorders; depression; comorbidity; long QT syndrome; paroxetine.

Apstrakt

Uvod/Cilj. Oboleli od hronične alkoholne bolesti vrlo često imaju depresiju i kardiomiopatiju. Lečenje sa nekim antidepresivima je povezano s produženim QT intervalom, ventrikularnim aritmijama i iznenadnom smrću. Cilj ove studije bio je da se utvrdi odnos između težine depresije, nivoa gama-glutamil transferaze (GGT) u serumu, kao markera oštećenja jetre, i mogućeg uticaja korišćenja paroksetina na trajanje QT intervala kod bolesnika kod kojih je započeto lečenje hronične alkoholne zavisnosti. **Metode.** U ispitiva-nje je bilo uključeno 147 osoba muškog pola, starijih od 18 godina, zavisnih od alkohola, kod kojih je na početku hospitalizacije na osnovu DSM-IV kriterijuma i pozitivne Hamiltonove skale za procenu depresije (HRSD) dijagnostikovan depresivni poremećaj. Od ukupnog broja ispitanika, njih 49 je metodom slučajnog izbora lečeno antidepresivom paroksetinom u dozi od 20 mg, jedanput dnevno, tokom 20 dana. Globalni QTc interval određivan je automatski. **Rezultati.** U uzorku od 147 depresivnih bolesnika sa alkoholnom zavisnošću, pre ordiniranja bilo kog antidepresivnog leka, primenom generalizovanog linearnog modela utvrđena je stati-

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stički značajna pozitivna korelacija između dužine QTc intervala i serumskih nivoa GGT, tj. intenziteta alkoholizma (p = 0.002), odnosno vrednosti HRSD skora, tj. intenziteta depresije (p = 0.021). I pored vulnerabilnosti bolesnika zbog oštećenja miokarda i poremećaja funkcionisanja jetre izazvanog konzumiranjem alkohola i, posledično, izmenjenog metabolizma lekova, nije utvrđeno produženje QTc intervala usled primene paroksetina. Dvadeset dana posle primene paroksetina dužina QTc interval iznosila je 401.43 ms, a pre njegove primene 403.31 ms. Razlika u dužini QTc intervala (nakon i pre ordiniranja paroksetina) iznosila je ΔQTc = - 1.88 ms (p = 0.524). **Zaključak.** Rezultati pokazuju da težina depresije i nivoi GGT u serumu pozitivno korelišu sa dužinom QT intervala. Sa druge strane, paroksetin nakon 20 dana primene, nije produžio QT interval.

Ključne reči:
alkoholizam; poremećaji izazvani alkoholom;
depresija; komorbiditet; sindrom produženog QT;
paroksetin.

Introduction

Depression and alcoholism are particularly connected. Clinical picture of the comorbidity of depression and alcoholism is manifested by significantly more severe disorder symptoms, longer duration of illness, reduced psychosocial functioning and higher suicidal risk in such patients. Many patients suffering from depression may become alcohol addicts because they try to "cure" bad mood and anxiety by alcohol. On the other hand, many things in the life of an alcohol addict have an effect on the increase of depression and bad mood, which is why a vicious circle from which it is really hard to escape is created. Treatment of patients with dual diagnosis of alcoholism and depression is carried out in several stages. The acute stage is directed at detoxification and stabilisation of depression. The phase of continued treatment is directed at symptoms and change of lifestyle. The maintenance phase is oriented towards the reduction of risk of relapse. Pharmacotherapy is more efficient when combined with counselling and self-help programmes. Antidepressants from the group of selective serotonin reuptake inhibitors (SSRI, e.g. sertraline, fluoxetine, paroxetine, escitalopram, citalopram, and others) and mirtazapine have a positive effect on patients suffering from alcoholism and coexisting depressive disorder as well as comorbid anxiety disorders [1–3].

Epidemiological, clinical and pharmacological research that should help clarify depression and prevent undesired effects of the antidepressant therapy on QT interval have faced problems from the very beginnings, which is of crucial significance for the reduction of serious consequences that these disorders can lead to individuals, their families and community as a whole [4]. Application of an adequate psychopharmacological treatment represents the central part of the therapeutic process of depressed alcoholic persons.

It is described that there is a positive correlation of the dependence of ethanol and paroxetine dosage with the reversible blockage of the voltage-dependent potassium channels of Purkinje cells of the heart which are responsible for the third phase of repolarization of the action potential, thereby causing the prolongation of QT interval [5, 6].

The aim of this study was to examine influences of depression intensity determined by the Hamilton Rating Scale for Depression, serum levels of serum gamma-glutamyltransferase (GGT), as a biomarker of the liver function, and the antidepressant drug paroxetine on the length of QT interval in depressed alcoholic patients.

Methods

This study included 147 male patients, older than 18 years of age, suffering from alcohol addiction and treated at the Department of Addictive Diseases of the Psychiatric Clinic, University Clinical Center of the Republic of Srpska in Banja Luka, Bosnia and Herzegovina, and the Psychiatric Clinic of the University Clinical Center in Novi Sad, Serbia, in whom depressive disorder was diagnosed at the very start of hospitalization, on the basis of the Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criterion [7] and the positive Hamilton Rating Scale for Depression [8]. Out of these 147 patients, 49 (by a method of random selection) were treated by antidepressant paroxetine. Due to necessity of applying an anxiolytic in relieving and preventing symptoms of alcoholic abstinence syndrome in patients, a benzodiazepine anxiolytic bromazepam in a dose of 3 mg (1, 1, 2) was applied during the study. Serum levels of GGT, as an indirect indicator of the intensity of alcoholism and liver cell lesions, as well as electrolyte status (sodium, potassium, calcium and magnesium) and values of creatine kinase myocardial isoenzyme (CK-MB) were determined in these patients at the beginning of the study and on the day 20 upon admission to the treatment. These parameters were determined in the serum by applying Olympus AU680 chemical analyser (Olympus America Inc.; Centerville, Pa., USA).

In order to be included in the study, patients had to satisfy the following criteria: to have a clinically diagnosed alcohol addiction and to satisfy the criteria under DSM-IV for depressive disorder. It was also necessary for them to have normal referential values in electrolyte findings (Na^+, K^+, Ca^{++}, Mg^{++}), not to have heart rhythm disorders or diagnosed heart diseases. The referential values of electrolytes were the working referential values that are used at the University Clinical Center in Banja Luka: Na^+ 130–147 mmol/L; K^+ 3.2–5.2 mmol/L; Ca^{++} 2.2–2.7 mmol/L, and Mg^{++} 0.5–1.1 mmol/L.

Patients who did not satisfy the above stated criteria, namely patients with diagnosed congenital long QT syndrome, Brugada syndrome, acute infective diseases, autoimmune and malignant diseases, as well as patients who took drugs which prolong QT interval, were not included in the study.

The study was approved by the Ethics Committee of the University Clinical Center in Banja Luka, and patients gave their written consent for participating in the study.

The existence of alcohol addiction and depression was assessed on the basis of anamnestically obtained data and clinical observation. DSM-IV criteria were used for the purpose of diagnosing alcoholic addiction and depression [7]. The HRSD [8] was used for quantifying the severity of depression. The version containing 17 items was used. The severity of depression was determined according to the following scoring system: a) 0–7 score is an indicator that depression is not present; b) 8–15 score speaks in favour of existence of minor (slight) depression; c) score ≥ 16 speaks in favour of existence of major (high) depression.

Antidepressant therapy, that is, paroxetine was applied in 49 patients, in a single morning dose of 20 mg, recommended by the drug manufacturer, during 20 days.

Long QT interval represents a marker of the development of ventricular arrhythmia and sudden death. ECG finding, including measurements of the length of QT interval, as well as GGT serum levels and the HRSD score, were made in patients at the beginning of the study (before the application of paroxetine) at 11 a.m., and on the day 20 after the treatment with the drug, also at 11 a.m. The stated time for ECG check-ups was chosen due to circadian changes in the heart electrophysiology [9]. Due to the impact of the sinus rhythm on the length of QT interval and for the adequate comparison among subjects, QT interval was corrected by the value of the heart frequency (the so-called QTc interval) [10]. Because of deferred adaptation of QT interval to values of the heart frequency, ECG measurement was done following the establishment of a stable heart frequency [11]. Measurement was done with patients in the resting (lying) position in the course of 20 seconds.

In our study, global QTc interval (12 leads) [12, 13] was determined by an automatic application of ECG device, type "Schiller Cardiovit AT-1", which uses "SCHILLER ECG Measurement and Interpretation Software for Children and Adult ECGs" (developed by SCHILLER AG, Altgasse 68, CH-6341 Baar, Switzerland, see http://www.schiller.ch). Global QTc interval represents the interval with the earliest QRS onset and the latest T end in any lead. Global QRS complex in our study was shorter than 120 ms, which excludes the impact of extended depolarisation of ventricles on the length of QT interval. The analysis included patients with technically regular ECG findings (without interference, background noise, 'wondering' of isoelectric line). Examination of automatic measurement by the coincidence of heart frequencies in V3 lead using classical method was done. Patients with double and biphasic T waves were not included in the study, while T wave amplitude was greater than 0.2 mV [12].

Measured/empirical data values were statistically processed in SPSS 16.0 programme package for Windows and Excel 2016. The methods of descriptive statistics and methods of statistic testing of hypotheses were used. Parametric methods were used as the first choice, whereas in case of undermining of the assumptions about the normality of distribution and homogeneity of variance, the relevant non-parametric methods were used. Control of variability and confounding factors was done by means of repeated measures test and application of multifactorial regression models

with determination of the degree of collinearity between the examined and set of independent variables.

For the purpose of examining the significance of differences in the length of QTc interval following the application of the antidepressant, paired-samples *t*-test was used. Dependence of the length of QTc interval on the serum levels of GGT and CK-MB, and values of the HRSD score was examined by means of multiple linear regression model, whereas in the case of undermined assumptions of the test, by means of generalised linear model of the subclass *LINEAR* and gamma with log link robust estimator. The effect of empirical values on the slope of the regression line (Cook's distance and leverage values) was also analyzed. Statistically significant conclusions were presented on the basis of 2-tailed *p*-values and the significance level $p < 0.05$.

Results

Descriptive values of examined parameters in depressed patients with alcohol dependence are shown in Table 1. By use of generalised linear mode, a statistically significant positive correlation between the length of QTc interval and the serum levels of GGT (Figure 1), that is, the intensity of alcoholism (regression coefficient B = 0.00007, $p = 0.002$), as well as values of the HRSD score, that is, the intensity of depression (regression coefficient B = 0.001, $p = 0.0021$) (Figure 2) was determined in 147 depressed alcoholic patients before administration of the antidepressant. We noticed statistically significant deviation of the residuals of multiple linear regression model from the normal distribution (Shapiro-Wilk test, $p = 0.041$, *skewness* = 0.261) as well as a mild heteroscedasticity of the residuals; therefore, we used a generalised linear model – subclass gamma with log link robust estimator. No statistically significant collinearity between independent variables (the lowest *eigenvalue* model value 0.080, the highest condition index 5.617) was observed. By removing the value with a high Cook distance and high *leverage* value from the generalised linear model, a statistically significant correlation was confirmed between the length of QTc interval and GGT serum levels (regression coefficient B = 0.00005, $p = 0.0029$) as well as with the HRSD score (regression coefficient B = 0.001, $p = 0.023$).

Table 1
Values of examined parameters in depressed patients with alcohol dependence

Parameter	Before paroxetine usage	After paroxetine usage
	mean ± SD	mean mean ± SD
HRSD score	18.51 ± 7.959	9.98 ± 5.234
GGT (U/L)	126.447 ± 63.1980	90.133 ± 44.1603
CK-MB (ng/L)	19.22 ± 2.816	19.66 ± 3.311

HRSD − Hamilton Rating Scale for Depression;
GGT − gamma-glutamyltransferase;
CK-MB − creatine kinase isoenzyme MB; SD − standard deviation.
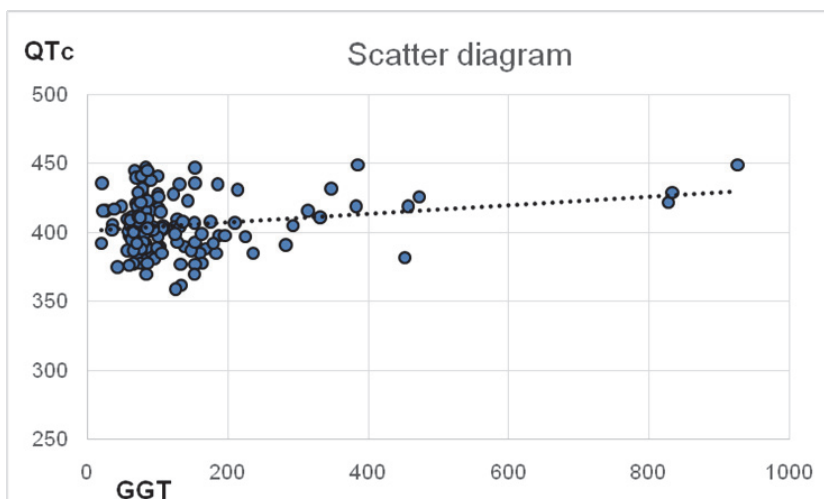
Fig. 1 – Scatter diagram showing correlation between values of gamma-glutamyl transferase serum levels (U/L) and QTc interval (ms).

Fig. 2 – Association between QTc interval and severity of depression.

Despite the assumptions of the multiple linear regression model being undermined, statistically significant differences were determined by applying that model too, and the same was used in order to determine the size of the effect of examined variables (GGT serum levels: partial eta² = 0.040, HRSD score: partial eta² = 0.034). A somewhat greater effect of GGT serum levels on the length of the QTc interval was observed.

Since creatine kinase isoenzyme MB (CK-MB) residuals deviated from the normal distribution and showed a negative asymmetry (*skewness)*, they were transformed by reflection into positive asymmetry (gamma distribution). The reflection was done in the way that empirical values of CK-MB were detracted from the maximal value of CK-MB, increased by a single unit (max CK-MB + 1). By using the generalised linear model, subclass gamma with log link robust estimator, a negative correlation between the serum levels of GGT and reflected values of creatine kinase isoenzyme MB levels (R_CK-MB) before the application of paroxetine (regression coefficient B = -0.0011, $p < 0.001$) was determined. The same correlation was also confirmed after removing values with huge Cook distance and high *leverage* values ($p < 0.001$). Therefore, we could conclude that higher values of

GGT serum levels were associated with higher degree of myocardium damage. No significant correlations between HRSD/R_CK-MB ($p = 0.925$), and HRSD/GGT ($p = 0.383$) were determined.

The length of QTc interval in 49 depressed alcoholic patients before the application of paroxetine was 403.31 ± 19.4 (362–441) ms.

No statistically significant deviation of the residuals of multiple linear regression model of dependence of the QTc interval length before the application of paroxetine from the normal distribution (n = 49, Shapiro-Wilk test, $p = 0.105$) was observed. Collinearity between the examined independent variables (the lowest *eigenvalue* 0.008, the highest condition index 21.113) was observed. Due to the present heteroscedasticity of residuals and low values of dependence of QTc interval on depression intensity ($p = 0.079$), the generalised linear model – subclass LINEAR with robust estimator was used. No statistically significant correlation between the length of QTc interval and serum levels of GGT (as a marker of alcoholism intensity) ($p = 0.983$), serum levels of CK-MB (as a marker of myocardium damage) ($p = 0.388$) was determined, but dependence on the HRSD score (as a marker of depression intensity) ($p = 0.045$) was established. However,

by inserting only one parameter into the stated model no statistically significant correlation between depression intensity and the length of QTc interval ($p = 0.063$) was confirmed, which was explained by the inflation of variance due to the collinearity of independent variables.

Due to the collinearity stated above, correlation between examined independent variables was analyzed in the group of patients who had not been taking paroxetine. Given that the assumptions related to the normality of distribution of the linear regression model residuals (Shapiro-Wilk test, $p = 0.001$, *skewness* = -1.258) were undermined, as well as because of the present heteroscedasticity, the generalised linear model, subclass gamma with log link robust estimator was used. A statistically significant negative correlation between the serum levels of GGT and R_CK-MB in these patients at the beginning of the study was established (regression coefficient B = -0.003, $p = 0.010$), that is, it was established that higher serum levels of GGT were associated (statistically significantly) with higher values of CK-MB. By excluding measured/empirical values with huge Cook distance and high leverage value (patients with GGT levels = 347.0 U/L and CK-MB levels = 19 ng/L), this correlation remained statistically significant and even greater ($p < 0.001$). No correlation between the HRSD score and R_CK-MB levels ($p = 0.097$), as well as the HRSD score and GGT levels ($p = 0.413$) was found.

The length of QTc interval in depressed alcoholic patients on the day 20 after the application of paroxetine was 401.43 ± 20.13 (366–446) ms.

No statistically significant deviation of the residuals of the multiple linear regression model of dependence of QTc interval length after the application of paroxetine from the normal distribution (n = 49, Shapiro-Wilk test, $p = 0.605$) was established. Collinearity between examined independent variables (the lowest *eigenvalue* 0.011, the highest condition index 18.066) was observed. Due to present heteroscedasticity of residuals, the generalised linear model, subclass *LINEAR* with robust estimator was used for the examination of the significance of differences. No statistically significant correlation between the length of QTc interval and serum levels of GGT (alcoholism intensity) ($p = 0.144$), as well as the

HRSD score (depression intensity) ($p = 0.345$) was established, but the correlation between the length of QTc interval and serum levels of CK-MB (myocardium damage) ($p = 0.027$) was found. However, by inserting only one parameter into the stated model, no statistically significant correlation between the myocardium damage and the length of QTc interval ($p = 0.154$) was confirmed, which was explained by the inflation of variance due to the collinearity of independent variables.

Due to stated collinearity, the correlation between examined independent variables was analyzed. A statistically significant correlation between the serum levels of GGT (alcoholism intensity) and the serum levels of R_CK-MB (myocardium damage) in patients suffering from alcohol addiction after the application of paroxetine (regression coefficient B = -0.007, $p < 0.001$) was determined. In other words, it was established, just as in the case when paroxetine had not been applied, that higher serum levels of GGT were statistically significantly associated with higher levels of CK-MB. No undermining of the assumption about the normality of distribution of the linear regression model residuals (Shapiro-Wilk test, $p = 0.130$) was observed, but due to the present heteroscedasticity of residuals ("fan in"), the generalised linear model, subclass *LINEAR* robust estimator was used. After exclusion of the empirical value with huge Cook distance and high leverage value from the model (patients with the serum levels of GGT = 253.2 U/L and CK-MB = 23 ng/L), the correlation remained statistically significant ($p < 0.001$). No statistically significant correlation between the HRSD score and the serum levels of R_CK-MB ($p = 0.501$), as well as the HRD score and the serum levels of GGT ($p = 0.988$) was established.

No statistically significant deviation of differences in the length of QTc interval from the normal distribution, both before and after the application of paroxetine, was present (dif QTc Shapiro-Wilk test: $p = 0.766$), due to which the paired-samples *t*-test was used for the examination of the significance of differences. No statistically significant difference in the length of QTc interval, before and 20 days after the application of paroxetine was established ($p = 0.524$) (Figure 3).
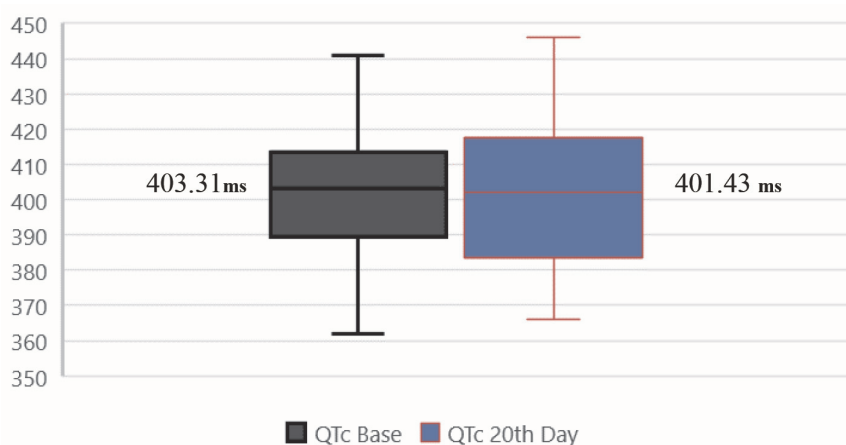
Fig. 3 – Influence of paroxetine administration during 20 days on the lenght of QTc interval in depressed patients with alcohol dependence.

Discussion

In our study, a statistically significant correlation between the HRSD score (depression intensity) and the length of QTc interval was established in 147 patients before the application of paroxetine. Therefore, we can claim that higher values of the HRSD score are statistically significantly associated with a longer QTc interval. In the same regression model, the statistically significant positive correlation between the length of QTc interval and the serum levels of GGT was also determined. These results were also confirmed after the exclusion of values with huge Cook distance and high leverage value from the model. A somewhat greater effect of the serum levels of GGT on the length of Qtc interval in relation to the HRSD score (GGT: partial eta² = 0.040, HRSD score: partial eta² = 0.034) was established.

The association of depression with higher values of QTc interval have also been determined in other studies [14, 15]. Besides higher values of QTc interval in patients with clinical depression, Minoretti et al. [15] point to higher values of QTc interval in healthy persons who are prone to the development of depression – with traits of neuroticism. This association has also been indirectly confirmed by observing higher death rates in depressed patients with acute coronary syndrome [16, 17]. Rainey et al. [14] did not notice longer values of QTc interval in patients suffering from depression and abusing psychoactive substances, whereas Whang et al. [17] established a longer QTc interval in depressed female persons with acute coronary syndrome (unstable angina pectoris and myocardial infarction without the elevation of ST segment), but not in men. Therefore, we would like to point out that our results of positive correlation of the HRSD score and the length of QTc interval refer to the population of patients who consume psychoactive substances (alcohol), as well as that the research pertains to male persons.

Even though the subject of our research is not to determine the frequency of the prolonged QT interval syndrome in persons suffering from alcohol addiction and depression in relation to healthy population, the correlation between the effect of alcohol and the extension of QTc interval has been confirmed by various studies. Thus, for example, Rossinen et al. [18] indicate that there is a direct effect of ethanol infusion on the extension of QTc interval independently from the presence of coronary arterial disease. A similar result is also stated by Gonzalez et al. [19] by presenting a case of QTc prolongation and heart rhythm disorder (*torsade de pointes*) in patients with acute alcoholic intoxication (F10.0). However, in that study, the associated/confounding factor was hypomagnesemia stated by authors. The correlation between alcoholism and extended QTc interval has also been confirmed by other studies [20–23]. In the study of Bär et al. [24], a statistically significant prolongation of QTc interval in male persons with the symptoms of alcoholic abstinence (n = 18) in relation to the "pair matched" control group was established, but not in the case of syndrome of dependence without the abstinence syndrome (n = 15). Authors explain the result in terms of extended repolarisation as a consequence of increased sympathetic tone or low levels of potassium, through

which they point to the goals of adjuvant therapy of the alcoholic abstinence syndrome.The correlation between abstinence and extended QTc interval is also stated by Koide et al. [21]. The frequency of QTc prolongation in persons with chronic alcoholism (n = 90) in their study amounted to 22%. The examination was carried out in the period of abstinence, on average 35 days from the day of quitting alcoholic beverages, while QTc interval was not correlated with values of serum electrolytes (Na⁺, K⁺, Ca⁺⁺, Mg⁺⁺). Main factors associated with extended QTc interval were greater daily consumption of alcohol and a longer period of abstinence. Even though the association of individual factors is not completely clear, authors assume that the damage of myocardium is the cause of extension of QTc interval. Krasemann [25] describes the phenomenon of ventricular tachycardia of a newborn delivered by a mother suffering from alcoholic addiction (on the third day upon birth). After a spontaneous normalisation of rhythm, extended QTc interval of the newborn (480 ms) was determined. Author concludes that the "abstinence syndrome of the newborn" is the cause of QTc interval extension.

Serum concentrations of GGT indirectly reflect the intensity of alcoholism. In our study, dependence of the QTc interval length on alcohol intensity (GGT serum levels) was determined in patients without abstinence syndrome, given that the same was controlled by applying drugs (bromazepam, a benzodiazepine anxiolytic). Dependence of QTc interval elongation on GGT serum levels are also stated by Borini et al. [20]. However, in their patients, disorders of electrolytes (hypokalemia) and hyperglycaemia were established. Authors point out that changes of ECG are a consequence of metabolic changes in persons with alcoholic dependence. We would like to remark that in our study the exclusion factors were disorders of electrolytes (Na⁺, K⁺, Ca⁺⁺, Mg⁺⁺), by which we excluded the possibility of the effect of electrolyte disorders on the extension of QTc interval.

There are also studies that negate the correlation between alcoholism and the extension of QTc interval. Pomini et al. [26] did not notice significant differences in the lenght of QTc interval between persons suffering from chronic alcoholism and persons who did not consume alcohol. Authors indicate that arrhythmogenic effect due to acute alcohol ingestion is not significant, but that further studies are needed. In this way they do not close the problem of researching subclinical alcohol cardiomyopathy. However, limitation of this study is a relatively small sample of the study and the control group: 12 persons with chronic alcoholism and 10 persons who do not consume alcohol.

Various causes are stated as the etiological factor that extends QTc interval in persons suffering from alcoholic addiction. Certain authors (as has been stated above) emphasize the dysfunction of autonomous nervous system of the heart with extended repolarisation as the main factor [22, 24], while others point to damage of myocardium [21]. In our study, in the group of alcohol-addicted patients, initially, a correlation between the serum level of CK-MB, that is, the degree of myocardial demage and the lenght of QTc interval ($p = 0.027$) was established. The same was not confirmed by inserting only one parameter into the stated model ($p = 0.154$). This

finding we explained by the inflation of variance due to present collinearity with other two independent variables (GGT serum levels and HRSD score). We remark that we often used R_CK-MB in the analysis, given that residuals of CK-MB diverged from the normal distribution and showed skewness, which is why the same were transformed by means of reflection into positive asymmetry and gamma distribution. Also, because of that, we used generalised linear model, subclass gamma with log link, in the course of statistical analysis. Reflection was also considered when interpreting coefficients of independent variables that had R_CK-MB as the dependent variable. In regression models with R_CK-MB as the dependent variable, we did not reflect independent variables, and the correlation between independent and dependent variables remained linear. In the course of the said transformation we noticed that a statistically significant intercept was not often established, which meant that the interpretation and comparison of the results were made harder. Occasional instability of the regression model (GGT/R_CK-MB) and not getting statistically significant correlations after the exclusion of values with huge Cook distance and high leverage value, are the consequence of measured high values of GGT serum levels in the examined population of patients. The highest recorded serum value of GGT amounted to 926.0 U/L in this study. However, this finding is not strange, given that in clinical practice we have often come across four-digit values of GGT serum levels. This is why we do not advocate the exclusion of extreme values from the regression model, because those values often draw attention to significant phenomena and correlations. We remark that in our study the results were confirmed after the exclusion of extreme values, and sometimes even the significance was greater (e.g. the correlation between GGT and R_CK-MB serum levels in the group of patients who had not been taking paroxetine.

Data from the literature that pertain to the assessment of the effect of paroxetine on the length of QTc interval are contradictory. Krulewicz et al. [27] point out in a study that included 449 children aged 7–18 years (placebo, n = 207; paroxetine dose of 10–50 mg daily, n = 200; and imipramine, n = 42) that paroxetine did not statistically extend QTcB (Bazett formula) and QTcF (Fridericia formula) in relation to placebo, in contrast to imipramine that prolonged QTcB, both in relation to placebo and paroxetine. Nelson et al. [28] indicate that duloxetine (serotonin-norepinephrine reuptake inhibitor – SNRI) (n = 736) and paroxetine (n = 359) did not significantly influence the QTc interval length in relation to placebo (n = 371). Paroxetine was used in a dose of 20 mg, in the period from 8 to 26 weeks. Yeragani and Rao [29] state that, in contrast to nortriptyline which due to its stronger anticholinergic effect exercises an impact on QTc interval, paroxetine does not show that effect on QTc interval in patients with panic disorder (n = 16).

On the other hand, Lim et al. [30] indicate that paroxetine in combination with flecainide (Ic antiarrhythmic) in persons with CYP2D6*10 gene allele, which determines microsomal cytochrome P450 metabolic enzymes of the liver, significantly extends QTc interval. Their study confirmed genetic

vulnerability of persons to effects of drugs that extend QTc interval. However, due to common administration with antiarrhythmic drugs, an isolated effect of paroxetine is not clear. It is also interesting to mention the study of Martin et al. [31], which undermines the previous result. Authors started with the assumption that paroxetine is a mild cytochrome P_{450} 3A4 (CYP3A4) inhibitor. The study examined combined effect of the drug with terfenadine (H1 antagonist) on the length of QTc interval. Terfenadine was used in a 60 mg dose, twice a day, and paroxetine was given in a 20 mg dose in the course of 15 days after the eighth day. QTcmax slightly changed the value (from 404 ms to 405 ms), and authors concluded that paroxetine did not change pharmacokinetics and cardiovascular effects of terfenadine. The limitation of this study could be a small sample: twelve male persons, and, as we know, terfenadine was discontinued due to extension of QTc interval. Gongadze et al. [6] indicate that due to a greater affinity for proteins of potassium channels coded by hERG gene, paroxetine extends QTc interval, whereas Erfurth et al. [33] show two cases of prolonged QTc interval syndrome and one case of severe bradycardia occurring due to application of paroxetine. There are indications that higher paroxetine doses (e.g. 50 mg) can cause QT elongation [34], but this question must be more explored. In our study, despite the vulnerability of patients due to heart damage and disorder of liver functioning due to alcohol consumption, as well as changed drug metabolism, no extension of QTc interval due to application of paroxetine was established. We found that the length of QTc interval 20 days after paroxetine administration was 401.429 ms and before paroxetine administration 403.307 ms. The average difference: global QTc on the day 20 – global QTc basic, amounted to -1.878 ms (95% confidence interval = -7.755 - 4.000 ms). Statistical probability of 2.5% that the increase in the length of QTc interval is greater than 4.0 ms after the application of paroxetine indicates that it is safe antidepressant in the examined population of patients (depressed alcoholic persons).

Conclusion

A statistically significant correlation of the HRSD score (depression intensity) and the length of QTc interval in patients suffering from alcohol addiction was established (higher values of the HRSD score were statistically significantly associated with longer QTc interval) as well as a statistically significant positive correlation of serum levels of GGT and the length of QTc interval.

Higher serum concentrations of GGT (as a parameter that indirectly reflects alcoholism intensity) were statistically significantly associated with higher serum levels of CK-MB, that is, the degree of myocardium damage.

Statistical probability of 2.5% that the increase in the length of QTc interval is greater than 4.0 ms after the application of paroxetine indicated that it is safe to apply this antidepressant in the examined population of patients (depressed alcoholic patients).

The presented results indicate that associated depression in patients suffering from alcohol addiction by far in-

creases the sensitivity of these patients to cardiotoxic effects of drugs that extend QTc interval. Because of that pharmacotherapy of depression should conduct with special attention in this population of patients. However, in this study paroxetine did not change the length of QTc interval in patients with alcohol dependence and associated depression.

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Investigation of short-term stability of parenteral nutrition nanoemulsions prepared under laboratory conditions

Ispitivanje kratkotrajne stabilnosti nanoemulzija za parenteralnu ishranu izrađenih u laboratorijskim uslovima

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Abstract

Background/Aim. The application of nanoemulsions (NE) in parenteral nutrition represents a very important advancement that marked the medicine and pharmacy of the twentieth century. Over the years, the technology of the production of NE and total parenteral nutrition (TPN) nanoemulsions or admixtures has undergone constant improvement. Representing the continuation of the previous research, this paper deals with nanoemulsions in a concentration of 20% that were prepared under laboratory conditions. The main emphasis was put on the possibility of detecting the potential presence of large droplets or agglomerates of droplets that could cause fatal effects. In addition, the quality assessment of the TPN admixture containing these nanoemulsions was performed. These results were compared with the results obtained from the TPN admixture prepared from the industrial emulsion (Lipofundin MCT/LCT 20%®). **Methods.** During the 30-day period of monitoring nanoemulsion physical-chemical characteristics, the volume diameters that define the width of the lipid droplet size distribution were determined using the laser diffraction method. In addition, TPN physical and chemical characteristics were monitored for 72 hours and included: measurements of the mean droplet diameter, the volume diameter, distribution of the droplet size, ie. polydispersity index (PDI), ζ-potential, and pH values. **Results.** Obtained results were in accordance with the literature data related to the quality of parenteral nanoemulsions (values of volume diameters ranged between 50 and 490 nm). TPN admixtures remained stable during the testing period, even in cases when TPN admixtures containing either a newly formed or an industrial nanoemulsion were tested. **Conclusion.** Characteristics of investigated nanoemulsions do not significantly alter under the ambient temperature storage. If the preparation principles and the component mixing order are followed, TPN admixture possessing satisfactory physical and chemical quality and stability can be obtained.

Key words:
parenteral nutrition, total; nanoparticles; emulsions; quality control.

Apstrakt

Uvod/Cilj. Primena nanoemulzija (NE) za parenteralnu ishranu predstavlja izuzetno značajno dostignuće koje je obeležilo medicinu i farmaciju dvadesetog veka. Tokom godina, tehnologija izrade NE i smeša za totalnu parenteralnu ishranu (TPN) stalno se usavršavala. Ovaj rad predstavlja nastavak prethodnog istraživanja i odnosi se na problematiku nanoemulzija (NE) koncentracije 20%, izrađenih u laboratorijskim uslovima. Osnovni akcenat stavljen je na mogućnost detektovanja eventualnog prisustva većih kapi ili njihovih aglomerata koji bi mogli da izazovu fatalne efekte. Pored toga, izvršena je i procena kvaliteta smeše za TPN sa NE. Rezultati su upoređeni sa rezultatima dobijenim praćenjem smeše za TPN izrađene od fabrički proizvedene emulzije (Lipofundin MCT/LCT 20%®). **Metode.** Primenom metode laserske difrakcije, praćenjem u periodu od 30 dana, dobijeni su rezultati koji se odnose na širinu raspodele veličina kapi NE, izraženu kao volumenski prečnik. Fizičko-hemijske karakteristike smeša za TPN određivane su tokom 72 sata i obuhvatale su: merenje srednjeg prečnika kapi, volumenskog prečnika, distribucije veličina kapi (PDI) i ζ-potencijala, kao i pH-vrednosti. **Rezultati.** Dobijeni rezultati bili su u skladu sa literaturnim podacima o kvalitetu parenteralnih NE (vrednosti volumenskih prečnika kretale su se između 50 i 490 nm). Tokom 72 h praćenja, TPN su ostale stabilne (i smeša za TPN sa NE izrađenom u laboratoriji, kao i TPN sa fabrički izrađenom NE). **Zaključak.** Tokom

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čuvanja u ambijentalnim uslovima, ispitivane karakteristike NE nisu se značajno menjale. Ukoliko se poštuju principi izrade i redosled mešanja komponenti, dobija se smeša za TPN sa zadovoljavajućim fizičko-hemijskim kvalitetom i stabilnosti.

Introduction

The discovery and development of parenteral nanoemulsions represent the milestone and a great achievement in various fields of medicine and pharmacy.

In that sense, one of the most important and most common use of nanoemulsions is for parenteral nutrition of patients with the nonfunctional gastrointestinal tract. Primarily, it has ensured significantly more comfortable and faster way of satisfying patients' requirements for energy, essential fatty acids and fat-soluble vitamins. All of this contributes to the faster recovery and prolongation of life of critically ill patients. For such a purpose, nanoemulsions are used either alone or as a component of the total parenteral nutrition (TPN) admixture.

The spectrum of diseases in which the TPN application is indicated is very wide, so it can be said that they are used in almost all areas of medicine. TPN admixtures known as the "All-in-One", are systems in which all the macronutrients (amino acids, glucose, lipids) and micronutrients (electrolytes, vitamins, oligoelements) are mixed and stored in the ethyl-vinyl-acetate (EVA) bag [1, 2].

From the pharmaceutical aspect, parenteral nutrition nanoemulsions are, by its nature, oil in water emulsions, so called O/W systems. The formation of these colloidal systems is not a spontaneous process, because the additional energy and a surfactant are needed for their production [3].

This research is conducted in line with the general physical and chemical aspects related to nanoemulsions. As far as their stability is concerned, it is widely known that nanoemulsions are thermodynamically unstable and kinetically stable.

In the first rough calculation, the mathematical model that proves the thermodynamic instability of nanoemulsions uses the expression for the change of the modified Gibbs function ("free energy") which is expressed through enthalpy (H), absolute temperature (T), entropy (S) and the work (W) done to increase the surface of oil droplets [4]:

$$\Delta G = \Delta H - T\Delta S + W$$

Analyzing the changes of particular terms in this equation during the nanoemulsion production process, it comes out that ΔG is a positive value. From the physical-chemical aspect, it is known that the system is moving toward the equilibrium position (stability) only in the case when the Gibbs function decreases [5–7]. Thus, the fact that stems from this is that nanoemulsions are unstable in terms of thermodynamics.

The assertion that nanoemulsions are kinetically stable refers to the information on the speed at which the destabilization processes occurs. Changes of critical nanoemulsion parameters take place very slowly, so the system remains in the achieved metastable state for a significantly long period of time.

Not only from the stability aspect, but also from the medical, ie. safety aspect, it is of particular importance to investigate whether the droplet size distribution of nanoemulsions contains droplets that can be fatal for a patient.

Considering capillary dimensions, the presence of particles larger than 5 μm would not be desirable as they can cause fat embolism [8, 9]. In addition, according to the United States Pharmacopeia (USP) Chapter 729 requirements, the mean droplet size of the parenteral nanoemulsion must be < 500 nm [10].

In the literature, the combination of several measurement techniques is recommended for measuring the mean size of lipid droplets (the hydrodynamic droplet diameter) [11, 12].

In order to detect the possible presence of larger droplets or agglomerates of droplets, the aim of this study was to assess, from the droplet size distribution aspect, the quality of produced nanoemulsions. During the experiment preparation stage and the result processing stage, the method of the 2^{4-1} fractional factorial design was used, and the results were compared with the results for droplet sizes of one of industrial nanoemulsions [13].

The next important aim of the research was to evaluate how nanoemulsion prepared under laboratory conditions affects characteristics of the TPN admixture when that nanoemulsion is mixed with above mentioned TPN admixture ingredients. Our further task was also to prepare the TPN admixture that contains an industrial nanoemulsion, and then to compare the obtained results.

Methods

Materials

The oil phase was composed of the following components: soybean oil, Lipoid Purified Soybean Oil 700 (SO) and egg phospholipids with 80% phosphatidylcholine, Lipoid® E80 – EP (both from Lipoid GmbH, Germany), fish oil, oleum jecoris (Ph. Eur. 7.5) – FO, Miglyol 812®, medium-chain triglycerides (MCT), and antioxidant (α-tocopherol), all from Caelo, GmbH, Hilden, Germany, and the second antioxidant, thioglycolic acid (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The water phase was composed of Lipoid Sodium Oleate B (Lipoid GmbH, Germany), Kolliphor® P 188 (Poloxamer 188) – Pl, (BASF, Ludwigshafen, Germany), glycerol (Ph. Eur.) and sodium hydroxide (Ph. Eur.) (Merck, Germany). Water used in the experiment was double distilled and obtained from the Milli Q-water purification system (Millipore, MA).

Selection of ingredients was carried out in accordance with their acceptability for parenteral administration.

In addition, for the production of TPN admixtures, the following solutions were used: the amino acid solution, Aminoven 10%® (Fresenius Kabi, Austria), Glucosi injection 50%® (S.A.L.F., Italy), a fat emulsion – Lipofundin® MCT/LCT 20% (Braun, Germany), electrolytes – Potassium chloride 7.45%® (Braun, Germany), Sodium chloride 10%® (Fresenius Kabi, Deutschland), Calcium-Sandoz 10%® (Novartis, Switzerland), Magnesium chloride injection 200 mg/mL® (Milan, Ireland), Glycophos® (Fresenius Kabi, Austria), as well as water for injection a 500 mL (Braun, Germany).

Methods

The oil and water phases were prepared separately. The oil phase (20% w/w) was heated to the temperature of around 65–70 °C, under mild stirring with a magnetic stir bar (IKA RCT Basic, Germany) at 800 rpm for 15 minutes until the surfactants were completely dissolved. In another plate, water soluble components were measured and heated to the same temperature as the oil phase during permanent stirring. Then, the water phase was added slowly to the oil phase with constant stirring, and that mixture was pre-emulsified by the Ultra-Turax T_{25} high shear mixer (Janke & Kunkel Ika- Labortechnik, Germany) at 13500 rpm for five minutes. In that way, a crude emulsion with the droplet size of around 2 μm was obtained.

In the second production phase (the high-pressure homogenization), the nanoemulsion droplets were obtained by processing a crude emulsion through the high-pressure homogenizer (Gea Niro Panda plus 2000, Italy). During that experimental phase, the pressure was 300 and 700 bars, while the number of homogenization cycles was four and ten. These values varied according to the mentioned 2^{4-1} fractional factorial design (Table 1).

The temperature of the entire homogenization process was maintained at 40 °C. The procedure was repeated as many times as defined by the experimental design (Table 1). Independent parameters included the type of oil phase (*X1*), the surfactant (egg phospholipids) with or without the second surfactant – Poloxamer 188 (*X2*), the number of homogenization cycles (*X3*) and the process pressure (*X4*), while the dependent parameter was the volume droplet size (*y*). In accordance with this type of design, it was possible to calculate only the selected interaction terms.

Table 1
Experimental matrix according to the 2^{4-1} fractional factorial design

Formulation	X_1	X_2	X_3	X_4
1	A1	1	4	300
2	A2	1	4	700
3	A1	2	4	700
4	A2	2	4	300
5	A1	1	10	700
6	A2	1	10	300
7	A1	2	10	300
8	A2	2	10	700

X_1 – oil phase (A1 – mixture of FO and SO, A2 – mixture of FO and MCT);
X_2 – surfactant (1 – EP, 2 – mixture of EP and Pl);
X_3 – number of cycles; *X_4* – pressure (bar).
For abbreviations see under Table 2.

Obtained values were fitted into the following model:

$$y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4$$

The signs b_0 to b_4 represent regression coefficients that demonstrate the influence of independent variables on the dependent variable; thereby b_0 is the intercept and b_1, b_2, b_3 and b_4 are the linear coefficients of the respective independent variables. On the other hand, b_{12}, b_{13} and b_{14} are regression coefficients that demonstrate the interaction between corresponding variables associated with respective model factor interactions (X_1X_2, X_1X_3, X_1X_4).

According to the design used in this study, the composition of prepared nanoemulsions is shown in Table 2.

The prepared nanoemulsions were subjected to the following tests: visual examination, centrifugation, then the repeated visual examination following the centrifugation, and finally the volume droplet size measurement by laser diffraction method.

In the course of testing, the samples were stored at the room temperature (about 25 °C). The dynamics of the nanoemulsion quality monitoring was the following: the first measurement was performed immediately after their preparation (0h), the next measurements were done after 10 and 30 days.

Table 2

Composition of nanoemulsion formulations

Formulation	Composition of nanoemulsion formulations (%, w/w)									
	SO	FO	MCT	EP	Pl	SOl	G	Toc	TA	Water to [g]
1	10	10	—	1.20	—	0.03	2.50	0.01	0.01	100
2	—	10	10	1.20	—	0.03	2.50	0.01	0.01	100
3	10	10	—	1.20	0.60	0.03	2.50	0.01	0.01	100
4	—	10	10	1.20	0.60	0.03	2.50	0.01	0.01	100
5	10	10	—	1.20	—	0.03	2.50	0.01	0.01	100
6	—	10	10	1.20	—	0.03	2.50	0.01	0.01	100
7	10	10	—	1.20	0.60	0.03	2.50	0.01	0.01	100
8	—	10	10	1.20	0.60	0.03	2.50	0.01	0.01	100

**SO – soybean oil; FO – fish oil; MCT – medium-chain triglycerides; EP –egg yolk phospholipids; Pl – Poloxamer 188;
SOl – sodium oleate; G – glycerol; Toc – α-tocopherol; TA – thioglycolic acid.**

Centrifugation of prepared nanoemulsions

The reason for centrifugation comes from the fact that the phase separation process can best be determined by exposing nanoemulsions to the high speed centrifugation, so all of the prepared nanoemulsions were subjected to the centrifugation at 3750 rpm/min for five hours, with the centrifuge radius of 10 cm ("Heraeus Megafuge 16 Centrifuge" – Thermo Fisher, Germany). The fact that this manner of centrifugation corresponds to the effect of one-year gravity refers to classical emulsions [14], but there are not any data on its possible application on the nanoemulsions.

Volume weighted diameters

The laser diffraction (LD) method was applied in this study for the detection of possible droplet agglomerates. For that purpose, the Cilas Granulometer device (1090 LD, France) was used with the measurement range of 20 nm–500 μm. In order to avoid the effects of multiple light scattering, each sample of prepared nanoemulsions was diluted with highly purified water to an appropriate concentration that the aparatus detected as optimal for the measurement. The values thus obtained refer to the volume distribution of particles and represent mean values of three repeated measurements. Using this method, the volume diameters were obtained by measuring the angle of light scattering based on the Mie theory. It should be mentioned here that the volume diameter is defined as the percentage of the presence of particle for a given volume that has a smaller size than a given value. The measurement of the industrial nanoemulsion droplet size (Lipofundin® MCT/LCT 20%) was performed in the same way.

The concept of the *Span* is introduced as an additional parameter for the width of the size distribution. The *Span* of the volume-based size distribution is defined as:

$$Span = [d(0.9) - d(0.1)] / d(0.5)$$

and gives an indication of how far the 10 percent *d(0.1)* and 90 percent *d(0.9)* points are apart, normalized with the midpoint *d(0.5)*[15]. The small *Span* value indicates the narrow size distribution.

Preparation and characterization of TPN admixtures

The preparation of TPN admixture from produced nanoemulsion was carried out as follows: after 30 days in storage, one nanoemulsion (NE I) selected by a random sampling was incorporated into the composition of the TPN admixture together with other ingredients. This nanoemulsion had the following characteristics: the mean droplet diameter 183.1 ± 1.6, the polydispersity index (PDI) 0.067 ± 0.006 and ζ-potential − 33.2 ± 1.4. The same procedure was followed for the industrial nanoemulsion (NE II – Lipofundin® MCT/LCT 20%). Its characteristics were: the mean droplet diameter 268.8 ± 4.5, PDI 0.073 ± 0.036 and ζ-potential − 29.7 ± 0.7. The composition of the both admixtures (the amount of macroingradients – amino acids, glucose, and fat,

as well as the amount of electrolytes – Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, PO_4^{3-}) is shown in Table 3. It was formulated on the basis of daily requirements of patients requiring intravenous nutrition[16].

Table 3
Composition of total parenteral nutrition (TPN) admixtures

Ingredients	Units	Volume
Aminoven 10%®	mL	25
Glucosi injectio 50%	mL	15
Lipid nanoemulsion (NE I or NE II)	mL	12.5
Water for injections to	mL	100
Potassium	mmol	3
Sodium	mmol	3
Magnesium	mmol	0.2
Calcium	mmol	0.2
Chlorides	mmol	5.9
Phosphates	mmol	0.25
Total volume of the mixture	mL	100
Energy value of the mixture	kJ	273
Nitrogen	g	0.4
Amino Acids	g	2.5
Glucose	g	7.5
Lipids	g	2.5

NE I – laboratory nanoemulsion; NE II – industrial nanoemulsion.

The admixtures were disposed into the sterile EVA infusion bag according to standard operating procedures [17, 18]. Samples of the prepared admixtures were taken from each bag at the defined time intervals (0h, and then after 24 and 72 hours storage at the temperature of 2–8 °C), the dynamics of which was determined based on the essential health needs of hospitalized patients. In practice, TPN admixtures are prepared on a daily basis except for weekends and holidays, when there is a need to use them 72 hours after the preparation (during that time the admixtures are kept at 2–8 °C). The monitoring of the TPN admixture quality included the visual examination, the measurement of the mean droplet diameter, PDI, the ζ-potential, the volume diameter of particle size and pH value.

In this case, the measurement of the mean droplet diameter, the PDI index and the ζ-potential was carried out using light scattering method. Furthermore, the diameter of particle size was additionally measured by the LD method. The determination of pH-value for admixtures was carried out by the potentiometric method using the calibrated pH-meter (Mettler Toledo, Seven Go, Swiss).

Results

Characterization of nanoemulsions

From the visual criteria view point, during and at the end of testing, all prepared nanoemulsions as well as TPN admixtures (which are also considered as nanoemulsions

from the pharmaceutical aspect), retained a milky-white appearance with a bluish shade, what is typical for these systems. In addition, neither the phase separation nor the formation of cream, coalescence, or the phase inversion was observed in the samples tested after centrifugation. The thin layer of oil was separated only in nanoemulsions numbered 1 and 3 (Table 2), but it was redispersed by stirring.

Volume weighted diameters

When the LD technique was used for particle sizing, d(0.1) diameter was found to be about 50 nm for all investigated samples (Table 4). Moreover, d(0.5) the range of values was from 130 to 290 nm, and finaly d(0.9) ranged from 220 to 490 nm. Particles above 500 nm were not observed what signified that obtained nanoemulsions were suitable for parenteral nutrition [10]. The Table 4 shows the values of the droplet volume diameter and Span of the prepared nanoemulsions. For example, if d(0.1) as in this table is 50 nm, it means that, in a given volume of the sample, 10% of the particles have a diameter of less than 50 nm, the d(0.5) of 160 nm indicates that 50% of the particles have a diameter of less than 160 nm, while d(0.9) of 400 nm indicates that 90% of particles has the diameter of less than 400 nm, etc. [15].

Table 4

Volume diameters (d) in nm and Span values of laboratory made nanoemulsions

Formulation*	Immediately after preparation				after 10 days				after 30 days			
	d(0.1)	d(0.5)	d(0.9)	Span	d(0.1)	d(0.5)	d(0.9)	Span	d(0.1)	d(0.5)	d(0.9)	Span
1	50	160	400	2.19	60	170	420	2.12	60	220	490	1.95
2	60	150	320	1.73	50	150	380	2.20	60	210	450	1.86
3	60	130	250	1.46	60	130	220	1.23	80	200	380	1.50
4	60	130	230	1.31	60	130	230	1.31	50	150	380	2.20
5	60	150	320	1.73	50	160	400	2.19	60	170	420	2.12
6	60	140	290	1.64	60	160	410	2.19	60	210	450	1.86
7	60	130	230	1.31	60	130	230	1.31	60	140	290	1.64
8	50	130	220	1.31	60	130	230	1.31	60	130	230	1.31

*see Table 2.

In Tables 5, 6 and 7, this issue was clarified by presenting the effect of independent variables and, especially their interactions on dependent variables, namely d(0.1), d(0.5), and d(0.9).

Table 5 clearly shows that none of the independent variables had a significant effect on the dependent variable, which, in this case, represented the droplet size distribution expressed in the form of d(0.1).

Based on the results presented in Table 6, it is evident that the surfactant type and amount had the greatest effect on d(0.5). Namely, when both surfactants are used, the value of d(0.5) was reduced. This effect was more pronounced after storage (after 30 days, it was –23.75). Other factors, as well as the interaction among the factors, did not significantly affect this level of droplet size distribution.

Table 5

Regression coefficients demonstrating the influence of independent variables and the interaction between corresponding variables on a volume diameter – d(0.1)

Variable	Immediately after preparation	10 days after preparation	30 days after preparation
X_1	+1.25	0	-3.75
X_2	+1.25	+2.5	+1.25
X_3	+1.25	0	-1.25
X_4	+1.25	-2.5	+3.75
X_1X_2	-1.25	0	-3.75
X_1X_3	-1.25	+2.5	+3.75
X_1X_4	-1.25	0	-1.25

X_1 – oil phase; X_2 – surfactant; X_3 – number of cycles; X_4 – pressure.

Table 6

Regression coefficients demonstrating the influence of independent variables and the interaction between corresponding variables on a volume diameter – d(0.5)

Variable	Immediately after preparation	10 days after preparation	30 days after preparation
X_1	-2.5	-2.5	3.75
X_2	-10	-15.0	-23.75
X_3	-2.5	0	-16.25
X_4	0	-2.5	-1.25
X_1X_2	+2.5	+2.5	-11.25
X_1X_3	0	+2.5	+11.25
X_1X_4	+2.5	0	-3.75

X_1 – oil phase; X_2 – surfactant; X_3 – number of cycles; X_4 – pressure.

Table 7

Regression coefficients demonstrating the influence of independent variables and the interaction between corresponding variables on a volume diameter – d(0.9)

Variable	Immediately after preparation	10 days after preparation	30 days after preparation
X_1	-17.5	-2.5	-8.75
X_2	-50	-87.5	-66.25
X_3	-17.5	2.5	-38.75
X_4	-5	-7.5	-16.25
X_1X_2	10	5	-6.25
X_1X_3	7.5	5	1.25
X_1X_4	10	0	-21.25

X_1 – oil phase; X_2 – surfactant; X_3 – number of cycles; X_4 – pressure.

According to obtained results, the surfactant type and amount had the greatest effect on d(0.9) as it was shown that with the increase of the value of that independent variable, the value of d(0.9) decreased. This effect was particularly pronounced 10 days after the preparation (the regression coefficient amounted to –87.5). The number of homogenization cycles significantly affected d(0.9), too. The negative sign in front of the regression coefficient indicates the antagonistic effect of this factor, ie. the fact that the increase in the number of homogenization cycles results in the decrease in the d(0.9) value.

The frequency distribution of nanoemulsion droplet sizes is presented by a cumulative frequency curve (Figure 1).

The abscissa shows a specific droplet size interval (in microns, μm), while the ordinate shows the percentage of certain fractions, that is, the percentage of droplets smaller than the monitored ones. The cumulative curve represents the determined droplet size as well as all the droplets smaller than the determined ones. In addition, the logarithmic scale is used on the abscissa to clearly present a wide range of results, while the linear scale is used on the ordinate.

Characterization of the total parenteral nutrition admixtures

The measurement results of the mean droplet diameter, the PDI, the ζ-potential and pH value of the TPN admixture containing a nanoemulsion prepared under laboratory conditions and the admixture containing an industrial nanoemulsion are given in Table 8, while the results of the droplet volume diameter measurement are shown in Table 9.
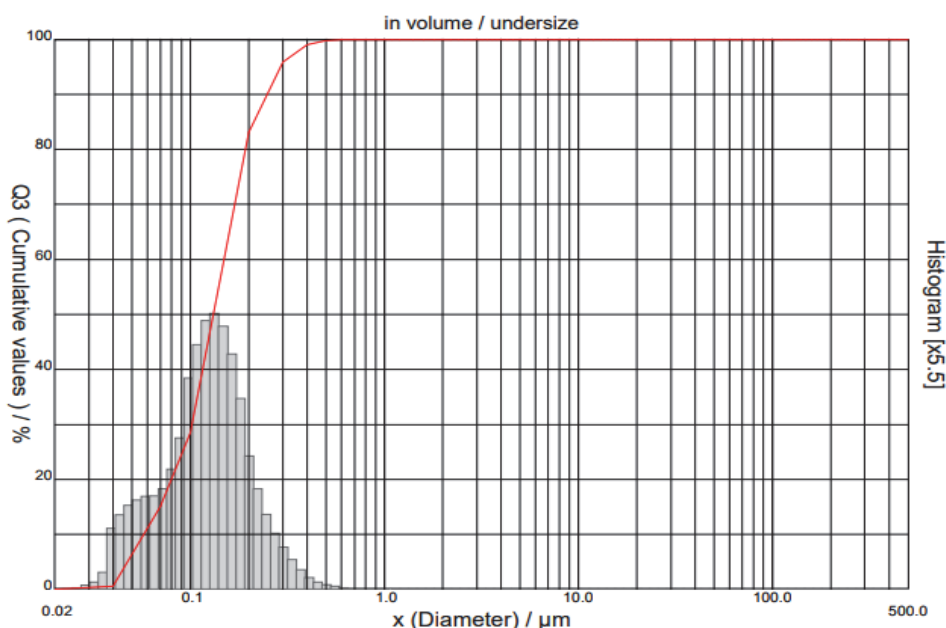
Fig. 1 – Cumulative frequency curve.

Table 8

Characteristics of the total parenteral nutrition (TPN) admixtures

TPN admixture	Mean droplet diameter (nm)			PDI			ζ-potential			pH-value		
	0h	24h	72h	0h	24h	72h	0h	24h	72h	0h	24h	72h
PN $_I$	185.67	214.51	271.03	0.111	0.099	0.202	-34.7	-43.2	-43.5	6.05	5.90	5.85
TPN $_{II}$	208.47	272.70	291.77	0.120	0.144	0.226	-47.3	-44.5	-38.5	5.60	5.45	5.60

TPN $_I$ – admixture that contains a laboratory-made nanoemulsion (NE I); TPN $_{II}$ – admixture containing an industrial nanoemulsion (NE II); PDI – polydispersity index.

Table 9

Volume diameters (d) in nm and Span values of the total parenteral nutrition (TPN) admixtures

TPN admixture	Immediately after preparation				After 24h				After 72h			
	d(0.1)	d(0.5)	d(0.9)	Span	d(0.1)	d(0.5)	d(0.9)	Span	d(0.1)	d(0.5)	d(0.9)	Span
TPN $_I$	60	160	320	1.62	60	210	380	1.52	60	210	410	1.67
TPN $_{II}$	50	150	380	2.20	60	280	410	1.25	60	240	480	1.75

TPN $_I$ – admixture that contains a laboratory-made nanoemulsion (NE I); TPN $_{II}$ – admixture containing an industrial nanoemulsion (NE II).

Discussion

Characterization of nanoemulsions for parenteral nutrition

Droplet size and droplet size distribution are the most important parameters that characterize the quality of nanoemulsions for parenteral use. With the LD method applied in this study, it is possible to see that the creation of agglomerate drops does not take place, indicating, thus, that there is no occurrence of neither coalescence nor Ostwald ripening, which represent the most common forms of nanoemulsion destabilization [19, 20]. Of particular importance here is to identify the presence of droplets larger than defined by the USP. The obtained results are in accordance with the USP 729 requirements (the mean droplet size less than 500 nm) [10, 21].

The distribution of droplet sizes represented the droplet volume diameter in all of the tested samples was relatively uniform, and the measured values were higher compared to the values obtained by the light scattering method [13]. The reason for that is that LD method gives the volume-based droplet size distribution, while the light scattering measurement is based on the intensity of light.

Analyzing the data given in the Table 4, it can clearly be seen that the values for droplet volume diameters measured in the nanoemulsion samples number 1, 2, 5 and 6 were the highest, particularly in cases d(0.9). So, their values immediately after the preparation were 400, 320, 320 and 290 nm, respectively, while at the end of the testing period they were 490, 450, 420 and 450 nm, respectively. Since these nanoemulsions contained a lower concentration of surfactants ie., that the only egg phospholipids were used for their production, it can be concluded that these facors had the greatest effect on the droplet volume diameters. Data presented in Table 4 also shows that the increase in the surfactant concentration significantly reduced the droplet size, what is in accordance with theoretical settings [22–24], as well as with the results obtained in our previous research [13]. According to the named after Derjagmin, Landau, Verwey, Overbeek (DLVO) theory, a surfactant forms a film around the emulsion droplet, and, with increasing the potential energy for repulsing droplets, provides its protection. On the contrary, the absence of a surfactant causes strengthening of the van der Waals attraction forces due to the increase in the potential energy associated with the gravitational potential energy. However, the increased values of volume diameters given in Table 4 were within stated limits required by the literature [10, 21].

The values obtained from *Span* measurement show that, during the testing, nanoemulsions numbered 7 and 8 had the narrowest width of the volume diameter distribution.

When analyzing the regression coefficients, it can be seen that the type and concentration of surfactant, ie. the factor X_2, had relatively the greatest impact on the volume diameter of nanoemulsion droplets. That is especially evident in the case of $d(0.9)$ analysis (Table 7). The X_2 value is negative, what means that droplets were smaller in size when the absolute value of the regression coefficient was higher, what was especially observed after monitoring nanoemulsions for 10 and 30 days.

In the case of d(0.1) analysis, Table 5 shows that none of the factors had a significant impact, while the analysis of d(0.5) given in Table 6 shows that the factor X_2 had the greatest impact. In addition, to make changes related to the volume diameter more obvious, the graphical presentation of one of the representative results is given for illustration purposes.

It is a common practice to use egg phospholipids as an emulsifier for the industrial parenteral nutrition nanoemulsions. However, there are no reports in the literature on the use of the combination of egg phospholipids and Poloxamer 188. It was interesting to examine what impact the combination of electrostatic emulsifier (egg phospholipids) and the stern emulsifier (Poloxamer 188) has on the emulsification and characterization of prepared nanoemulsions, what was actually done in this research.

It was shown that when those two emulsifiers were combined (samples no. 3, 4, 7 and 8), the volume diameters, and therefore, the Span values were lower (Table 4). According to the authors' knowledge, there are no data in the available literature on the production and testing of short-term stability of nanoemulsions for parenteral nutrition, which would be prepared under laboratory conditions.

Characterization of the total parenteral nutrition admixtures

Problems associated with the stability of TPN admixtures are complex and occur due to their complex composition. In addition, it is known that electrolytes, particularly polyvalent cations, can cause the occurrence of various forms of physical and chemical instability. The fact that these complex systems may also contain more than 50 components indicates a greater possibility of the occurrence of a number of instabilities and different unwanted incompatibilities.

Determination of conditions under which the stability of the TPN admixture is maintained is considered a serious problem because seemingly unnoticeable changes can take place in them slowly and over a long period of time. In addition, if a phase separation occurs, the problem that arises is the formation of seemingly homogeneous emulsion by redispersion of separated droplets of the size that may be fatal for a patient.

The results obtained by measuring the basic physical values that are characteristic for the TPN admixtures will be further discussed. Measurement results in our study confirm the stability of prepared admixtures for the total parenteral nutrition (TPN$_I$ and TPN $_{II}$). As indicated, the mean droplet diameter, the polydispersity index, the ζ-potential, the volume diameter of droplets, and pH value were measured.

As for the mean droplet size, the results show that there are no significant differences between values obtained by measurements immediately after the TPN admixture preparation and those obtained after 24 and 72 hours. C omparison of the mean droplet sizes of the TPN admixture produced with the nanoemulsion prepared under laboratory conditions (TPN$_I$), and the TPN admixture containing the industrial nanoemulsion (TPN$_{II}$) shows that the values of mean droplet diameters of the admixture containing the industrial nanoemulsion (NE II) were higher.This can be explained by the

fact that the industrial nanoemulsion NE II (Lipofundin® MCT/LCT 20%) was produced much earlier than the one produced under the laboratory conditions. (NE I). This shows the effect of so-called „natural aging" on the quality of nanoemulsion, and, by that, on the quality of the TPN admixture. Generally speaking, when the "natural aging" of nanoemulsions is concerned, their stability is explained by the ability of very small droplets to reduce the effect of the gravitational force through the Brownian motion. However, the nanoemulsion stability can be compromised in another way, that is, by the influence of some external factors (so-called induced aging). The most common factors are: the type and concentration of electrolytes that are added to the admixture, the admixture pH value, the temperature and other.

On the day when the TPN admixture formulations (TPN$_I$ and TPN$_{II}$) were prepared, they contained droplets with the average size of 185.67 nm and 208.47 nm, and with a very narrow PDI amounting up to 0.111 and 0.120. The mean droplet diameters after 24 hours of the admixture storage at the room temperature (214.51 and 272.70 nm) were slightly different from those measured at the time zero. Furthermore, the admixtures were stored in the refrigerator, and after 72 hours, the mean diameter of the lipid droplets was measured again (271.03 and 291.77 nm, respectively). It is observed that even these values did not exceed the established USP limits, ie. they all were less than 500 nm [10, 21].

As it is known from the literature, the values obtained by measuring the PDI provide information of the deviation from the average droplet size. In all the measurement intervals, these values were in accordance with theoretical settings which, from the perspective of the droplet size distribution, define the quality of parenteral nanoemulsions. Namely, it is considered that if the PDI was less than 0.25, such a preparation is suitable for the parenteral administration [25]. This indicates that all the admixtures were homogeneous.

Apart from the fact that the ζ-potential should not always be a key indicator of the colloidal system stability [26], it is known that if these values are higher than 30 mV, such a preparation can be used for the parenteral nutrition. In general, the values of ζ-potential are conditioned by the amount of added electrolytes (especially cations), and pH values. The reduction of pH value points to the release of free fatty acids what leads to the increased ζ-potential negative values (it is considered a favorable factor that affect the increase in stability) [27, 28]. However, in this case, it cannot be taken into account.

There are no large differences between pH values of prepared TPN admixtures. During the testing period, the values obtained by measuring pH value of the TPN$_I$ admixture ranged from 6.05 to 5.85, ie. pH value was found to be slightly acidic after 72 hours. As for the TPN$_{II}$ admixture, the values after the admixture preparation and after 72 hours were 5.6. This decrease in pH value was insignificant, and triglycerides did not decompose into free fatty acids.

It is well known that at low pH values (about 2.5), the phase separation of the admixture takes place. Glucose solutions have acidic pH (3.5 to 6.5), and, therefore, should not be directly mixed with the fat emulsion because low pH values cause the reduction of the fat drop surface potential, what would further lead to the emulsion phase separation. Because of that, glucose solutions are, firstly, mixed with amino acid solutions, which by its buffering capacity resist changes in pH values, and then a nanoemulsion is added to such a mixture. Here, it should be said that amino acids exert their protective influence on the stability by mechanical impact, ie. through insertion into the intermediate layer between the oil and water phase of the emulsion, and, thus, prevents the integration of drops [12, 16]. By slow homogenization of the prepared admixture, an equilibrium dispersion state is created (homogeneous admixture).

Values obtained by measuring the volume diameter and *Span* of the tested TPN admixtures also confirmed their stability during 72 hours. During that period, neither droplet agglomerates were formed nor droplets with a diameter greater than 500 nm were detected.

Conclusion

The research results showed that no droplet aggregates were observed in the short-term period of monitoring of the prepared nanoemulsions (immediately after, and 10 and 30 days after preparation), that is, the processes of destabilization did not occur. Namely, the values of volume diameters – d(0.1), d(0.5), and d(0.9) were within the established USP limits (≤ 500 nm). Results for the nanoemulsions prepared in the laboratory setting had approximately the same values as for the industrial nanoemulsion.

Regarding the preparation and characterization of TPN admixtures, no significant differences were found among parameters (mean droplet diameter, PDI, ζ-potential, pH value, volume diameter) measured during the 72-hour monitoring period.

Finally, the study showed that nanoemulsions can be successfully produced under laboratory conditions. These nanoemulsions with their composition and physical-chemical characteristics obtained from the short-term monitoring period are suitable for parenteral feeding. They can also be used as a component of the TPN admixture that is safe for the administration in the hospital setting.

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Correlation between central venous and mixed venous oxygen saturation in the elective abdominal aortic aneurysm surgery

Korelacija između saturacije kiseonikom centralne i mešane venske krvi u elektivnoj hirurgiji aneurizme abdominalne aorte

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Abstract

Background/Aim. The concept of utilizing central venous oxygen saturation ($ScvO_2$) to calculate cardiac index (CI) remains controversial and neither precise nor generally applicable conclusion has been reached yet. We evaluated the relationship between $ScvO_2$ and mixed venous oxygen saturation (SvO_2) in elective surgery of the abdominal aorta. The adequacy of their interchangeability was tested by comparing cardiac indices (CI) calculated by two methods in patients that underwent major vascular surgery. The aim of this study was to test the correlation between $ScvO_2$ and SvO_2 in different time frames, in patients undergoing elective abdominal aortic aneurysm (AAA) surgery as well as to determine if the use of $ScvO_2$ for calculating CI by the modified Fick equation, could be feasible and accurate surrogate for the values obtained by pulmonary artery catheter (PAC). **Methods.** This prospective observational study included 125 consecutive patients that underwent elective AAA surgery. The $ScvO_2$ and SvO_2 data, as well as CI values, were obtained and compared from samples taken in three different time frames: immediately after induction of general anesthesia (T_0), immediately after admission in the intensive care unit (ICU; T_1), and 8 h after admission in the ICU (T_2). The Fick equation, used for CI estimation from $ScvO_2$ (CI-F), for the purpose of this study, was simplified according to Walley. **Results.** There was good linear correlation between $ScvO_2$ and SvO_2 in all time frames and linear regression study revealed strongest coefficient of determination ($R^2 = 0.661$) in T_2 time-frame. There was no correlation between CI-F (i.e. CI calculated from $ScvO_2$ by modified Fick equation) and CI (measured by PAC from SvO_2) in any time-frame. **Conclusion.** The results of our study confirm that $ScvO_2$ is a reliable substitute for SvO_2 among patients undergoing elective surgery of the AAA. However, $ScvO_2$ cannot be used as a surrogate to true SvO_2 in the calculation of CI.

Key words:
aorta, abdominal; aortic aneurysm; monitoring, physiologic; oxygen; oximetry.

Apstrakt

Uvod/Cilj. Koncept korišćenja saturacije kiseonikom centralne venske krvi ($ScvO_2$), umesto saturacije mešane venske krvi (SvO_2), za izračunavanje srčanog indeksa (CI), ostaje kontroverzan s obzirom na to da još uvek nema pouzdanih podataka koji bi ukazivali da jedna saturacija može biti adekvatna zamena drugoj. Odnos između ova dva parametra testirali smo upoređivanjem vrednosti CI izračunatih na dva načina, kod elektivno operisanih bolesnika zbog aneurizme abdominalne aorte (AAA). Cilj rada bio je testiranje korela-cije između $ScvO_2$ i SvO_2 u različitim vremenima merenja kod bolesnika podvrgnutih elektivnim operacijama AAA, kao i utvrđivanje mogućnosti korišćenja $ScvO_2$ za izračuna-vanje CI, modifikovanom Fick-ovom jednačinom, kao adekvatne zamene vrednostima CI dobijenih merenjem putem plućnog arterijskog katetera (PAC). **Metode.** Prospektiv-nom opservacionom studijom bilo je obuhvaćeno 125 kon-sekutivnih bolesnika podvrgnutih elektivnim operacijama AAA. Podaci o $ScvO_2$ i SvO_2, kao i vrednosti CI dobijeni su uzimanjem uzoraka krvi i merenjem u tri različita vremena: posle uvoda u opštu anesteziju (T0), odmah posle prijema u

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jedinicu intenzivnog lečenja (JIL) (T1), i osam sati posle dolaska u JIL (T2). Za izračunavanje CI upotrebljena je pojednostavljena Fick-ova jednačina po Walley-u, u kojoj smo koristili ScvO$_2$ (CI-F). **Rezultati**. Nađena je dobra linearna korelacija između vrednosti ScvO$_2$ i SvO$_2$ u svim vremenima merenja, a linearna regresiona studija pokazala je najjači koeficijent determinacije (R2 = 0.661) u T2 vremenskom okviru. Nije bilo korelacije između CI-F (CI izračunat iz ScvO2 modifikovanom Fick-ovom jednačinom) i CI (meren PAC-om) u bilo kom vremenskom okviru. **Zaključak.** Rezultati studije potvrđuju da ScvO$_2$ može biti pouzdana zamena za SvO$_2$ kod bolesnika podvrgnutih elektivnim operacijama AAA. Međutim, ScvO$_2$ se ne može koristiti kao surogat za pravu SvO$_2$ u izračunavanju CI.

Ključne reči:
aorta, abdominalna; aorta, aneurizma; fiziološke funkcije, praćenje; kiseonik; oksimetrija.

Introduction

Measurement of mixed venous oxygen saturation (SvO$_2$) is useful indirect index of the entire body tissue oxygenation [1]. However, risk/benefit of the pulmonary artery catheter (PAC) placement remains controversial, and thus, its use has became somewhat unpopular [2, 3]. Routine use of the PAC in critically ill patients does not influence mortality and is associated with higher costs and complication rates [4, 5]. Insertion of a central venous catheter (CVC) in the superior vena cava (SVC), via the right internal jugular or subclavian vein, on the other side, remains standard of care in critically ill patients [6]. Monitoring of central venous oxygen saturation (ScvO$_2$) may be, therefore, the safer alternative to SvO$_2$.

Despite recent renewed interest in clinical applicability of serial ScvO$_2$ measurements, there are no published data in the available literature describing the pattern of ScvO$_2$ changes during major vascular surgery or possible relationships with outcome [7, 8].

The aim of this study was to test the correlation between ScvO$_2$ and SvO$_2$ in different time frames, in patients undergoing elective abdominal aortic aneurysm (AAA) surgery. Additionally, we wanted to determine if calculating cardiac index (CI) using ScvO$_2$, by the modified Fick equation, could be feasible and accurate surrogate for the values obtained by PAC.

Methods

This prospective observational study included 125 consecutive patients, scheduled for the elective AAA surgery, between July 2015 and April 2016, at the Clinic for Vascular and Endovascular Surgery, the Clinical Center of Serbia in Belgrade.

Patients with aortoiliac occlusive disease (Leriche's syndrome), cardiac or dialysis access shunt (fistula or graft) and emergent cases (ruptured AAA) were excluded from the study.

The study protocol was approved by the Ethics Committee of the Clinical Center of Serbia. Written informed consent was obtained from all patients before enrollment.

All operations were performed with combined (peridural and general endotracheal) anesthesia. Patients were premedicated with 5 mg im. midazolam (Dormicum®, Roche) 45 min prior to anesthesia. Peridural catheter (Perifix, B. Braun Melsungen AG) was inserted under local anesthesia at Th$_{10}$–L$_1$, or L$_1$–L$_2$, or L$_2$–L$_3$ levels, with a patient in left recumbent position. Induction proceeded with 0.2 mg/kg midazolam and 0.6 mg/kg rocuronium bromide (Esmeron®, Merck Sharp & Dohme). Patients were connected to an anesthesia apparatus (Primus, Dräger) and anesthesia was maintained with gas mixture O$_2$/N$_2$O (FiO$_2$ = 0.5) and sevoflurane (Sevorane®, AbbVie) in concentration of 0.8–1.5 vol%, along with rocuronium bromide in a total dose of 100 mg. For analgesia, 6–8 mL of 0.5% levobupivacain was given every 1.5 h–2 h via the peridural catheter. Operations were completed without any use of iv. analgsics.

Median laparotomy and transperitoneal approach to the abdominal aorta (AA) and classical inguinal approach to the femoral arteries were utilized. Abdominal aortic cross clamping was done below or above the origin of renal arteries, and occasionally above the origin of *truncus coeliacus*. Reconstruction of AA included interposition of either tubular Ao graft interposition (GI) or "Y" Dacron graft (Ao-biilliac – Aii, Ao-bifemoral – AFF).

Postoperative analgesia was maintained with a bolus dose of 6–8 mL of 0.25%, levobupivacain, every 8 h, via the peridural catheter. Lungs were mechanically ventilated (Evita, Dräger).

Invasive monitoring included radial artery cannulation (Becton Dickinson off-on), for the measurement of systemic blood pressure and serial blood sampling for gas analyses (Radiometar ABL 90 flex).

Insertion of the CVC (Arrow) was performed via the right internal jugular or subclavian vein and position of its tip in SVC, for ScvO$_2$ measurements, subsequently verified by chest radiograph. In addition, PAC (Swan-Ganz catheter, Arrow, 7F) was also inserted for SvO$_2$, CO (cardiac output), and CI measurements. Thermodilution CO and CI were obtained in triplicate and averaged. Samples from CVC and PAC were taken simultaneously in following time-frames: immediately after induction of general anesthesia (T$_0$), immediately after admission in the ICU (T$_1$), and 8 h after admission in the ICU (T$_2$).

The Fick equation, used for CI estimation from ScvO$_2$ (CI-F), for the purpose of this study, was simplified according to Walley [10]

$$CI \approx 100/Hgb \times 1/(SaO_2 - SvO_2)$$

where: CI as previously explained (L/min/m^2); Hgb = hemoglobin (g/L); SaO$_2$ = arterial oxygen saturation (%) and ScvO$_2$ (%).

Statistical analyses were performed using SPSS software v.23.0 (SPSS Inc., Chicago, IL, USA). Descriptive data for all groups and variables were expressed as mean ± stan-

dard deviation (SD) for continuous measures, or percent of a group for discrete measures.

A normal distribution was tested using the Koglo-morov-Smirnov test. If the data were normally distributed, RM-ANOVA was used. Nonparametric data were analyzed using Fridman test. *Post hoc* analysis was performed using Bonferroni test (parametric data) and Wilcoxon test (non-parametric data).

Correlation of the CVC and PAC parameters was tested with Pearson's (parametric data) and Spearman's correlation coefficients (nonparametric data).

All reported p values were two-sided; differences were considered significant when p value was < 0.05.

Results

Preoperative and intraoperative patient characteristics are summarized in Table 1. It is noteworthy emphasizing that majority of patients were in the seventh decade of life, with significant male predominance. Almost 95% were hypertensive and more than a half had some form of coronary artery disease. Intraabdominal reconstruction (ie. GI and Aii) with infrarenal clamp was possible in more than 90% cases.

Values of observed parameters ($ScvO_2$, SvO_2, CI, CI–F), obtained in three different time frames, are summarized in Table 2. Significant changes were registered for all of them, but inter-group significance was present only for $ScvO_2$ and SvO_2.
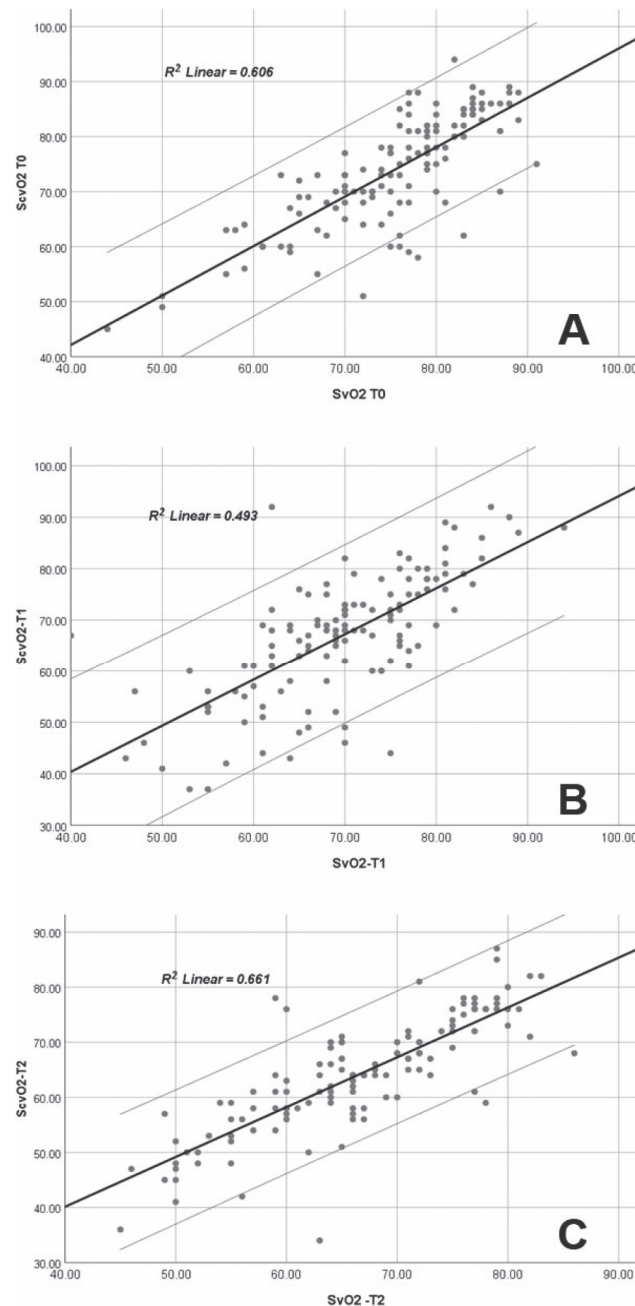
Fig. 1 – Correlation between central venous oxygen saturation ($ScvO_2$) and mixed venous oxygen saturation (SvO_2) in different time frames: A) immediately after induction of general anesthesia (T0); B) immediately after admission in the Intensive Care Unit (ICU); C) 8 h after admission in the ICU (T2).

Table 1

Demographic and clinical characteristic of patients (n = 125)

Characteristics	Values
Gender, n (%)	
male	108 (86.4)
female	17 (13.6)
Age (years), mean ± SD (Med; min-max)	66.39 ± 6.49 (66.0; 49–86)
BMI (kg/m^2),(mean ± SD (Med; min-max)	26.36 ± 3.85 (26.10; 14.70–36.50)
BSA (m^2), mean ± SD (Med; min-max)	2.00 ± 0.21 (2.03; 1.28–2.51)
Comorbidities, n (%)	
hypertension	118 (94.4)
DM	16 (12.8)
COPD	29 (23.2)
carotid surgery	14 (11.4)
CVI	17 (13.6)
CRF	14 (11.2)
CABG	11 (8.8)
valvular surgery	2 (1.6)
AP	46 (36.8)
PCI	14 (11.3)
Ao reconstruction, n (%)	
Ao-II	51 (40.8)
Ao-FF	10 (8.0)
Ao GI	64 (51.2)
Infrarenal cross clamp, n (%)	114 (91.2)
Proximal clamp time (min), mean ± SD (Med; min-max)	21.94 ± 8.09 (21.0; 9–53)
Total clamp time (min), mean ± SD (Med; min-max)	49.73 ± 20.21 (45.0; 17–118)

BMI – body mass index; BSA – body surface area; DM – diabetes mellitus; COPD – chronic obstructive pulmonary disease; CVI – cerebro-vascular insult; CRF – chronic renal failure; CABG – coronary artery bypass grafting; AP – angina pectoris; PCI – percutaneous coronary intervention; Ao-II – aortobiiliac bypass; Ao-FF – aortobifemoral bypass; Ao-GI – abdominal aortic graft interposition; Med – median.

Table 2

Analysis of selected parameters measured by central venous catheter (CVC) and pulmonary artery cateter (PAC) in different time frames

Parameters	Values	p-value [a,b]	Intergroup comparison[c,d]
ScvO$_2$ (%), mean ± SD			
(Med; min-max)	73.79 ± 10.12 (74.5; 45–94)	[a]0.000*	[c.1]0.000*
T0	66.82 ± 12.24 (68; 37–92)		[c.2]0.000*
– T2	63.94 ± 10.35 (64; 34–87)		[c.3]0.044*
SvO$_2$ (%), mean ± SD			
(Med; min-max)			
T0	75.31 ± 8.76 (77; 44–91)	[a]0.000*	[c.1]0.000*
T1	69.52 ± 9.59 (70;40–94)		[c.2]0.000*
T2	66.33 ± 9.30 (66; 45–86)		[c.3]0.000*
CI, mean ± SD			
(Med; min-max)			
T0	3.31 ±1.09 (3.01; 1.50–7.0)	[b]0.000*	[d.1]0.097
T1	3.34 ± 0.97 (3.20; 1.70–6.8)		[d.2]0.001*
T2	3.62 ± 0.79 (3.60; 1.30–5.90)		[d.3]0.000*
CI-F, mean ± SD			
(Med; min-max)			
T0	3.03 ± 1.05 (2.81; 1.27–5.93)	[b]0.024*	[d.1]0.118
T1	2.83 ± 1.02 (2.62; 1.21–6.12)		[d.2]0.001*
T2	2.64 ± 0.88 (2.49; 1.3–5.45)		[d.3]0.041*

ScvO$_2$ – central venous oxygen saturation; SvO$_2$ – mixed venous oxygen saturation; CI – cardiac index; CI-F – ScvO$_2$ for calculating CI, by modified Fick equation; T0 – immediately after induction of general anesthesia; T1 – immediately after admission in the Intensive Care Unit (ICU); T2 – 8 h after admission in the ICU; Med – median.
*statistical significance; [a]RM ANOVA; [b]Fridman-s test; [c]Bonferroni test; [d]Wilcoxon-s test ([1]p = To and T1 comparison; [2]p = To and T2 comparison; [3]p = T1 and T2 comparison).

Correlation between $ScvO_2$ and SvO_2 in different time frames is shown in Table 3. Since we established statistically significant correlation between observed parameters, a linear regression study was performed (Figure 1) and the strongest coefficient of determination ($R^2 = 0.661$) was found in T2 time-frame (Table 3, Figure 1C). These results confirmed that $ScvO_2$ could be reliable surrogate for SvO_2, particularly 8 h after admission in the ICU.

Table 3
Correlation of central venous catheter (CVC) and pulmonary artery catheter (PAC) parameters: $ScvO_2$ and SvO_2

Time-frame	Linear correlation	R^2	p-values
T1	0.779	0.606	0.000*
T2	0.702	0.493	0.000*
T3	0.814	0.661	0.000*

$ScvO_2$ – central venous oxygen saturation; SvO_2 – mixed venous oxygen saturation; T0 – immediately after induction of general anesthesia; T1 – immediately after admission in the Intensive Care Unit (ICU); T2 – 8 h after admission in the ICU.
*statistically significant.

Unlike expected, there was no correlation between CI-F (i.e. CI calculated from $ScvO_2$ by the modified Fick equation) and CI (measured by PAC from SvO_2) in any time-frame (Table 4).

Table 4
Correlation of central venous catheter (CVC) and pulmonary artery catheter (PAC) parameters: CI and CI-F

Time-frame	Spearman's correlation coefficient (ρ)	p-values
T0	0.085	0.346
T1	0.148	0.100
T2	0.069	0.444

CI – cardiac index; CI-F – CI, calculated by modified Fick equation.

Discussion

Interchangeability of $ScvO_2$ and SvO_2 values has been a matter of debate, primarily because of different sampling points and venous blood pools they represent (ie. entire body for SvO_2 and upper part of the body for $ScvO_2$) [9].

Complex relationship of these two parameters is different in healthy and diseased persons. Thus, $ScvO_2$ is slightly lower than SvO_2 in healthy individuals (76% vs. 78%, respectively), but in persons with cardiovascular instability, this relationship changes [10].

The most valuable information is trend of either $ScvO_2$ or SvO_2 changes upon applied treatment. Renewed interest in $ScvO_2$ monitoring came from the fact that lots of complications related to PAC insertion have been documented in the literature [11]. Intravascular pressure could not provide an adequate insight in the intravascular volume, which is, in turn, the only cardiac preload equivalent [12]. Sandham et al. [2] found no correlation between PAC guided therapy and outcome in non-cardiac surgical patients.

Scheinman et al. [11] compared $ScvO_2$ and SvO_2 levels in different hemodynamic states. They found no significant difference in stabile patients and patients with heart failure (54.7% vs. 56.9%, $p > 0.1$; and 61.8% vs. 58.2%, $p > 0.1$, respectively). In patients with circulatory shock, this difference was significant (58.0% vs. 47.5%, $p < 0.001$), due to poor left ventricular function and renal impairment [12, 13].

The degree of correlation between $ScvO_2$ and SvO_2 was tested by numerous studies, regardless of patient's hemodynamic status. By doing so, it was unable to find the reasons for poor correlation observed.

This main shortcoming comes from the fact that CO distribution changes in critically ill patients, thus affecting $ScvO_2$ and SvO_2 relationships [14–17]. Unlike previous, studies performed under experimentally controlled conditions found good correlation between $ScvO_2$ and SvO_2, regardless of their absolute values [18, 19]. Also, some studies emphasized the importance of similarity of trends between two parameters, while others deny the reliability of $ScvO_2$ [20–22].

If we keep on mind that $ScvO_2$ depends on: hemoglobin levels, SaO_2, CO, oxygen consumption (VO_2), body temperature, analgesic level and metabolic state, keeping all, except selected one constant, than $ScvO_2$ value reflects the changes of the remaining. The relationship between $ScvO_2$ and SvO_2 is not simple. In healthy persons, absolute values of these parameters are similar, which is not necessarily true in critically ill patients. Absolute values of $ScvO_2$ may be pathological even when it is high or low [23].

Attempts to calculate CI from $ScvO_2$ is not a new concept [24]. In experimental studies, with dogs in different cardio-respiratory conditions, Reinhart et al. [20] found a good correlation ($r = 0.97$) between CI calculated using two different methods. Goldman et al. [24] 1968, performed similar study in human subjects. Since then, a lot of studies on human subjects in different medical conditions were designed to correlate $ScvO_2$ and SvO_2 [25, 26].

During hypovolemic circulatory disturbances, CI and $ScvO_2$ showed better correlation with the extent of blood loss, than central venous pressure (CVP), pulmonary capillary wedge pressure (PCWP), arterial pressure and heart rate. Interestingly, in spite of different absolute values, the trends of $ScvO_2$ and SvO_2 changes paralleled observed hemodynamic changes. Orthostatic hypotension is commonly used, as a model of the cardiovascular disturbances associated with hypovolemia in humans [25, 27]. Median $ScvO_2$ fell from 75% to 60%, paralleling CO decrease from 4.3 to 2.7 L/min, at the onset of presyncope symptoms. However, unlike in experiments, in series of major trauma victims, there was no strong correlation of $ScvO_2$ and SvO_2 with the extent of blood loss [27, 28].

In septic patients, different trials could not find firm correlation between absolute values of $ScvO_2$ and SvO_2 [29, 30], probably due to modified blood flow distribution and oxygen extraction (O_2 ER) by brain and splanchnic tissues [30, 31]. In spite of this, variations in these two parameters usually occured in a parallel manner [29, 32].

Maybe the most extensively studied were the patterns of SvO_2 and $ScvO_2$ changes in cardiac failure and myocardial infarction. Goldman et al. [24] correlated derangements of $ScvO_2$ with severity of myocardial dysfunction and subsequent response to treatment, finding that levels below 45% usually indicate the onset of cardiogenic shock. While decrease of $ScvO_2$ levels depicts the severity of disease [11], trends are associated with CO and response to treatment [33–35].

There are few papers describing SvO_2 monitoring during the aortic surgery [36, 37]. Application and removal of aortic and femoral clamps produces complex SvO_2 changes. Clamp removal and lower body reperfusion produce significant SvO_2 decrease, not necessarily reflecting a need to change cardiovascular management. However, there are very few or no data, regarding $ScvO_2$ monitoring during the abdominal aortic surgery.

Kopterides et al. [37] investigated the significance of CVC tip position. When positioned 15 cm away from the inlet of the right atrium, $ScvO_2$ overestimated SvO_2 by 8%. However, when the tip of the CVC was advanced deeper in the right atrium, $ScvO_2$ becomes an excellent surrogate, overestimating SvO_2 by only 1%.

Our study enrolled patients without pulmonary artery and superior vena cava (SVC) catheterization under fluoroscopic guidance. So, both measurements, neither $ScvO_2$ nor SvO_2, were obtained under direct visualization of the catheter tips. Our subsequent analyses of the central line tip positions, in the ICU, showed that most of them were located in SVC or proximal right atrium (RA) or SVC-RA junction. This implies that blood samples were actually obtained from different places. We used the X-ray confirmation of the CVC tip position in the ICU, to exclude the patients in whom CVC was accidentally placed in the innominate vein. Thus, we intended to test the correlation between $ScvO_2$ and SvO_2 within more limited variations of $ScvO_2$ values. It should be emphasized that it was our intention to adapt on "real-life" situation, without changing established perioperative protocols for the purposes of this study. On the other hand, PAC parameters (SvO_2 and thermodilution CI) were obtained in triplicate and then averaged. Although our results confirmed statistically significant linear correlation between $ScvO_2$ and SvO_2, almost paradoxically, the same was not true with CI-F and CI. The most logical explanation is that, in fact, we used "different mathematics". Walley's simplification of the Fick formula, using $ScvO_2$ values to calculate CI-F, could not meet correlation criteria with thermodilution CI values obtained by PAC, using SvO_2. The ability of $ScvO_2$ measurement to estimate SvO_2 is useful but still imperfect, depending on CVC catheter placement, patient anatomy and physiologic state. Importantly, $ScvO_2$ is an increasingly less reliable substitute for SvO_2 as the cardiac performance is worsened. This should always be kept in mind when interpreting $ScvO_2$ measurements. When true SvO_2 is essential, PAC placement remains the gold standard, since it provides more data than just a calculation of CI and many patients may still benefit from it. In that sense, significant linear correlation between $ScvO_2$ and SvO_2 in our study could be seen as a result of standardized and reliable team work, resulting in absence of significant perioperative hemodynamic disturbances and mayor blood loss, allowing early detubation (within two hours postoperatively) and stabile spontaneous breathing in all patients.

Limitations of the study

This study has some limitations which have to be pointed out.

Accuracy of $ScvO_2$ measurement depends on CVC catheter placement, patient anatomy and physiologic state. Positioning of PAC and measurements was not always done by the same physician.

Conclusion

The results of our study confirm that $ScvO_2$ is a reliable substitute for SvO_2 among patients undergoing elective surgery of the abdominal aorta. It seems, when applied appropriately, that measurements of either $ScvO_2$ or SvO_2 may provide a valuable guide to circulatory management in the early postoperative period. However, this is not always true. In our study $ScvO_2$ cannot be used as a surrogate to true SvO_2 in the calculation of CI. Further studies are needed to confirm our findings. In practice, $ScvO_2$ seems especially useful in combination with vital signs and other relevant parameters.

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Introducing Nasal Obstruction Symptom Evaluation (NOSE) scale in clinical practice in Serbia: validation and cross-cultural adaptation

Uvođenje *Nasal Obstruction Symptom Evaluation* (NOSE) skale u kliničku praksu u Srbiji: validacija i kros-kulturalna adaptacija

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Abstract

Background/Aim. The Nasal Obstruction Symptom Evaluation (NOSE) scale is widely used in clinical practice for assessment of quality of life in patients with nasal obstruction. It has been validated in several countries up to date. The aim of this study was to validate and cross-culturally adapt the NOSE scale for Serbian population. **Methods.** The Serbian version of the NOSE scale (NOSE-s) was prepared through forward and backward translation, committee review, and pretesting. Validation process was carried out on 50 patients diagnosed with the nasal septal deviation (the study group) and 50 ear, nose and throat (ENT) patients with other non-rhinological diagnosis (the control group). **Results.** The NOSE-s instrument demonstrated good reliability (Cronbach α coefficient 0.81). Stability and reliability of the NOSE-s questionnaire were confirmed by test-retest procedure showing no statistically significant difference in obtained responses (Goodman-Kruskal gamma coefficient 0.83). Item and total scores were significantly higher in the study group than in the control group indicating the very good inter-group discrimination ($p < 0.001$). Inter-item and item-total correlations were similar to the original NOSE instrument. Three months after septoplasty, a mean NOSE-s score in patients was 19.2 ± 12.8. Calculated standardized response mean of 1.7 showed high sensitivity to change. **Conclusion.** The Serbian version of the NOSE scale is simple, valid and reliable instrument for estimating the nasal obstruction. Therefore, it can be recommended for application in rhinological practice and research in Serbian speaking population.

Key words:
nasal obstruction; quality of life; surveys and questionnaires; translations; serbia.

Apstrakt

Uvod/Cilj. *Nasal Obstruction Symptom Evaluation* (NOSE) skala se koristi u kliničkoj praksi za procenu kvaliteta života bolesnika sa nazalnom opstrukcijom. Do sada je validirana u nekoliko zemalja. Cilj ove studije je bio da se validira i kulturalno adaptira NOSE skala za korišćenje u srpskoj populaciji. **Metode.** Srpska verzija NOSE scale (NOSE-s) je pripremana na sledeći način: prevodom na srpski jezik, potom povratnim prevodom na engleski jezik, komisijskim pregledom prevoda i pretestiranjem skale. Proces validacije sproveden je u grupi od 50 bolesnika sa postavljenom dijagnozom devijacije nosne pregrade (studijska grupa) i među 50 bolesnika koji su se lečili na Klinici za uho, grlo i nos, kod kojih je postavljena dijagnoza nekog drugog ne-rinološkog problema (kontrolna grupa). **Rezultati.** NOSE-s instrument je pokazao dobru pouzdanost (Cronbach α coefficient 0.81). Stabilnost i pouzdanost NOSE-s upitnika su potvrđeni test-retest procedurom pokazujući da nema statistički značajne razlike u dobijenim odgovorima (*Goodman-Kruskal gamma coefficient* 0.83). Skor pojedinačnih pitanja, kao i ukupan zbir su bili viši u studijskoj grupi bolesnika nego u kontrolnoj grupi, pokazujući da postoji razlika između grupa ($p < 0.001$). Međusobna veza između pojedinačnih pitanja i pojedinačnog pitanja i ukupnog zbira je bila sličnih vrednosti kao i kod originalne skale. Tri meseca nakon septoplastike prosečan NOSE-s skor je bio 19.2 ± 12.8. Izračunata je i vrednost *standardized response mean* (1.7) koja je pokazala visoku senzitivnost upitnika na promenu. **Zaključak.** Srpska verzija NOSE skale je jednostavna za korišćenje, validna i pouzdana za procenu nosne opstrukcije. Zbog toga je peporučujemo za upotrebu u svakodnevnoj rinološkoj praksi kao i u budućim kliničkim istraživanjima u populaciji koja govori srpski jezik.

Ključne reči:
nos, opstrukcija; kvalitet života; ankete i upitnici; prevođenje; srbija.

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Introduction

The sensation of blockage or insufficient airflow through the nose is one of the most common reasons why patients seek medical help from an otorhinolaryngologist [1, 2]. Among numerous conditions that may manifest with nasal obstruction (adenoidal hyperplasia, (non)allergic rhinitis, chronic rhinosinusitis, nasal polyposis, turbinate hypertrophy), nasal septal deviation is a frequent diagnosis [3, 4]. Recent epidemiological studies reported that 10,000–95,000 septoplasties are performed in developed countries every year [1, 5]. However, objective assessment of nasal obstruction is controversial, and generally accepted measurement tool is still lacking [6]. Hence, patients' subjective evaluation of symptom severity stayed valuable source of information. Consequently, health-related-quality-of-life (HRQoL) instruments that estimate patients' health status and symptom severity are considered reliable and valid health-related measurement tools [7].

The nasal obstruction symptom evaluation (NOSE) scale is the HRQoL questionnaire specifically designed to assess quality of life in patients with nasal obstruction. This instrument consists of five obstruction-related questions that evaluate severity of complaints experienced during the last month. The NOSE instrument uses 5-point Likert scale scoring system for each item (not a problem, very mild problem, moderate problem, fairly bad problem, and severe problem). The raw score is then multiplied by 5 so that the total score ranges from 0 (no problem with nasal obstruction) to 100 (the most severe problem with nasal obstruction). The NOSE scale has been confirmed as a valid, reliable and sensitive to change in patient's clinical status [6]. The original version of the NOSE scale was primarily applied to test patients prior to and after septoplasty. Additionally, its application is recommended for comparison of the effects of different treatment modalities (medical vs. surgical, different surgical techniques) as well as for evaluation of symptom severity between different groups of patients (eg. patients with and without nasal polyposis) [6]. Furthermore, the NOSE scale has been more widely used, for example, to evaluate the outcomes after nasal valve surgery, functional rhinoplasty, and radiofrequency turbinate reduction [8–10].

The NOSE scale has been accepted and validated in a few countries up to date [11–19]. The aim of the current study was to translate, culturally adapt, and validate NOSE scale for Serbian population.

Methods

Study design

The validation of the Serbian version of the NOSE (NOSE-s) instrument was designed as a prospective instrument-validation study. Consent to perform cross-cultural adaptation of the NOSE instrument into Serbian language was obtained from the author of the original version of the scale.

Ethical approval

The study was approved by the Ethics Committee of the School of Medicine, No. 29/V-1. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and national research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Cross-cultural adaptation process

Standard techniques for cross-cultural adaptation and validation of HRQoL instruments were applied [20–22]. Two independent Serbian native-speakers with an academic knowledge of English performed forward translations. Both translated versions were reconciled into a single version by an expert committee. Subsequently, two persons performed independent back translations of this version of the questionnaire. The first person was an English native speaker with a medical education, who was also fluent in Serbian language. Another person was a bilingual speaker, the English teacher whose first language is Serbian. None of the back translators had insight into the original scale. These versions were further adjusted into a single version. The expert board reviewed all reports once again and created the pre-final version of the scale. This version was pretested on a group of 30 randomly selected patients. Each patient completed the pre-final version of the NOSE-s scale. According to technique suggested by Reichenheim and Moraes [23], meaning of each question was explored by asking patients to rephrase them. Proper understanding and approval of the instrument was surveyed by achieving more than 90 percent of understanding [23]. Thus, the final version of the NOSE-s scale was created.

Sample size

Patient selection was carried out at the Department of Diagnostic Radiology, Faculty of Dental Medicine, University of Belgrade, due to high frequency of patients and in order to better represent general population. Patients were consecutively gathered for the study group (n = 50) and the control group (n = 50), respectively. The size of each group was calculated using a general rule of thumb, which is a common procedure to determine sample size for psychometric validation of questionnaires [6, 11–13, 20]. This rule recommends inclusion of 10 subjects per each item of the instrument [20].

The study group was selected among patients who were clinically diagnosed with nasal septal deviation by an otolaryngologist and referred to the computed tomography (CT) examination of the nose and paranasal sinuses. All patients had symptoms of chronic nasal obstruction persisting 4 weeks after trial of medical therapy. Patients with the history of surgery (septoplasty, septorhinoplasty, septoplasty combined with a paranasal sinus surgery), craniofacial syndromes, facial bone trauma, adenoid hypertrophy, sleep apnea syndrome, acute or chronic sinusitis, sinonasal malignancy, radiotherapy of the head and neck, and uncontrolled asthma, were not included in the study.

Patients enrolled in the control group were referred to the CT examination of the head and neck by ear, nose and throat (ENT) specialist. These patients did not complain of any rhynological symptoms and had no nasal septal deviation, which was additionally confirmed by CT scans. None of these patients had developmental facial anomalies, history of facial trauma, and/or sinonasal malignancy.

Patients from both groups were sex and age matched. All participants were older than 18 years, and gave written informed consent for participation in the study.

The NOSE-s scale testing

In order to avoid possible investigator influence on patients' responses, the NOSE-s questionnaires were self-administered. The time needed to complete the questionnaire was measured for each patient. The test-retest procedure was carried out among 30 randomly selected patients from the study group within two weeks. A total of 40 patients from the study group underwent septoplasty, while 10 patients refused surgical intervention. Three months after surgery, 33 patients completed the NOSE-s questionnaire again. The rest of seven patients were lost to follow-up.

Statistical analysis

Data were statistically analyzed by descriptive (mean, standard deviation, range, frequencies) and analytical methods. Internal consistency was assessed by Cronbach's alpha coefficient. The value higher than 0.81 was considered satisfactory [24]. Test-retest reliability was evaluated by Goodman-Kruskal gamma coefficient. Discriminant validity between groups was evaluated by Mann–Whitney U test. Spearman's coefficient (r) was used to correlate item-item and item-total score. The statistically significant degree of correlation was considered if the coefficient r was higher than or equal to 0.40. In order to evaluate response sensitivity of the questionnaire, standardized response mean was computed by dividing the mean score change by the standard deviation of the change. A value of approximately 0.2 demonstrated low sensitivity to change, while a value of 0.5 demonstrated a moderate sensitivity, and 0.8 demonstrated high sensitivity to change. A p-value < 0.05 was considered as significant. All statistical analyses were performed using SPSS Statistical Software 17.0 (SPSS, Inc., Chicago, IL).

Results

The final version of the NOSE-s scale is displayed in Table 1. The mean time required to fulfill the questionnaire was 2.5 ± 0.5 min and 2.0 ± 0.5 min for the study group and the control group, respectively.

The internal consistency analysis demonstrated good reliability of the NOSE-s questionnaire at the level of Cronbach's alpha coefficient of 0.81. The mean time between test-retest administrations was 11.4 days (5–14 days). The obtained value of Goodman-Kruskal gamma coefficient of 0.83 ($p < 0.001$) suggested a good test-retest reliability. Test reproducibility was presented by standardized response mean of 0.18, which confirmed low sensitivity to change after retesting.

Average scores for each item obtained in both groups are shown in Table 2. All values (single items and the total score) were significantly higher in patients from the study group when compared to the control group ($p < 0.001$), which demonstrated excellent inter-group discrimination.

Table 1

The Serbian version of the Nasal Obstruction Symptom Evaluation (NOSE-s) scale

Over the past 1 month, how much of a problem were the following conditions for you?
У последњих месец дана, колики проблем су Вам представљале следеће тегобе?
Please circle the most correct response
Молимо Вас да заокружите одговор који најбоље описује Ваше тегобе

Symptom	Not a problem Без тегоба	Very mild problem Веома благе тегобе	Moderate problem Средње изражене тегобе	Fairly bad problem Изражене тегобе	Severe problem Веома изражене тегобе
1. Nasal congestion or stuffiness Осећај запушености носа	0	1	2	3	4
2. Nasal blockage or obstruction Осећај непроходности носа	0	1	2	3	4
3. Trouble breathing through my nose Отежано дисање кроз нос	0	1	2	3	4
4. Trouble sleeping Лош сан	0	1	2	3	4
5. Unable to get enough air through my nose during exercise or exertion Отежано дисање кроз нос приликом изражене физичке активности	0	1	2	3	4

Table 2
**Comparison of item and total scores between groups (items presented as mean ± standard deviation; range of patients'
responses shown in parentheses)**

Item	Study group mean ± SD	Control group mean ± SD	*p*-value	Skewness	Kurtosis
Nasal congestion	2.0 ± 1.1 (0–4)	0.2 ± 0.4 (0–2)	< 0.001	-0.176	-0.582
Nasal obstruction	1.8 ± 1.1 (0–4)	0.1 ± 0.4 (0–2)	< 0.001	-0.253	-0.607
Trouble breathing	1.6 ± 1.2 (0–4)	0.1 ± 0.1 (0–1)	< 0.001	0.026	-1.221
Trouble sleeping	0.9 ± 1.1 (0–4)	0	< 0.001	1.207	0.884
Trouble breathing during exercise	2.5 ± 1.3 (0–4)	0.2 ± 0.5 (0–2)	< 0.001	-0.595	-0.717
Total raw score	8.9 ± 4.4	0.6 ± 0.8	< 0.001	–	–
Total score × 5	44.3 ± 22.3	2.9 ± 3.9	< 0.001	–	–

SD – standard deviation.

Table 3
Inter-item and item-total correlations (Spearman's correlation coefficient)

Item	Nasal congestion	Nasal obstruction	Trouble breathing	Trouble sleeping	Trouble breathing during exercise
Nasal congestion					
Nasal obstruction	0.646				
Trouble breathing	0.368	0.611			
Trouble sleeping	0.170	0.310	0.466		
Trouble breathing during exercise	0.386	0.537	0.673	0.383	
Total score	0.653	0.776	0.852	0.571	0.811

Table 3 displays construct validity of the NOSE-s questionnaire assessed through inter-item and item-total correlation coefficients. The item "Nasal congestion or stuffiness" correlated significantly only with the item "Nasal blockage or obstruction" (r = 0.646). The item "Nasal blockage or obstruction" correlated significantly with all other items except with the "Trouble sleeping" (r = 0.310). Moreover, the item "Trouble breathing" was significantly associated with all but the first item ("Nasal congestion or stuffiness") (r = 0.368). The fourth item ("Trouble sleeping") correlated significantly with the "Trouble breathing" (r = 0.466) and not with other items. Finally, the item "Trouble breathing during exercise" was not significantly associated with items "Nasal congestion or stuffiness" (r = 0.386) and "Trouble sleeping" (r = 0.383). Additionally, each item correlated significantly with the total score.

Preoperative NOSE-s score of the patients that underwent septoplasty was 53.75 ± 16.8. Three months after septoplasty, a mean NOSE-s score in patients was 19.2 ± 12.8. Calculated standardized response mean of 1.7 showed high sensitivity to change.

Discussion

Development of an entirely new HRQoL instrument is a time consuming and expensive process. Instead, researchers often use previously validated and published instruments that are recognized as valuable tools for self-assessment of symptom severity. Achievement of the equivalence between the original and the target version of the HRQoL instrument is an important and necessary step prior to application in a new population. This process requires translation, cross-cultural adaptation, and psychometric validation according to well-established principles [20, 22]. The entire process enables detection of the impact of a disease or patients' response to the applied therapy in a uniform way in each adopted version of the instrument. In addition, standardized questionnaires allow result comparison across studies. Moreover, thorough process of cultural adaptation enables inclusion of immigrant population in health studies, and omits a bias in quality of life studies [20].

The NOSE scale was developed and validated in order to assess quality of life in patients with nasal obstruction [6]. In general, the main point of the NOSE scale is to evaluate nasal obstruction in any disease [14]. This questionnaire has been validated in several countries up to date [11–19]. Given that the number of patients involved in these studies usually ranged from 100 to 116 [11, 13, 15, 18, 19], our sample size can be considered as optimal when compared with previous studies.

All patients enrolled in the current study completed the NOSE-s scale without any difficulty, showing that it was not burdensome for them. The psychometric properties of the NOSE-s instrument are consistent with the original questionnaire confirming high reliability and validity of the instrument. Internal consistency of the NOSE-s scale was similar to values reported in previous studies that ranged from 0.74 to 0.97 [6, 11–19].

Among five nasal obstruction related symptoms that NOSE scale evaluate, only trouble sleeping was close to one

end of the Likert's scale (Table 2). This result could be explained by consecutive patient sampling used in our study. Patients who were diagnosed with the nasal septal deviation and referred to the CT examination during sampling period were included in the study regardless of the obstruction severity. The predominance of patients with no or very mild sleeping trouble contributed to the low mean value of the item. If the study group contained more patients with severe nasal obstruction and thus severe sleeping trouble, it would certainly shift the mean score of the item 4 to the greater values.

Considering a short period (5 to 14 days) during which test-retest was made, significant changes in a clinical status of patients were not expected. Given that underlying patient's status did not change during this period and the fact that scores of the scale remained constant, our results demonstrated that the NOSE-s instrument measured a true state of the patient health. Calculation of standardized response mean confirmed our expectations and showed low sensitivity to change, suggesting good stability and reproducibility of the NOSE-s scale.

The comparison between the study group and the control group showed very good inter-group discrimination. Patients with a nasal septal deviation had scores significantly higher than controls. This indicates that the NOSE-s scale is a sensitive to detect the presence or absence of the nasal obstruction, which is consistent with the original NOSE instrument [6] and other validation studies [11–19]. Construct validity of the NOSE-s questionnaire was also in accordance with the original version of the instrument [6] as with other validation studies too [14, 15, 18]. All items correlated significantly with each other and with the total score, except the "trouble sleeping" with the "nasal congestion or stuffiness" and the "nasal blockage or obstruction". Additionally, our results demonstrated that the NOSE-s scale is also sensitive to detect change in the health status in patients treated with septoplasty.

The Serbian version of the NOSE instrument is the first validated rhinological scale that could be used in clinical studies on Serbian speaking territory. Additionally, there are nearly 3 million Serbs living abroad (1.2 million in the United States of America and Canada, half million in Germany, 300,000 in Austria, 207,000 in Australia and New Zeeland, 12,000 in France and Sweden each) and about half a million labor migrants from Serbia in the European Union [25]. Given that some of them are not fluent in the language of the country they are living in, this questionnaire also allows them to be involved in clinical studies.

Although validity and reliability of the Serbian version of the NOSE scale was in accordance with the original NOSE scale, the lack of criterion validity testing could potentially limit our study. Another limitation refers to lack of multi trait multi method matrix approach. Questionnaires in our study were self-administrated in order to omit interviewer related bias and provide honest answers, as suggested in the literature [26, 27]. Since second turn testing by investigator was not performed, multi trait multi method matrix could not be made.

Conclusion

The equivalence between Serbian version and the original version of the NOSE scale was provided. Serbian speaking population gained culturally adapted and validated, feasible and intelligible questionnaire that is user-friendly both for patients and for health professionals. Patients found it understandable and not burdensome, while doctors considered it an important source of information with handy statistical data processing. An opportunity to use the already developed and validated NOSE-s instrument is created, which allows this important and frequently used HRQoL instrument to be applied in clinical practice and research. Additionally, the application of the NOSE-s scale would also enable comparison with the results obtained in studies conducted in other speaking areas and cultures.

Acknowledgement

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The influence of the skin tumors excision width in the postoperative facial asymmetry

Uticaj širine ekscizije tumora kože lica na postoperativnu asimetriju

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Abstract

Background/Aim. Planning an elliptical excision of the facial skin, caused by lines of minimum tension, is very important in order to achieve good aesthetic results. The width of the tumor excision affects the possibility of a direct closure of the post-excision defect. The aim of the study was to determine the minimum width of excision that does not affect postoperative symmetry of the face, in relation to the preoperative one, using an objective scanning method with a line laser scanner. **Methods.** The study included 50 patients of both sexes, older than 50 years, who had verified facial skin tumor and established medical indication for surgical elliptical excision and direct suture. All patients had laser scanning preoperatively, and then seven days and 90 days postoperatively, giving x, y, and z coordinates of 5 cephalometric points on the face, which determined the shape of the examined region. Patients were divided into three groups depending on the width of the excision (< 10 mm, 10–15 mm, > 15 mm). The shape of the examined region among different width of excision was compared, preoperatively, 7 days and 90 days postoperatively, using Procrustes analysis and analysis of the coordinates of cephalometric points. **Results.** Taking into account preoperative and postoperative x, y and z coordinates of the cephalometric points, statistically significant differences between the group of patients with the width excision < 10 mm and the other two groups (excision width 10–15 mm and > 15 mm) were found. **Conclusion.** The width of the skin tumors excision < 10 mm does not affect the postoperative facial asymmetry when a post-excisional defect is closed by direct suture.

Key words:
facial neoplasms; reconstructive surgical procedures; postoperative period; facial asymmetry; lasers; cephalometry.

Apstrakt

Uvod/Cilj. Planiranje elipsastih ekscizija na koži lica, uslovljeno linijama minimalne tenzije, veoma je bitno u postizanju dobrih estetskih rezultata. Širina ekscizije tumora utiče na mogućnost direktnog zatvaranja postekscizionog defekta. Cilj rada bio je određivanje najmanje širine ekscizije koja ne utiče na postoperativnu simetriju lica, u odnosu na preoperativnu simetriju, primenom objektivne metode skeniranja lica linijskim laser skenerom. **Metode.** Istraživanjem je bilo obuhvaćeno 50 ispitanika oba pola, starijih od 50 godina, kod kojih je verifikovan tumor kože lica i postavljena medicinska indikacija za hiruršku elipsastu eksciziju i direktnu suturu. Svi ispitanici su skenirani laser skenerom preoperativno, a potom 7 dana i 90 dana postoperativno. Na taj način dobijene su x, y i z koordinate pet kefalometrijskih tačaka na licu, koje su određivale oblik ispitivane regije. Ispitanici su podeljeni u tri grupe u zavisnosti od širine ekscizije (< 10 mm, 10–15 mm, > 15 mm). Upoređivan je oblik ispitivane regije između različitih širina ekscizije, preoperativno, kao i 7 dana i 90 dana postoperativno, primenom Prokrustove analize i analize koordinata kefalometrijskih tačaka. **Rezultati.** Posmatrajući preoperativne u odnosu na postoperativne x, y i z koordinate kefalometrijskih tačaka, nađena je statistički značajna razlika u obliku ispitivane regije između grupe ispitanika sa širinom ekscizije < 10 mm u odnosu na ostale dve grupe ispitanika (širina ekscizije 10–15 mm i > 15 mm), **Zaključak.** Širina ekscizije tumora kože lica < 10 mm ne utiče na postoperativnu asimetriju kada se postekscizioni defekt zatvara direktnom suturom.

Ključne reči:
lice, neoplazme; hirurgija, rekonstruktivna, procedure; postoperativni period; facijalna asimetrija; laseri; kefalometrija.

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Introduction

The basic postulates in plastic and reconstructive surgery with a consequent good aesthetic result are remodeling, restoring lost or establishing a new position. Face, as the most exposed part of the body, has always been a region of the greatest challenges for the plastic surgeon. In order to achieve good aesthetic results on site, planning the excision is conditioned on knowledge of the lines of minimum tension [1, 2].

In assessing the aesthetic results, plastic surgeons have used the symmetry of objectification, although they eventually realized that harmony is an additional important moment in the evaluation. Historically speaking, many methods for assessing symmetry were developed [3–5].

Unfortunately, geometry with mathematical precision could not be applied in clinical practice, since geometric and biological laws affect the renewal of tissue. On the other hand, using geometry is the most precise, the best, and the most objective way of assessing symmetry [6].

Although the use of geometry in measuring and estimating the obtained results is the most accurate method, it was very difficult to find the best way to use geometry. It is necessary to decide what is measured, how it is measured, and how to evaluate the measurement results. In the initial development of morphometry, representing the method of shape measurement, the orientation points were first determined, after which the distance between the orienting points was measured. In order to compare the distances, it was necessary to standardize the position of the orientation points or to perform scaling of the measured shapes. In that way, all the measured shapes would be of the same size. The distance between the orientation points of measured shapes would be the measurement of two shapes' difference. The big problem was to determine the position of the reference plane so that no errors in the measurement occurred as a result of an inadequate position of the plane in terms of rotation along the vertical or horizontal axis of rotation. Even when all the stated difficulties in measuring were corrected, the question arose as to how to compare the obtained results, because the positions of measured points, or the distance between the measured points, did not provide information about the whole shape of the measured region. It was necessary to standardize the positions of all measured points and find a way to statistically compare the shapes of the measured region in several patients. But, the statistical analysis of the position of the measured points individually presented a more detailed analysis of the shape, presenting which exact points changed the shape of the measured region. All of the above conditions could be satisfied with the use of a line laser in scanning faces because it is the most precise first step in measuring the position of the points. After that, it is necessary to do the scaling and positioning of the level of the measured face in order to define the coordinate start, after which the orientation points receive their x, y and z coordinates [7].

In addition to this, a major problem was in defining the shape variables, determining the statistical significance of the difference between the measured shapes and in assessing the asymmetry between the shapes. The above problem was re-solved using the Procrustes analysis, which involved translating, rotating and scaling scanned shapes, in order to bring them to a level so that the orientation points could be measured. After that, the Procrustes distance was determined, representing the square root of the square of the difference sum between the analog measured points, presenting the difference between the two forms. In statistical data processing, ANOVA and MANOVA with post-hoc tests for coordinates of measured points, ie. Procrustes distance between measured shapes, which are characterized by measured points, is a selection method for an adequate estimation of the difference between the measured shapes. This methodology has wide application in many morphometric analyzes, and can also be applied in plastic surgery as the most objective method for assessing the level of asymmetry, on the basis of the statistical significance of the difference between the patient groups in relation to the independent variable and among the same groups of patients at different time intervals in relation to the time of the operation. The described methodology can also be used in measuring other regions of the body [8].

Laser light in the assessment within two points in the space base was used ten years ago for the first time in the world for the formation of a low-budget hardware-software package for the three-dimensional scanning and editing models [9].

Laser light today has a great application in estimating the distance between two points in space. It has found its place in many scientific disciplines and spheres of life, mostly in forensics, construction, anthropology, and traffic. When we talk about the application of laser light in morphological analyses in medicine and dentistry, nowadays it is most commonly used in the prosthetics [10, 11].

In accordance with the basic principles of plastic and reconstructive surgery, including aesthetic surgery, when the morphological aspect is one of the essential elements in the assessment of postoperative results, the use of laser light in a precise assessment of morphological characteristics and the relationship of anatomical entities can find its important place and role in the scientific research, as well as in the clinical practice [12, 13].

The aim of the study was to determine the minimum width of excision that does not affect the postoperative symmetry of the face, in relation to the preoperative one.

Methods

The study included 50 patients of both sexes, older than 50 years, who had verified facial skin tumor and established medical indication for surgical elliptical excision and direct suture.

All patients had laser scanning preoperatively, and 7 days and 90 days postoperatively, giving the three-dimensional (3D) coordinates (x, y and z) of five cephalometric points on the face (nasion, endocanthal central point, pronasale, lower palpebral point, endocanthion).

The excision width was determined, as an independent variable, in order to compare the obtained results with different excision widths.

The width of excision implied the sum of the tumor width and the width of the excised healthy skin on both sides of the tumor, in the widest part of excision. Measurement of excision width was done before surgery, after scanning with a line laser scanner, and also we measured x, y and z coordinates of cephalometric points before the surgery. After the surgery, on the 7th and 90th day, we measured x, y, and z coordinates of cephalometric points.

Patients were divided into three groups depending on the excision width (< 10 mm, 10–15 mm, > 15 mm).

Equipment for three-dimensional scanning (recommended by the Institute for Robotics and Process Control, University of Braunschweig, Germany) was consisted of a red laser line laser (first class, 650 nm adjustable focus) and auto focusable camera Logitech QuickCam Pro 9000, a resolution of 1600 × 1200 pixels, and laser projection lines were recorded on the faces of patients included in the study. The equipment was connected during the scan to a computer and data processing was done using a professional licensed Laserscanner software. This result was the virtual model with 400.000–600.000 points having defined x, y and z coordinate, to the accuracy of 0.2 mm [14].

Using 3D coordinates, by Procrustes analysis of five cephalometric facial points, we determined Procrustes distance, given by Procrustes coordinates, as a measure of the shape variability of the examined region. Procrustes distance was determined using the MorphoJ program (MorphoJ, version 1.06d, 2014), while all other statistical analyses were done in the SPSS program (SPSS 23, IBM, 2015).

Our methodology in estimating the level of asymmetry of the face after the excision of facial skin tumors has not been applied in our institution until now, and according to the available literature, the complete methodology described has not been applied in plastic surgery regarding the evaluation of postoperative asymmetry at different widths of facial skin tumor excision.

Results

The mean values of Procrustes distances between facial coordinates in patients with facial tumors excised by using three excision widths (< 10 mm, 10–15 mm, > 15 mm), preoperatively, 7 days after surgery and 90 days after surgery, were presented in Figure 1.
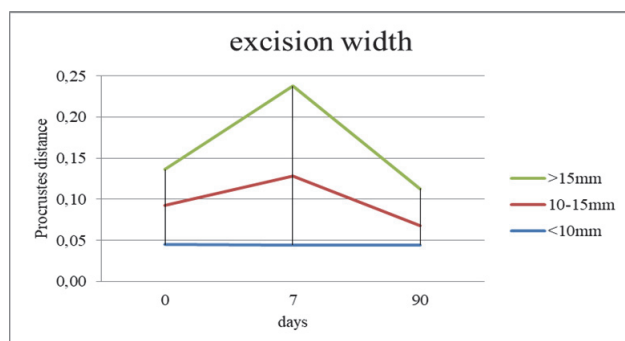
Fig. 1 – Procrustes distances among the different excision widths in patients with facial skin tumors in all scanning times (0, 7, 90 days).
Excision width: < 10 mm, 10–15 mm, > 15 mm.

We found a very similar value of Procrustes distances in the group with excision width < 10 mm, in all three scanning times. Seven days postoperatively, we found the highest value of Procrustes distances in two groups (10–15 mm, > 15 mm), while 90 days postoperatively, the value of Procrustes distances was lower than preoperatively, in the same groups. Preoperatively, median of Procrustes distances was lower than mean in the group with excision width < 10 mm and 10–15 mm, while median was higher than mean in the group with excision width > 15 mm; 7 days postoperatively median was lower in the group with excision width < 10 mm and in > 15 mm, and higher in the group with excision width 10–15 mm; 90 days postoperatively median was lower than mean in all three groups.

The statistical significance of Procrustes distance differences between different excision widths in all of three scanning times was analyzed using ANOVA and post-hoc Tukey test, and presented in Table 1. We found statistically highly significant difference among all three excision width groups, 7 and 90 days postoperatively.

Table 1
Procrustes distances (Pd) between different excision widths in all three scanning times (0, 7, 90 days)

Pd (mm)	Days		
	0	7	90
	p-value		
< 10 vs. 10–15	0.259	0.000	0.000
10–15 vs. > 15	0.070	0.000	0.000
< 10 vs. > 15	0.793	0.000	0.005

Excision width: < 10 mm, 10–15 mm, > 15 mm.

The statistical significance of Procrustes distance differences of different excision width in all of three scanning times was analyzed using ANOVA and post-hoc Tukey test, and presented in Table 2. We found statistically highly significant difference in two groups (10–15 mm and > 15 mm) between all scanning times.

Table 2
Procrustes distances (Pd) of different excision width between all three scanning times (0, 7, 90 days)

Pd (mm)	Days		
	0 vs. 7	7 vs. 90	0 vs. 90
	p-value		
< 10	0.098	0.902	0.098
10–15	0.000	0.000	0.000
> 15	0.000	0.000	0.000

Excision width: < 10 mm, 10–15 mm, > 15 mm.

MANOVA results of statistically significant difference among the coordinates of all three excision widths in all three scanning times (0, 7, 90 days) were presented in Table 3.

We found statistically highly significant difference between 0 and 7 days, and between 7 and 90 days, in two groups (10–15 mm and > 15 mm), in $x2$, $y2$–4, in $z4$ in > 15 mm (0–7 days), in $z2$ and $z4$ in > 15 mm (7–90 days), and in $x2$, $y2$ and $y4$ in 10–15 mm and > 15 mm, in 0–90 days. There was no statistically significant difference in the group with excision width < 10 mm between all the scanning times.

Table 3
Influence of different excision width (< 10 mm, 10–15 mm, > 15 mm) on x, y and z coordinates in all scanning times
(0, 7, 90 days)

Coordinate	0 vs.7			7 vs. 90			0 vs. 90		
	< 10 mm	10–15 mm	> 15 mm	< 10 mm	10–15 mm	> 15 mm	< 10 mm	10–15 mm	> 15 mm
					p-value				
x1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
x2	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.001	0.000
x3	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
x4	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
x5	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
y1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
y2	0.956	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000
y3	1.000	0.000	0.000	1.000	0.000	0.000	1.000	1.000	1.000
y4	1.000	0.000	0.000	1.000	0.000	0.000	1.000	0.000	0.000
y5	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
z1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
z2	1.000	0.083	0.000	1.000	0.083	0.000	1.000	1.000	1.000
z3	1.000	0.946	1.000	1.000	1.000	1.000	1.000	0.946	1.000
z4	1.000	0.083	0.000	1.000	0.083	0.000	1.000	1.000	1.000
z5	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Discussion

Previous studies have shown a large number of methods for the evaluation of facial asymmetry, ranging from digital photography, and ending with a three-dimensional laser scanning faces [15–17].

The introduction of laser light in the estimation of two points distance in space was the basis used for the first time in the world ten years ago for the creation of a low-budget hardware-software package for three-dimensional scanning and editing of the model. Laser light today has a great application in estimating two points distance in space and has found its place in many scientific disciplines and spheres of life, mostly in forensics, construction, anthropology, and traffic. When we talk about the application of laser light in morphological analyses in medicine and dentistry, it is most commonly used to date in the prosthetics [18–20]. In accordance with the basic principles of plastic and reconstructive surgery, including aesthetic surgery, when the morphological aspect is one of the essential elements in the assessment of postoperative results, the use of laser light in a precise assessment of morphological characteristics and the relationship of anatomical entities can find its important place and role, regarding both scientific and clinical aspect. The historically observed use of laser light in determining the morphology of three-dimensional objects has not essentially changed since the first days. The forms of laser light application were the only modifications, resulting in measurements of greater precision. The most widely used laser light was initially red, but due to its contrast and laser length, it was eventually concluded that the laser green and blue light gave more accurate results in the measurement. Having done further analysis of measurement accuracy improvement, scien-

tists have concluded that the brightness of the room and the observation object are very important because, in this way, a more precise measurement result is obtained with better contrast. In addition to this, the precision of the optics is an essential part of the camera that records the movement of the laser line through the object of observation [21–23].

In accordance with the basic postulates in plastic and reconstructive surgery, the application method that allows an objective assessment of asymmetry is necessary regarding the purpose of objective assessment and postoperative facial asymmetry results. When talking about the characteristics of the skin cover, elasticity and skin quality are the most important, as well as the presence of possible comorbidity with skin exposure [24, 25].

Analyzing the impact of the excision skin tumor width in the postoperative facial asymmetry, we found no statistically significant difference in the operated region shape between the groups of patients with various excision width preoperatively. However, there was the statistically highly significant difference among all the groups of patients, postoperatively in all scanning times. We can conclude that excision width has a role in the postoperative facial asymmetry. Besides, there was no statistically significant difference of operated region shape in the group with excision width < 10 mm, between all the scanning times. On the other hand, there was statistically highly significant difference in the groups with excision width 10–15 mm and > 15 mm, between all the scanning times. Thus, we can conclude that excision width < 10 mm does not cause postoperative asymmetry in patients with excised facial skin tumors.

Analyzing x, y and z coordinates of cephalometric points in the region of medial cheek, we found that there was no statistically significant difference among them in the

group of patients with excision width < 10 mm, reagarding different scanning times, in relation to the same results when we analyzed the shape of operated region, but x2, y2 and y4 were changed postoperatively in the groups 10–15 mm and > 15 mm. That could be explained by high concavity and small surface, as morphologic characteristics of medial canthal region. In this region, the excision of higher width moved the landmarks more significantly than in other regions of the face.

The elasticity of the skin certainly influenced the decision on the suture line tension, but the overall morphological result was taken into account, and not only in the area of the suture line. In order to achieve a total good morphological-aesthetic result, it would be necessary to use the inductive-deductive, bi-directional and comprehensive approach. The quality of the skin in a similar way affected the overall morphologic-aesthetic re-

sult as well as the elasticity, but it was a more dominant biological aspect in the healing phase of the wound [24, 25].

Conclusion

The determination of the x, y and z coordinates of the face, most accurately and most objectively, can be done with laser scanning. A modern method in morphometric analyses of scanned faces implies Procrustes analysis, as well as single coordinated measurements determining the level of statistically significant differences in shape.

The obtained results show that excision width of less than 10 mm does not affect the postoperative facial symmetry in the region of the medial cheek when post excisional defect is closed by direct suture.

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O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl) propanoate dihydrochloride enhances influx of effective NK and NKT cells in murine breast cancer

O,O'-dietil-(S,S)-etilendiamin-N,N'-di-2-(3-cikloheksil)propanoat dihidrohlorid povećava influks efektivnih NK i NKT ćelija u karcinomu dojke miša

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Abstract

Background/Aim. *O,O′*-diethyl-(*S,S*)-ethylenediamine-*N,N′*-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) has been found to possess promising cytotoxic activity against various tumor cell lines. Also, DE-EDCP reduces tumor progression by several mechanisms such as triggering tumor cell death and inhibition of cell proliferation. The aim of present study was to further evaluate anti-tumor activity of DE-EDCP by investigating effects on migratory potential of tumor cells and anti-tumor immune response. **Methods.** Migratory potential of DE-EDCP was evaluated by scratch wound assay. Female BALB/c mice were inoculated with 4T1 breast cancer cells and treatment with DE-EDCP started five days following orthotopic tumor implantation. The frequency and phenotype of tumor-infiltrating natural killer (NK) and natural killer T (NKT) cells were analyzed by flow cytometry. **Results.** DE-EDCP inhibited migratory potential of highly metastatic 4T1 cells.

DE-EDCP facilitated accumulation of CD3^{+}CD49^{+} NKT cells and CD3^{-}CD49^{+} NK cells in tumor microenvironment. DE-EDCP treatment led to significant decrement of tumor infiltrating anergic NKT cells expressing cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), killer cell lectin like receptor G1 (KLRG-1) and programmed cell death protein-1 (PD-1). Mice given DE-EDCP had significantly increased percentages of tumoricidal fas ligand (FasL) positive NK cells. **Conclusion.** DE-EDCP inhibits murine breast cancer progression through direct effects on tumor cells and by facilitating anti-tumor immunity. DE-EDCP enhances accumulation, promotes tumoricidal phenotype and maintenances responsiveness of NK and NKT cells in 4T1 murine breast cancer.

Key words:
breast neoplasms; carcinoma; mice, inbred balbc; antineoplastic agents.

Apstrakt

Uvod/Cilj. *O,O′*-dietil-(*S,S*)-etilendiamin-*N,N′*-di-2-(3-ciklo-heksil)propanoat dihidrohlorid (DE-EDCP) poseduje značajnu citotoksičku aktivnost na linije različitih tumorskih ćelija. DE-EDCP redukuje rast i metastaziranje karcinoma dojke tako što indukuje ćelijsku smrt i inhibira progresiju će-

lijskog ciklusa. U cilju dalje analize antitumorske aktivnosti DE-EDCP ispitan je njegov uticaj na migratorni potencijal, kao i na antitumorski imunski odgovor. **Metode.** Uticaj DE-EDCP na mobilnost malignih ćelija ispitan je testom migracije (*scratch wound assay*). Model karcinoma dojke je indukovan ortotopskom transplantacijom malignih ćelija 4T1 u singene ženke BALB/C miševa. Nakon pojave palpabil-

nog tumora, životinje su tretirane sa DE-EDCP. Protočnom citometrijom određivana je procentualna zastupljenost *natural killer* (NK) i *natural killer* T (NKT) ćelija, kao i njihov funkcionalni fenotip. **Rezultati.** DE-EDCP je inhibirao migratorni potencijal 4T1 malignih ćelija sa izraženom metastatskom sposobnošću. DE-EDCP je povećao influks CD3+CD49+ NKT i CD3-CD49+ NK ćelija u tumorsku mikrosredinu tretiranih ženki miševa u poređenju sa farmakološki netretiranim životinjama. DE-EDCP je smanjio procent anergičnih NKT ćelija koje eksprimiraju inhibicione receptore: *cytotoxic T-lymphocyte-associated protein 4* (CTLA-4), *killer cell lectin like receptor G1* (KLRG-1) i *programmed cell death protein-1* (PD-1). Kod miševa tretiranih sa DE-EDCP uoče-

na je povećana zastupljenost tumoricidnih NK ćelija koje eksprimiraju *fas ligand* (FasL). **Zaključak.** DE-EDCP inhibira progresiju mišjeg karcinom dojke što je posledica kako direktnih efekata ispitivane supstance na tumorske ćelije, tako i pojačanog antitumorskog imunskog odgovora. Drugim rečima, DE-EDCP, osim što povećava influks, podstiče tumoricidni fenotip i zadržava responzivnost NK i NKT ćelija u mišjem karcinomu dojke.

Ključne reči:
dojka, neoplazme; karcinom; miševi, visoko srodjeni balbc; antineoplastici.

Introduction

Ester derivatives of (*S,S*)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic acid were synthesized as ligands for ethylenediamine-based platinum complexes [1]. These platinum(IV) complexes exhibit higher tumoricidal activity toward several cancer cell lines compared to cisplatin as a conventional chemotherapeutic drug. The cytotoxicity of platinum(IV) complexes could be at least partly related to their organic ligands with lack of relationship between the cytotoxic capacity and the alkyl side-chain length of these ligands. In line with this observation, it was further demonstrated that ligand *O,O'*-diethyl-(*S,S*)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl) propanoate dihydrochloride (DE-EDCP) alone exerted similar or even higher cytotoxic activity compared to cisplatin against various human and mouse cell lines [1,2]. Taken together, it seems that DE-EDCP exerted highly potent cytotoxic activity against murine melanoma cells [1] and human promyelocytic leukemia cells [2]. Next, cytotoxicity of DE-EDCP was demonstrated on various leukemic cell lines [3]. According to obtained IC_{50} values, human promyelocytic leukemia cells were highly sensitive to DE-EDCP. The cytotoxic effect of DE-EDCP against human leukemic-60 (HL-60) cells was accompanied with increased production of superoxide and depolarization of mitochondrial membrane [3]. Results of this study indicated that DE-EDCP treatment caused caspase-independent apoptosis of HL-60 cells by presentation of phosphatidylserine on cell membrane and fragmentation of DNA [3].

Recently, we demonstrated that DE-EDCP attenuated murine breast cancer progression by facilitating apoptosis and inhibiting proliferation of tumor cells [4]. DE-EDCP in cell line of murine breast cancer (4T1) tumor cells reduced expression of anti-apoptotic Bcl-2 while increased expression of pro-apoptotic Bax and caspase-3. Also, DE-EDCP treatment blocks cell cycle progression in 4T1 tumor cells by increasing expression of cyclin-dependent kinase inhibitors p16, p21 and p27 with subsequent decrease in the expression of cyclin D3 and Ki-67, and arresting 4T1 cells in G0/G1 phase of cell cycle [4]. Further, DE-EDCP reduced the malignant potential of tumor cells by reducing expression of signal transducer and activator of transcription 3 (STAT3) and downstream regulated molecules, NANOG and SOX2 in 4T1

cells. Recent study also reported that DE-EDCP reduces melanoma growth mainly by inducing expression of key proapoptotic genes [5]. Melanoma cells treated with DE-EDCP underwent caspase-dependent apoptosis as a result of mitochondrial dysfunction and increased accumulation of reactive oxygen species. In both murine breast cancer and melanoma models, DE-EDCP was well tolerated *in vivo* without obvious side effects [4,5].

Activity of natural killer (NK) cells, as well as natural killer T (NKT) cells, represents the major mechanism of innate immunity against tumors [6–9]. NK cells lyse tumor cells without prior sensitization and represent the first line of defense against established tumors [6–8]. NKT cells, by production of various immunoregulatory cytokines, link innate and adaptive immune response [9]. It has been reported that NKT cell reduce tumor progression, mostly by enhancing cytotoxicity and interferon-gamma (IFN-γ) production of NK cells and CD8+T cells [10].

The aim of this study was to further evaluate anti-tumor activity of DE-EDCP in 4T1 murine breast cancer model. We investigated the effects of DE-EDCP on migratory potential of tumor cells as well as modulation of anti-tumor immune response.

Methods

Cell culture and reagents

The cell line of murine breast cancer was purchased from American Type Culture Collection (ATCC, USA). 4T1 cells were grown in suspension in complete Dulbeccos Modified Eagles Medium (DMEM) in a 5% CO_2 incubator with standard conditions [11]. Tumor cell suspension with > 90% viability was prepared using 0.25% trypsin and 0.02% Ethylendiaminetetraacitic acid (EDTA) in phosphate buffered saline (PBS, PAA Laboratories GmbH). In all *in vitro* and *in vivo* experiments only cell suspensions with > 95% viable cells were used. In order to determinate the viability of tumor cells trypan blue was used.

The organic compound DE-EDCP, was prepared according previously described procedure [1,2]. As a referent cytostatic, cisplatin (CDDP, *cis*-diamminedichloroplatinum(II)/*cis*-[PtCl$_2$(NH$_3$)$_2$]) (Sigma-Aldrich) was used.

Scratch wound assay

The wound healing assay was performed as previously reported [12]. After 4T1 cells were seeded into 6-well plates, they were allowed to growth to about 90% confluence in present of complete medium. After 4T1 achieved appropriate confluence, a plastic tip was used to make a scratch on the cell monolayer [13]. The wound area was washed three times with PBS and the 4T1 cells were incubated with DE-EDCP (15.63 µM) or cisplatin (CDDP) (15.63 µM) for 4 and 15 hours. The 4T1 cells migrated into the wound surface and the average distance of the migrating cells was observed using inverted microscopy. All data were analyzed from three independent experiments performed in triplicate using ImageJ software and the results are presented as the mean ± standard deviation (SD) [14].

Animals

Female (8–12 weeks old) BALB/c mice were used in *in vivo* experiment. Experimental animals were equalized in weight and randomized in the experimental or control groups. The mice were housed in a temperature-controlled environment with a 12-hour light-dark cycle, fed *ad libitum* and observed daily. All experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Animal model and drug treatment

BALB/c mice were inoculated with 3×10^4 4T1 tumor cells orthotopically into the fourth mammary fat pad [11, 15]. Pharmacology treatments started when tumors were palpable five days after implantation of 4T1 cells. Tumor bearing mice received intraperitoneal injection of either DE-EDCP (10 mg/kg/dose - five consecutive doses + two days pause + five consecutive doses; ten doses in total); CDDP (3 mg/kg/dose; three times per week; nine doses in total) or 0.9% NaCl. Mice were sacrificed on 18th day of the experiment.

Flow cytometric analyses of tumor-infiltrating NK and NKT cells

After three experimental groups of mice were sacrificed on 18th day of the experiment, primary tumor was isolated from mice and single cell suspensions of primary tumors were obtained by enzymatic digestion, as previously described [16]. Fluorochrome-conjugated monoclonal antibodies specific for CD3 (145-2C11), CD49b (HMa2), CD178/FasL (MFL3), CD152/CTLA-4 (BNI3), PD-1 (PDCD1/922), KLRG-1 (2F1) or their respective isotype controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA) were used. Expression of cell surface antigens was analyzed with Flow Cytometer (BD Biosciences, San Jose, CA) and the data were analyzed using FlowJo (Tree Star). Data are presented as means ± SD of two individual experiments, each carried out with six mice per experimental group.

Statistical Analysis

The data were analyzed using software package IBM SPSS Statistics version 20. First the normality of data distribution was tested by Kolmogorov-Smirnov or Shapiro-Wilk test. The two-tailed Student's *t*-test or Kruskal-Wallis test followed by Mann-Whitney *U* test were used. All data in this study were expressed as the mean ± standard deviation. Values of $p < 0.05$ were considered as statistically significant.

Results

DE-EDCP reduces migration of 4T1 cells

It is well known that cell migration is the first step in the invasive-metastatic cascade [17]. We recently reported that DE-EDCP reduces murine breast cancer growth and metastasis [4]. Herein, we add the effect of DE-EDCP treatment on 4T1 cell migration examined by wound healing assay using non-lethal concentration (15.63 µM) for 4 and 15 hours. Migration assay revealed that scratch wound area in wells with untreated 4T1 cells had a significant diminution (approximately 65%), while wound area of 4T1 cells treated with DE-EDCP was significantly wider in comparison to control cells 4 hours following treatment ($p = 0.003$; Figure 1). The same phenomenon was observed 15 hours after scratch ($p = 0.019$; Figure 1). In addition, significant effect of CDDP on the reduction of 4T1 cell migration was achieved after 15 hours (Figure 1).

DE-EDCP administration facilitates accumulation of NKT and NK cells within tumor microenvironment

We further analyzed the effect of DE-EDCP on local antitumor immune response. The obtained data revealed that DE-EDCP significantly increased the percentages of $CD3^+CD49^+$ NKT cells in tumor tissue when compared to vehicle-treated mice ($p = 0.03$; Figure 2, left panel). Of note, the frequencies of NKT cells were increased in mice treated with CDDP, but it did not reach statistical significance (Figure 2, left panel). DE-EDCP also increased the percentages of tumor-infiltrating $CD3^-CD49^+$ NK cells ($p = 0.032$; Figure 2, right panel). CDDP did not significantly affect accumulation of NK cells in tumor tissue (Figure 2, middle panel). We did not reveal effect of DE-EDCP administration, or CDDP, on intratumoral accumulation of $CD3^+CD49^-$ T cells (Figure 2, right panel).

DE-EDCP affects functional phenotype of tumor-infiltrating NKT and NK cells

Further, we analyzed functional phenotype of tumor-infiltrating NKT and NK cells. Apart from CDDP, DE-EDCP did not affect the presence of tumoricidal NKT cells expressing FasL (Figure 3). However, DE-EDCP significantly decreased the presence of NKT cells expressing inhibitory markers such as CTLA-4, KLRG-1 and PD-1 in comparison with vehicle and CDDP treated mice (Figure 3).

In contrast to DE-EDCP, CDDP significantly increased the percentage of PD-1 positive NKT cells when compared to vehicle-treated mice (Figure 3).

Furthermore, mice treated with DE-EDCP, but not CDDP, exhibited significantly increased percentages of tu-

moricidal FasL$^+$ NK cells compared to vehicle-treated mice (Figure 4). There were no significant differences in the expression of inhibitory KLRG-1, CTLA-4 and PD-1 among NK cells from both DE-EDCP and CDDP treated mice (Figure 4).
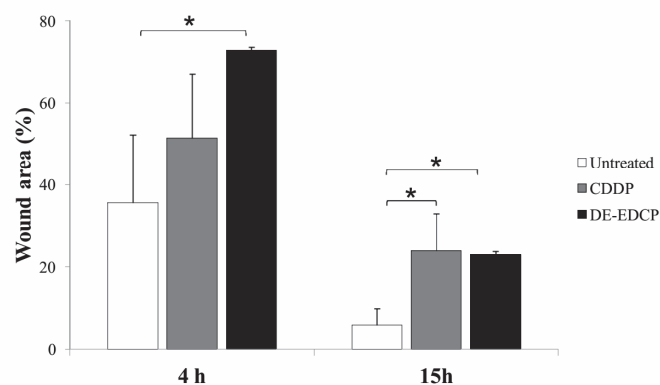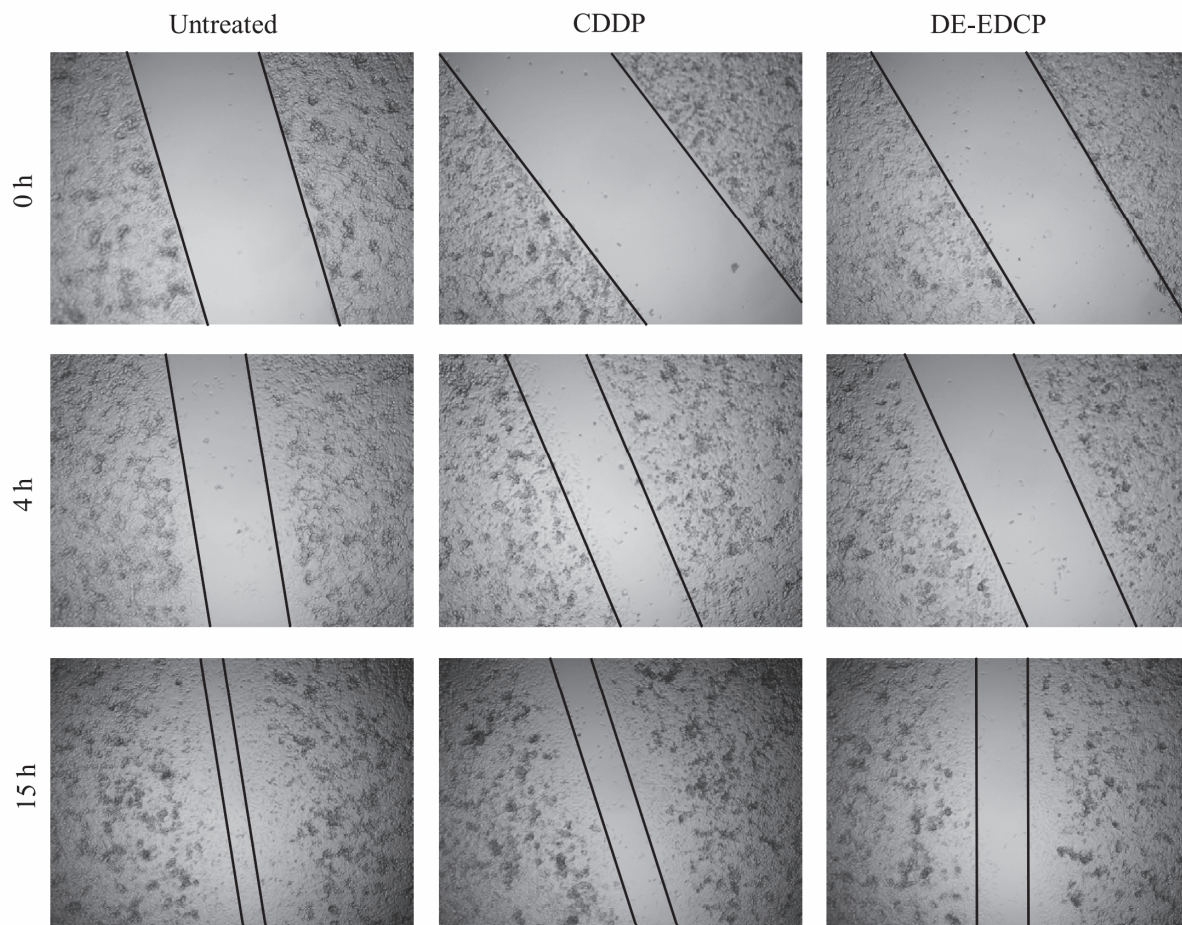
Fig. 1 – Inhibitory effect of O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) on murine breast cancer cell migration.

The scratch wound assay of 4T1 cells treated with DE-EDCP (15.63 µM) or cisplatin (CDDP) (15.63 µM) for 4 hours and 15 hours. Representative images of wound closure in the control, CDDP and DE-EDCP treated cell line of murine breast cancer (4T1) cells. The images were captured three times at different areas and the results were analyzed by ImageJ software. Cell migration was quantified measuring the mean cell-free gap distance between the edges of the scratch area. Data are presented as mean of wound area ± standard deviation (SD). Mann-Whitney U test was performed and significant differences are reported (*$p < 0.05$).
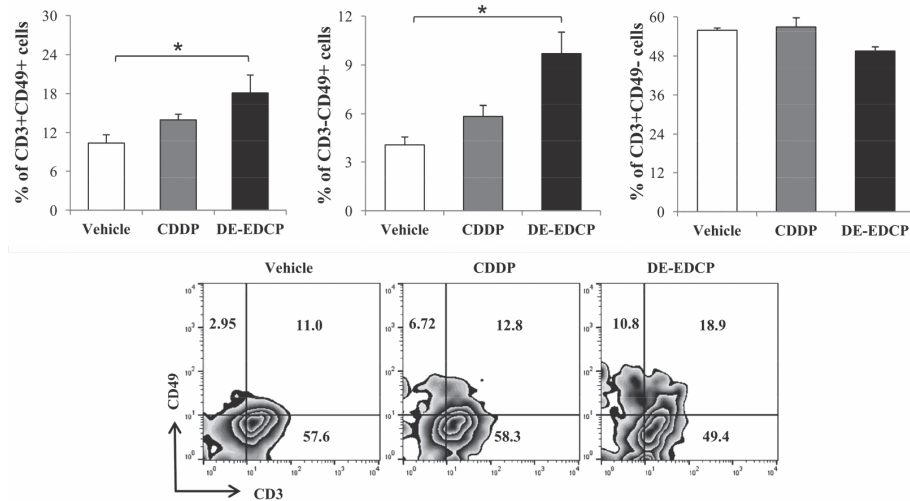
Fig. 2 – O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) increases influx of natural killer T (NKT) and natural killer (NK) cells in tumor microenvironment.
The graphs and representative sorting cells based on flow cytometry data (FACS) plots showing the percentages of NKT, NK and T cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as mean ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney U test, (*$p < 0.05$).
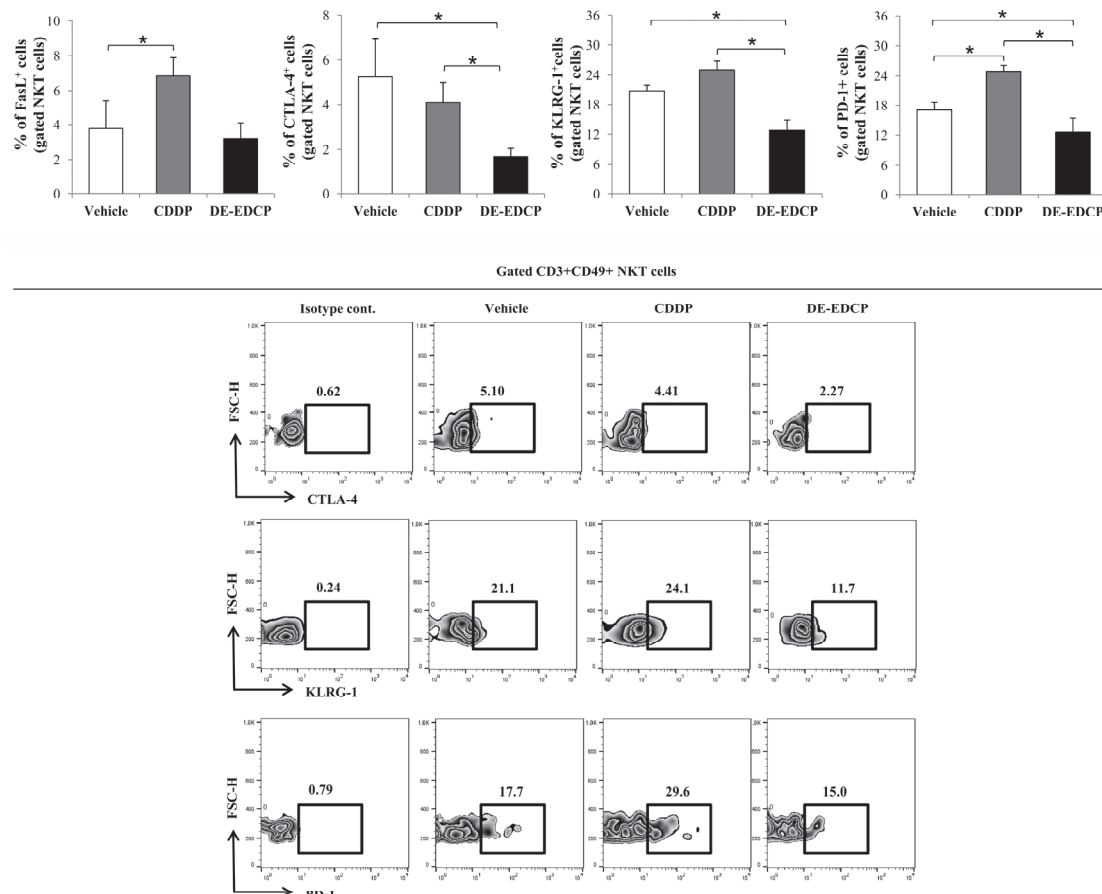
Fig. 3 – O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) affects the functional phenotype of natural killer T (NKT) cells in tumor tissue.
The graphs and representative sorting cells based on flow cytometry data (FACS) plots show the percentage of fas ligand (FasL[+]), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4^{+}), killer cell lectin like receptor G1 (KLRG-1^{+}) and programmed cell death protein-1 (PD-1^{+}) NKT cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as means ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney U test (*$p < 0.05$).

Gated CD3-CD49+ NK cells
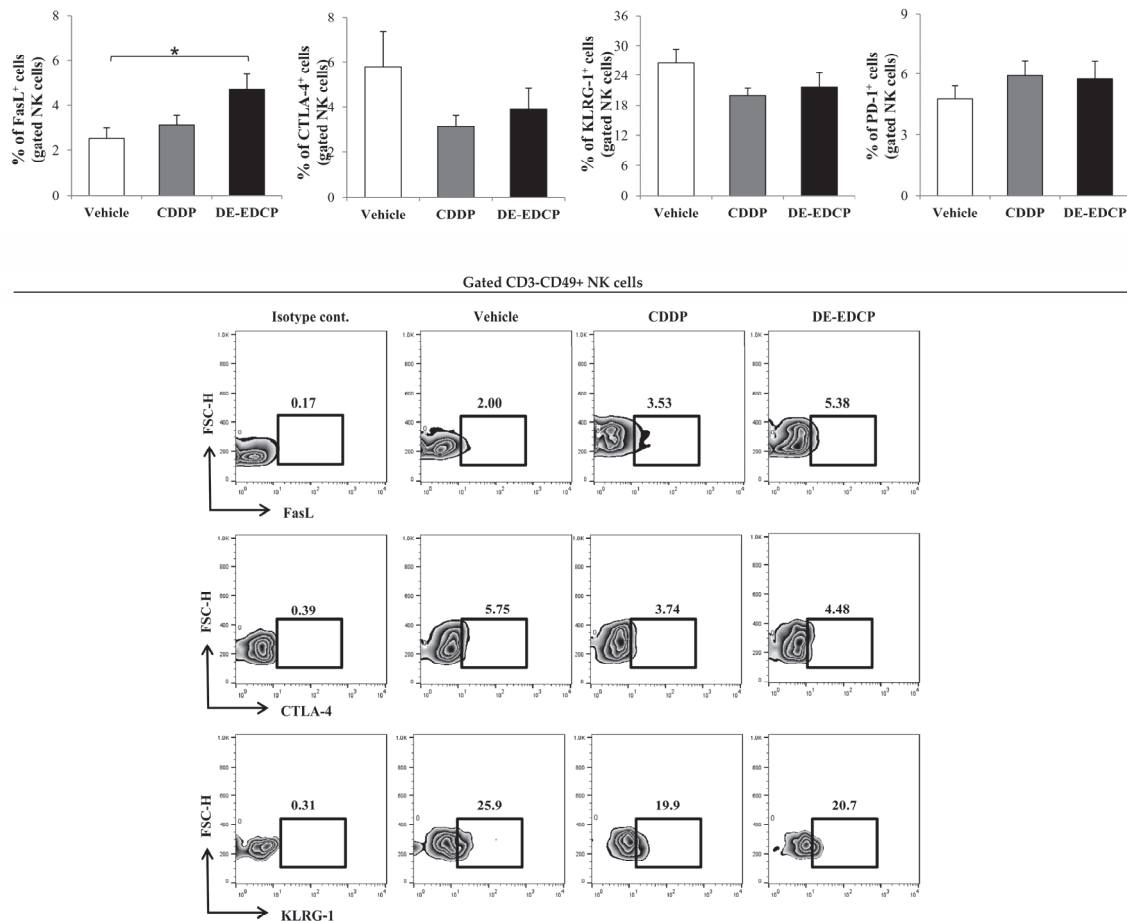
Fig. 4 – O,O'-diethyl-(S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate dihydrochloride (DE-EDCP) affects the phenotype of natural killer (NK) cells in tumor tissue.

The graphs and representative flow cytometry data (FACS) plots show the percentage of fas ligand (FasL[+]), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4^{+}), killer cell lectin like receptor G1 (KLRG-1^{+}) and programmed cell death protein-1 (PD-1^{+}) NK cells derived from tumor tissue of vehicle-treated, cisplatin (CDDP)-treated and DE-EDCP-treated mice 18 days after cell line of murine breast cancer (4T1) cell inoculation. Data are presented as means ± standard deviation (SD) of two individual experiments, each carried out with six mice per group. Statistical significance was tested by Mann-Whitney *U* test (*$p < 0.05$).

Discussion

Cell migration is a prerequisite for tumor invasion and metastasis [17], and can be a potential therapeutic target for tumor treatment. For this purpose we used 4T1 cells, known as cells with high metastatic potential [18]. Herein, our results indicated that DE-EDCP effectively inhibits the migration of 4T1 cells as evaluated by wound healing assay (Figure 1). We previously reported that DE-EDCP decreased expression of signal transducer and activator of transcription 3 (STAT3) in 4T1 cells, as well as NANOG and SOX2 which are downstream targets of STAT3 signaling pathway [4]. STAT3 has impact on cell invasion and motility [19]. For instance, knockdown of STAT3 compromised the proliferation and migration of Michigan Cancer Foundation-7 (MCF7) human breast cancer cells [20]. In addition, overexpression of NANOG promoted the migration and invasion of MCF7 cells [21]. Similarly, NANOG regulated cell migration in ovarian cancer [22]. Furthermore, SOX2 silencing has also been found to prevent

migration of MDA-MB-231 human breast cancer cells [23]. In agreement with these findings, it appears that DE-EDCP inhibits tumor cell migration via downregulation of STAT3, NANOG and SOX2 expression. Therefore, inhibition of 4T1 cell migration seems to be the additional beneficial effect of DE-EDCP on breast cancer progression.

In addition to obvious direct effects on tumor cells, we further hypothesized that DE-EDCP might influence tumor progression by modulating anti-tumor immune response. To the date, it was found that DE-EDCP inhibited production of IFN-γ and IL-17 by cells derived from spleen and lymph nodes of mice and rats [24]. However, the effects of DE-EDCP on anti-tumor immune response are still unknown. In this study, we explored the effects of DE-EDCP on anti-tumor innate immunity in the weakly immunogenic and highly metastatic 4T1 murine mammary cancer model. We showed that DE-EDCP facilitated influx of CD3^{+}CD49^{+} NKT cells and CD3^{-}CD49^{+} NK cells in tumor microenvironment (Figure 2). CDDP treatment slightly increased influx of these

cells, however the increment did not achieve statistical significance so we can only assume that CDDP antitumor effects in particular tumor model were achieved by some other mechanisms. NK cells are innate immune effector lymphocytes that play an important role in the protection against tumor. NK cells infiltrate solid tumors thus contributing to favorable prognosis in cancer patients [25]. Apart from NK cells, activated NKT cells are involved in elimination of tumor cells either directly or indirectly by engagement of other immune cells [26–27]. Furthermore, recently an association between numbers of tumor-infiltrating NKT cells with better clinical outcome was found [28]. NKT cells react quickly to stimuli and have a remarkable capacity to produce an array of cytokines and chemokines in order to modulate both innate and adaptive immune response [29].

In addition to increased influx of NKT and NK cells in breast cancer tissue, the obtained data revealed that DE-EDCP affects functional phenotype of these cells. It is well-established that both cell types, in particular NK cells, directly eliminate target tumor cells by at least two mechanisms, producing perforins and granzymes as well as the engagement of cell death receptors. Cell death receptor Fas and its ligand FasL are important players in initiation of target cell apoptosis [30]. Fas-FasL interaction induces receptors trimerization, activation of adaptor protein fas-associated protein with death domain (FADD) which results with activation of caspase-8 and consequent initiation of apoptosis [31]. Our results revealed that DE-EDCP treated mice had significantly increased percentages of FasL^{+} NK cells, but not NKT cells, indicating their enhanced tumoricidal potential (Figures 3 and 4). These data are in line with our previously described results revealing that DE-EDCP treatment increased percentage of apoptotic (TUNEL^{+}) tumor cells in breast cancer tissue [4]. NKT cells directly eliminate CD1d-expressing tumor cells [32]. 4T1 cells express minimal surface levels of CD1d [33]. Therefore, there is low possibility that NKT cells directly eliminate 4T1 cells. However, NKT cells could produce IL-2 further stimulating NK cells to kill the NKT cell-resistant tumor cell targets [34]. Teng et al. [33] showed that CD8^{+} T cells and IFN-γ are crucial for 4T1 tumor eradication. However, other studies revealed that antitumor activity based on cytotoxicity of CD8^{+} T cells plays a nonessential role in 4T1 breast tumor model [11, 35]. Innate immunity cells, especially NK cells, occupy a central place in the control of growth and metastasis of weakly immunogenic mouse 4T1 breast tumor [11, 35]. The influence of DE-EDCP on the functional status of NK cells in the tumor microenvironment indicates that the DE-EDCP effects on the innate immune response may be an additional anti-tumor mechanism of action. At this point, we may suppose that higher percentage of tumor- infiltrating NK cells and higher expression of FasL on NK cells after DE-EDCP treatment may lead to Fas-FasL interaction of tumor and NK cells and, consequently, cause tumor cell death.

Also, we can only assume that DE-EDCP might stimulate NKT cells in tumor microenvironment to produce various cytokines thus enhancing tumoricidal capacities of NK cells. This can be an additional mechanism of DE-EDCP-mediated diminishing of tumor progression.

Killer cell lectin-like receptor G1 (KLRG-1) is C-type lectin-like inhibitory receptor expressed mostly on NK cells, cytotoxic T cells and long-lived effector NKT cells [36, 37]. KLRG-1 regulates homeostasis and maturation of NK cells [38]. High KLRG-1 expression correlates with low proliferative capacity [38, 39] and increases apoptosis of NK cells [40]. DE-EDCP significantly decreased percentage of NKT cells, but not NK cells, expressing inhibitory receptors KLRG-1 (Figures 3 and 4). The programmed cell death-1 receptor (PD-1) is immune checkpoint inhibitor expressed on the surface of immune effector cells, including T cells, NK and NKT cells [41–43]. Marked increase in PD-1 expression after α-GalCer stimulation indicated NKT cell anergy [44, 45]. Herein, we observed significantly decreased percentage of PD-1^{+} NKT cells following DE-EDCP treatment (Figure 3) thus contributing to NKT cell responsiveness. Next, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) is another immune checkpoint molecule with crucial role in decline of immune response and maintaining immune homeostasis [46, 47]. CTLA-4 is expressed on tumor-infiltrating NK cells in mice [48]. The obtained data revealed that DE-EDCP also reduced the frequencies of CTLA-4^{+} NKT cells (Figure 3). Both PD-1 and CTLA-4 blockade, as well as their combination, have proven to be very effective in animal models of melanoma and some breast cancer models [49–52].

Conclusion

In addition to our previously published data regarding the beneficial effects of DE-EDCP on 4T1 breast cancer progression, here we add the evidences that DE-EDCP inhibits 4T1 cell migration and promotes anti-tumor immune response mediated by NK and NKT cells. DE-EDCP enhances accumulation, promotes tumoricidal phenotype and maintenances responsiveness of NK and NKT cells in 4T1 murine breast cancer model.

Acknowledgments

Declaration of interest

The authors declare that they have no competing interests.

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Antioxidant status and clinicopathologic parameters in patients with Parkinson's disease

Antioksidativni status i kliničko-patološki parametri kod obolelih od Parkinsonove bolesti

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Abstract

Backgroun/Aim. Constant production of free radicals and antioxidants (AO) in cells is a part of normal cellular function. Their imbalance might take a part in pathophysiology of many diseases, including Parkinson's disease (PD). Evaluation of the disease status, prooxidant-antioxidant balance (PAB) and antioxidants are being widely estimated. The aim of this study was to examine potential interaction between several AO variables: glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and PAB, and clinicopathologic features of patients with PD, particularly the Hoehn and Yahr (H&Y) stage. **Methods.** A multivariate analysis of variance (MANOVA) was conducted to analyze mean differences between clinicopathologic characteristics (gender, age at examination, duration of the disease, and the H&Y stage) and AO variables of PD patients and those of age/sex matched healthy controls. The study included 91 patients with idiopatic PD patients and 20 healthy persons. **Results.** The multivariate effect size was estimated at 0.269 ($p < 0.001$), implying that 27.0% of the variance of the dependent variables was accounted for the H&Y stage. Univariate tests showed that there were significant differences ($p < 0.001$) across the H&Y stage of all AO variables. The H&Y stage remained significant predictor after controlling for the second variable, the disease duration ($p < 0.001$, $\eta^2 = 0.249$), and there were still significant differences across the H&Y stage of all variables, with effect size (η^2) ranging from 0.132 ($p = 0.011$) (lnGSH) to the still high values of 0.535 (lnPAB), 0.627 (lnSOD) and 0.964 (lnCAT). **Conclusion.** The results indicate that higher level of oxidative stress in blood of PD patients is possibly related to the PD stage. Along with reduction of SOD and GSH levels, CAT activity was elevated in comparison to these values in healthy subjects. Furthermore, PAB was shifted toward oxidative stress.

Key words:
parkinson disease; disease progression; free radicals; antioxidants; demography.

Apstrakt

Uvod/Cilj. Ćelijska homeostaza zasniva se na konstantnoj produkciji slobodnih radikala i antioksidanasa (AO). Svako narušavanje njihove ravnoteže može dovesti ili učestvovati u patofiziološkim promenama mnogih bolesti, uključujući i Parkinsonovu bolest (PB). Kako bi se pratio status bolesti, koristi se veliki broj parametara, uključujući i prooksidativni-antioksidativni balans (PAB) i AO, koji ujedno predstavljaju i fokus ispitivanja ove studije. Stoga, cilj ove studije je bilo ispitivanje potencijalne interakcije između AO varijabli: glutation (GSH), superoksid dismutaza (SOD), katalaza (CAT) i PAB i kliničko-patoloških osobina PB bolesnika, najviše Hoehn i Yahr (H&Y) stepena bolesti. **Metode.** Multivarijantna analiza varijanse (MANOVA) korišćena je za analizu međusobnih razlika između kliničko-patoloških karakteristika (pola, starosti, dužine trajanja bolesti i H&Y stepena bolesti) i AO varijabli bolesnika sa PD sa onima od zdravih osoba. Studija je uključila ukupno 111 ispitanika, 91 bolesnika kojima je dijagnostifikovana idiopatska PB i 20 zdravih osoba. **Rezultati.** Multivarijantni efekat je bio procenjen na 0,269 ($p < 0,000$), što implicira da se 27,0% varijanse za-

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visne varijable odnosi na H&Y stepen bolesti. Univarijantni test je pokazao da postoji statistički značajna razlika ($p < 0,001$) kroz H&Y stepen bolesti svih AO varijabli. H&Y stepen bolesti ostao je značajan prediktor i nakon uvođenja druge varijable, dužine trajanja bolesti ($p < 0,001$; $\eta^2 = 0,249$). Pokazano je da je ostala značajna razlika kroz H&Y stepen bolesti za sve varijable, tako da se jačina odnosa dve varijable kretala od 0,132 (lnGSH) do i dalje visokih vrednosti: 0,535 (lnPAB), 0,627 (lnSOD) i 0,964 (lnCAT). **Zaključak.** Rezultati pokazuju da je visoki nivo oksidativ-
nog stresa u krvi obolelih od PB verovatno povezan sa stepenom bolesti. Zajedno sa smanjenjem aktivnosti SOD i nivoa GSH, aktivnost CAT se povećava u poređenju sa ovim vrednostima kod zdravih osoba. Pored toga, PAB ukazuje na povećani oksidativni stres kod obolelih od PB.

Ključne reči:
parkinsonova bolest; bolest, progresija; slobodni radikali; antioksidansi; demografija.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease, histologically characterized by progressive loss of dopaminergic neurons in *substantia nigra pars compacta* (*SNpc*) and formation of Lewy bodies [1]. It is manifested by cardinal features such as bradykinesia, rigidity, tremor and postural instability, and good response to levodopa (L-dopa) is often used to support the diagnosis of PD [2]. Although the exact mechanism of PD pathogenesis still remains unclear, studies have indicated that oxidative stress (OS), inflammation, mitochondrial dysfunction and proteasomal inhibition are the major factors that accelerate dopaminergic neurodegeneration [3].

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant (AO) defense capacity. ROS are generally short-lived and highly reactive molecules derived from oxygen [4], varying in their site of formation, physiological function, reactivity and biological half-life. They include free radicals, such as hydroxyl and superoxide radicals, and non-radicals including hydrogen peroxide (H_2O_2) and singlet oxygen [5]. Maintenance of the physiological level of ROS is basically regulated by antioxidant enzymes (AOE) and small antioxidant molecules [6].

Antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPxs), glutathione reductases (GRs) and glutathione-S-transferases (GSTs), while non-enzymatic antioxidants are represented by glutathione (GSH), ascorbic acid (vitamin C), α-tocopherol (vitamin E), flavonoids, etc. [7]. The main function of SOD is catalyzing the breakdown of highly reactive superoxide anion into oxygen and the less reactive H_2O_2, which is further decomposed to water and oxygen by CAT or GPx [8]. Disturbance of AOE activity is strongly implicated in a variety of age–related brain disorders [9].

Glutathione is the major small AO molecule [6], with the concentration of 1–3 mM in the brain cells [10]. It is highly abundant in the cytosol (1–11mM), nucleus (3–15 mM) and mitochondria (5–11 mM) [11]. In some studies, a much lower concentration of 2 μM was found in blood plasma [10]. GSH can reduce superoxide radicals, hydroxyl radicals, and peroxynitrites, reacting alone or with other enzymes, such as GPx or GST [12].

Other than individual molecules, one of the important parameters for oxidative stress evaluation is a prooxidant-antioxidant balance (PAB), which determines a state of dy-
namic balance between free radicals that are produced and those utilized (scavenged) [13].

Similar to other diseases, a disturbed AO balance renders PD patients more vulnerable to OS. Thus, to further evaluate its degree, the present study investigated PAB and AO enzymes (SOD, CAT), as the first line of defense against ROS, and GSH level in the blood of PD patients, compared to healthy subjects. Furthermore, the relation of AO parameters with clinicopathologic features of PD patients such as gender, age, duration of the disease, and the Hoehn and Yahr (H&Y) staging was estimated.

Methods

Participants

The study comprised 91 patients with idiopathic PD, and 20 healthy controls, originated in the Republic of Serbia. All blood samples were collected at the Neurology Clinic, Clinical Center of Serbia in Belgrade. The study was performed in compliance with the ethical principles of the Declaration of Helsinki and all applicable national laws and regulations. The study protocol was approved by the Ethics Committee of the Clinical Centre of Serbia, Belgrade, and written informed consent was obtained from each patient prior to study engagement. All patients had idiopathic PD diagnosed in accordance with UK brain bank criteria [14]. Inclusion criteria were disease duration (up to 25 years), age (30–75 years), the Hoehn and Yahr (H&Y) stage (I–IV), receiving symptomatic PD therapy and a stable dose of L-dopa for longer than 3 months. Patients with current evidence of a recent diagnosis of malignancy, marked autonomic disturbances, a renal insufficiency or failure, hepatitis, serious and/or unstable gastrointestinal, hematologic or other medical disorders, as well as subjects using antipsychotics were excluded from the study. The clinicopathologic features of patients including age, gender, disease duration and the H&Y stage of the disease is given in Table 1.

Blood sampling and biochemical measurements

Venous blood samples were collected from each patient using conventional techniques into Vacutainer (BD Diagnostics, Plymouth, UK) tubes with K_2EDTA as an anticoagulant. For PAB measurement, one batch was centrifuged at 1,500 g, for 10 min, at 4°C, within 30 min of collection. Plasma was carefully separated and stored at -80°C until further processing.

Table 1
Demographic and clinical data of patients with
Parkinson's disease PD

Characteristic	Values
Gender, n (%)	
male	60 (65.9)
female	31 (34.1)
Age at examination (years), mean ± SD	62.7 ± 9.7
< 59	28 (30.8)
59–70	44 (48.3)
> 70	19 (20.9)
Age at disease onset (years), mean ± SD	53.8 ± 9.1
Disease duration (years), mean ± SD	8.8 ± 6.2
< 3	18 (19.8)
3–8	35 (38.5)
> 8	38 (41.8)
H&Y stage, n (%)	
1	9 (9.9)
2	31 (34.1)
3	27 (29.7)
4	24 (26.4)

H&Y – Hoehn and Yahr.

For enzyme activity measurements, the second batch of unfrozen blood was used. All blood samples were diluted with cold dH$_2$0 1:3 (v/v), vortexed and centrifuged for 1 min (10,000 g, 15 min, 4 °C). Supernatants were collected and kept at -80 °C till the assay.

For GSH measurement the blood was prepared as recommended by the kit producer (BIOXYTECH® GSH-420™, OXIS International Inc., Foster City, CA, USA).

Assays

Total SOD activity was measured using Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The reaction between superoxide radicals (O$_2^-$) and tetrazolium salt, generated by xanthine oxidase, results in the development of formazan dye, with max absorbance on 450 nm. SOD inhibits this reaction by dismutation of O$_2^-$ and one unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. Measurements were performed in a microplate reader (Wallac 1420 Victor[2], Perkin Elmer Inc., Waltham, MA, USA).

Total GSH concentration was determined by the BIOXYTECH® GSH-420™ Assay (OXIS International, Inc., Foster City, CA, USA). The measurement of total GSH concentration was performed in three colorimetric reaction steps. Tris (2-carboxyethyl) phosphine (TCEP) as a reducing agent, reduces all oxidized glutathione present in the sample. During the second step, chromogen (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate) reacts with thiols in the sample and forms thioethers. Addition of base (NaOH) raises reaction mixture pH over 13 and chromophoric thione is formed as a result of β–elimination specific to the GSH-thioether. GSH concentration is directly proportional to the absorbance at 420 nm.

Catalase activity measurement was performed according to the method by Beutler[15]. The reaction mixture was prepared from 50 μL of a Tris-HCl buffer (1 M Tris-HCl, 5 mM EDTA, pH 8.0), 900 μL of a substrate (10 mM H$_2$O$_2$), 30 μL of dH$_2$0,

and 20 μL of the sample. Decomposition of H$_2$O$_2$ was monitored spectrophotometrically (UV Line 9400, SI Analytics GmbH, Mainz, Germany) at 230 nm, 3 min at 37 °C. One unit of CAT activity is defined as the amount of the enzyme which degrades 1 μmoL of H$_2$O$_2$ per min under the assay conditions. The extinction coefficient for H$_2$O$_2$ is 0.071 mM^{-1}cm^{-1}.

Prooxidant-antioxidant balance

Evaluation of PAB was performed as described previously[16]. Following the incubation for 2 min at room temperature in dark, 200 μL of working solution (1 mL TMB cation solution with 10 mL TMB solution) was added to a 96-well microtiter plate and mixed with 10 μL of plasma sample, standard or blank (dH$_2$O). The mixture was incubated in a dark place for 12 min, at 37 °C and the reaction was stopped by adding 100 μL of 2 N HCl. The values of PAB in plasma samples were determined at 450 nm, with a reference wavelength of 620 or 570 nm, by comparing optical density (OD) of a sample to the standard curve. PAB values are expressed in arbitrary units (HK).

Statistical analysis

The statistical analyses were performed by the GraphPad Prism and SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SD. General linear model (GLM) was used to test the differences between AO and clinicopathological variables, followed by Dunnett and Scheffe *post hoc* tests. Since examined variables had not passed the normality of the distribution (Shapiro-Wilks test), data were previously log-transformed. Pearson's correlation analysis was performed to test the correlation between AO/clinicopathological variables. The *p*-value < 0.05 was considered statistically significant.

Results

The average age of healthy controls was 57.5 ± 8.5 years, and for PD patients it was 62.7 ± 9.7 years, with a predominance of males (65.9%). The H&Y stage 1 was the least present (in 9.9% of the patients) (Table 1). The activity of AO enzymes (SOD, CAT), the GSH level and PAB are shown in Figure 1.

A multivariate analysis of variance (MANOVA) was conducted to test mean differences between the H&Y stage and AO variables. Prior to conducting the analysis, the Pearson's correlation was performed between the dependent variables in order to test the correlation assumption (Table 2) and significant pattern of correlations was observed amongst all of the dependent variables. Since the Box's M value of 110.06 (*p* < 0.001) indicated significant difference between the covariance matrices, the Pillais' Trace test was used. The MANOVA effect (Pillais' Trace = 1.07, F = 9.103; *p* < 0.001) showed significant differences among the H&Y stage groups on the linear combination of the dependent variables. The multivariate effect size was estimated at 0.269, implying that 27.0% of the variance of the examined AO parameters was accounted for the H&Y stage.
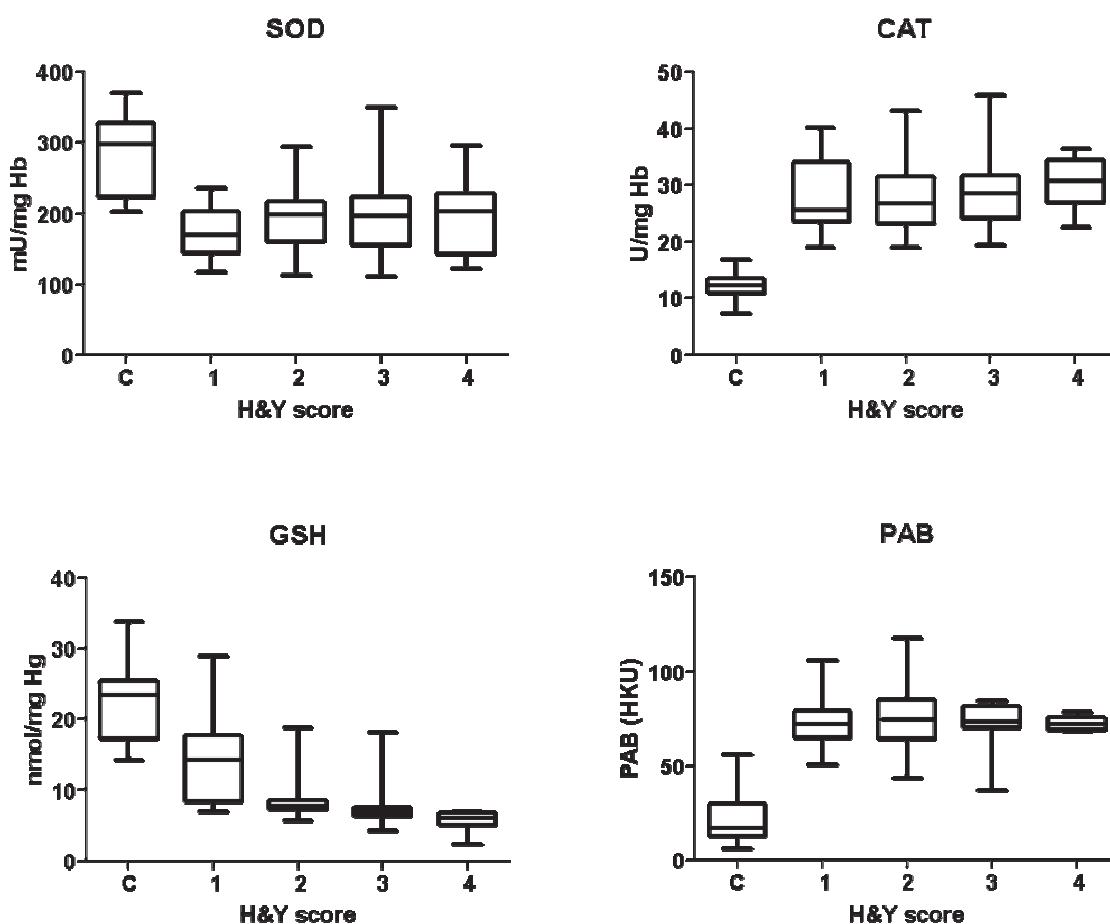
Fig. 1 – Superoxide dismutase (SOD) and catalase (CAT) activity, glutathione concentration (GSH), and prooxidant-antioxidant balance (PAB) in the blood of healthy controls (C) and patients with Parkinson disease of different Hoehn and Yahr (H&Y) scores. Boxes represent values between 25th and 75th percentiles. Medians are given inside the boxes; Whiskers extend between min. and max. values.

Table 2
Pearson's correlation between antioxidant (AO) parameters

Parameters	Pearson's correlation coefficient			
	lnGSH	lnSOD	lnCAT	lnPAB
lnGSH	1	-0.498	0.581	0.595
p (2-tailed)		0.000	0.000	0.000
n	111	111	111	111
lnSOD		1	-0.922	-0.793
p (2-tailed)			0.000	0.000
n		111	111	111
lnCAT			1	0.864
p (2-tailed)				0.000
n			111	111

GSH – glutathione; SOD – superoxide dismutase;
CAT – catalase; PAB – prooxidant/antioxidant.

The homogeneity of variance assumption was tested for the AO variables and two (lnGSH and lnPAB) of the four Levene's F tests were statistically significant ($p < 0.05$). Prior to conducting a series of follow-up ANOVAs, the Bon-ferroni procedure was used to protect against Type I error, adjusting the alpha level to $p < 0.001$. Univariate tests showed that there were significant differences ($p < 0.001$) across the H&Y stage on all AO variables, with effect size (η^2) ranging from 0.365 (lnGSH) to the extremely high values of 0.744 (lnPAB), 0.861 (lnSOD) and 0.988 (lnCAT).

Finally, the series of post-hoc analyses (Dunnett and Scheffe test) were performed to examine individual mean difference comparisons across all H&Y stages and all four AO variables. The results revealed that high effect size observed by univariate analysis was the consequence of the mean differences in AO values between H&Y stages and control values (Dunnett test, $p < 0.001$). Scheffe test did not reveal a significant mean difference in AO values among any of H&Y stages.

In the next step, to test whether H&Y stage remained significant after controlling for the next clinical variable, the disease duration was added as a covariate to the model. The MANCOVA analysis of the effect of the H&Y stage on all AO parameters was still significant (Pillais' Trace = 0.998, F = 7.560; $p < 0.000$), η^2 0.249. Univariate

tests showed that there were still significant differences across the H&Y stage of all AO variables with effect size (η^2) ranging from 0.132 ($p = 0.011$) (lnGSH) to the still high values of 0.535 (lnPAB), 0.627 (lnSOD) and 0.964 (lnCAT) (Table 3a).

There was no significant association between AO parameters and gender (Pillais' Trace = 0.033; F = 0.713; $p = 0.585$; $\eta^2 = 0.033$) or age (Pillais' Trace = 0.70; F = 1.558, $p = 0.193$; $\eta^2 = 0.070$).

Table 3
General linear model (GLM) analysis of the associations between: a) H&Y stage and antioxidant AO parameters; b) H&Y stage and AO parameters after controlling for disease duration

GLM analisys		lnGSH	lnSOD	lnCAT	lnPAB
a)	F	3.462	38.268	607.374	26.187
	p	0.011	$p < 0.001$	$p < 0.001$	$p < 0.001$
	eta^2	13.2%	62.7%	96.4%	53.5%
b)	F	0.042	3.523	0.650	0.790
	p	0.837	0.064	0.422	0.377
	eta^2	0.000	3.7%	0.7%	0.9%

H&Y – Hoehn and Yahr; GSH – glutathione, SOD – superoxide dismutase, CAT – catalase, PAB – prooxidant/antioxidant balance, eta – quantified variance component.

Discussion

Oxidative stress has long been implicated in pathophysiological mechanisms underlying various neurodegenerative diseases, including PD. Investigation of different oxidant/AO parameters have yielded inconsistent results and it is still challenging to assess these parameters in peripheral blood of patients with PD. The current study is focused on the association of specific AO variables (GSH, SOD, CAT, and PAB) and clinicopathologic features of patients with PD, particularly H&Y stage.

Among all ROS-scavenging enzymes, SOD is often regarded as the first line of defense and there is sufficient evidence relating superoxide anion to human diseases, such as PD [17]. The results of our study showed decreased SOD activities in PD patients compared to healthy subjects, which is in accordance with the findings of some authors [18–21] while the others [22–25] reported increased SOD activity or no significant change at all [26, 27]. It is known that AO enzymes are regulated through the AO system to cope with acute or mild OS; however, severe or prolonged OS may induce consumption and decrease of enzyme activity. The decrease of SOD observed in our study might involve inactivation of SOD by ROS or some posttranslational modifications [28]. This observation is comparable with the fact that reduced activity of blood SOD is detected in many chronic diseases such as obstructive pulmonary disease [29], renal failure [30], as well as in some neurological disorders [31]. Chronic OS has already been speculated to cause antioxidant consumption and thus a decline in antioxidant levels [32]. Another possible reason for decreased SOD level could be in mutations that not only provoke a decline in its activity but also induce self-aggregation of mutated SOD proteins – an initial cause of neuron malfunction leading to the disease, as already shown in a cell culture model of amyotrophic lateral sclerosis [33]. The confirmation of such assumptions requires more extensive research in the field of molecular events related to this disease.

The term OS describes the condition where free radicals production exceeds a capacity of AO system. Studies indicated different findings of erythrocyte CAT activity in PD patients in which no significant changes [27, 34] or deficit [18, 21] of CAT were recorded in comparison with healthy subjects. PD patients involved in the present study had elevated CAT activity compared to healthy controls, and there were no differences between H&Y stages and the disease duration. Similar results were obtained in the research of Younes-Mhenni et al. [22], who have not observed the correlation between the duration of illness and CAT activity.

Several studies have shown contrasting results. Sudha et al. [27] observed no significant changes of erythrocyte antioxidants in PD patients while Abraham et al. [21] reported decreased AO enzymes activity in PD patients compared to controls. Considering that CAT is crucial in removing H_2O_2 at higher concentrations [35] (GPx is predominant at physiologically low levels of H_2O_2 [36]), elevation of CAT activity in the blood of PD patients confirms the general conclusion of this study that PD patients are exposed to chronic oxidative stress [37].

It is hypothesized that the adjustment of the AO system is based on shifts in AO activities rather than on the formation of new AO resources. Thus, for some aspects of the issue, it may be more useful to study whole groups of radical scavengers rather than focusing on individual molecule species [38]. PAB can be considered as a measure of an imbalance between oxidants (H_2O_2, tert-butylhydroperoxide, chloramine T and HClO) and antioxidants (vitamin C, trolox, GSH, uric acid, bilirubin, albumin, and ceruloplasmin) [39]. In our study, PAB shifted forward the OS indicating that PD patients had an elevated level of OS compared with healthy subjects, regardless of the H&Y stage.

The physiological roles played by the GSH include maintenance of thiol redox potential, clearing metabolic waste, and as a reservoir for amino acids [40]. Since GSH is involved in antioxidant defense and regulation of cellular metabolic functions ranging from gene expression, DNA, and protein synthesis to signal transduction, cell proliferation and apoptosis [41], its depletion might have a wide impact on many physiological and pathological processes. For instance, GSH deficiency has long been implicated in PD degeneration [42]. A recent report even suggests that whole blood GSH may have the utility as a biomarker in PD progression as it was statistically associated with PD status [43]. Accordingly, in our study, a blood concentration of GSH in PD patients was significantly decreased compared to healthy controls, and such tendency was more pronounced through H&Y stages. These findings are important as the changes in the level of GSH have consequences to numerous molecular processes as well

as the progression of the disease. Furthermore, it should be emphasized that the exact cause of GSH reduction has not been fully clarified, however, it is known that the most common ways for reducing GSH involve its consumption by GPx, conjugation reaction with proteins [44] and 4-hydroxynonenal (4-HNE) [45] and translocation of GSH/GSSG across the plasma membrane [46]. In order to compensate for this decrease, the possible ways of therapeutic compensation of GSH are investigated. They include intranasal [47], intravenous, and liposomal [48] GSH augmentation, and some of them showed a promising effect in the treatment of PD disease [49].

Conclusion

Obtained results show that some of the examined AO parameters in blood of PD patients are possibly related to the PD stage. We observed a correlation of H&Y stage with PAB and AO parameters. The reduction of GSH level was associated with higher H&Y stage while PAB, SOD and CAT activity changed regardless of the H&Y score.

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Disclosure statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Translation to Serbian and transcultural adaptation of the oral health-related quality of life [OHQoL-UK(W)] instrument

Prevod na srpski jezik i kulturološka adaptacija instrumenta za merenje kvaliteta života u vezi sa oralnim zdravljem [OHQoL-UK(W)]

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Abstract

Background/Aim. Measuring health-related quality of life is of great help to clinicians when they have to choose optimal therapy for their patients or estimate its effects. The aim of this study was to translate the oral health-related quality of life [OHQoL-UK(W)] questionnaire from English to Serbian, to make necessary cultural adaptations of the translation, and to test its reliability in a sample of adult Serbian patients. **Methods.** After obtaining permission from the authors, translation and cultural adaptation of the OH-QoL-UK(W) was made according to the International Society for Pharmacoeconomics and Outcomes Research (IS-POR) guidelines. Reliability of the Serbian translation was tested on a sample of 250 patients through calculation of Cronbach's alpha, as a measure of internal consistency. **Results.** Serbian translation of the OHQoL-UK(W) had very similar degree of internal consistency (Cronbach's alpha 0.947), and correlated satisfactorily with the visual analogue scale (VAS) score and inversely with the Decay-missing-filled teeth (DMFT) index. Factorial analysis revealed only one factor, as in the original scale. **Conclusions.** Serbian translation of the OHQoL-UK(W) is reliable instrument for measuring oral health-related quality of life in adult dentistry patients.

Key words:
oral health; surveys and questionnaires; serbia; quality of life.

Apstrakt

Uvod/Cilj. Merenje kvaliteta života u vezi sa zdravljem može mnogo da pomogne kliničarima kada biraju terapijsku opciju za svoje bolesnike ili utvrđuju njen efekat. Cilj ove studije bio je da se prevede upitnik za merenje kvaliteta života u vezi sa oralnim zdravljem [OHQoL-UK(W)] sa engleskog na srpski jezik, da načini neophodne izmene zbog kulturoloških razlika, i da testira pouzdanost prevoda na uzorku odraslih bolesnika u Srbiji. **Metode.** Posle dobijene dozvole od autora za prevođenje i kulturološku adaptaciju OHQoL-UK(W) instrumenta, to je učinjeno prema vodiču Međunarodnog društva za farmakoekonomiju i proučavanje ishoda lečenja (ISPOR). Pouzdanost prevoda na srpski jezik ispitana je na uzorku od 250 stomatoloških bolesnika kroz izračunavanje Cronbach-ove alfe, kao mere unutrašnje saglasnosti. **Rezultati.** Prevod OHQoL-UK(W) instrumenta na srpski jezik imao je veoma sličnu vrednost Cronbach-ove alfe (0.947) kao original, a vrednosti prevedenog instrumenta su dobro korelirale sa vrednostima na vizuelnoj analognoj skali (VAS) i inverzno sa vrednostima DMFT (*decayed, missing and filled teeth*) indeksa. Faktorska analiza je otkrila samo jedan faktor, kao što je pokazano i kod originalnog upitnika. **Zaključak.** Prevod OHQoL-UK(W) upitnika na srpski jezik pouzdan je instrument za merenje kvaliteta života povezanog sa oralnim zdravljem kod odraslih stomatoloških bolesnika.

Ključne reči:
oralno zdravlje; ankete i upitnici; srbija; kvalitet života.

Introduction

Measuring health-related quality of life is of great help to clinicians when they have to choose optimal therapy for their patients and estimate its effects [1]. During the last few decades several instruments for measuring oral health-related quality of life were developed in English language, like the General Oral Health Assessment Index (GOHAI) and the Oral Health Impact Profile (OHIP) [2]. One of such instruments is the oral health-related quality of life [OHQoL-UK(W)] questionnaire with 16 items, constructed and validated in adult population of Great Britain [3]. The OHQoL-UK(W) has high internal consistency (Cronbach's alpha 0.94) and each item asks about opinion of patients about "ef-

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fect" (good, bad or none) of oral health on certain aspect of quality of life and "impact" or extent of this effect (none, little, moderate, great or extreme impact on quality of life) [4, 5].

There are a few instruments for measuring oral health-related quality of life available in Serbian language [e.g. translation of the Orthognatic Quality of Life Questionnaire (OQLQ) and of the Oral Impacts on Daily Performance (OIDP)] [6, 7]. While the first instrument had very good psychometric results, the latter showed minimal internal consistency (Cronbach's alpha was only 0.75), and was tested on small sample with 44 patients only. Besides, the OIDP instrument lacks questions about effect of oral health on professional and financial aspects of quality of life, as well as on self-confidence of the patients [8].

Increasing number of instruments for measuring oral health-related quality of life available in Serbian language would help clinicians to estimate this important outcome with more precision and adjust their treatment plans accordingly. The aim of this study was to translate the OHQoL-UK(W) questionnaire from English to Serbian, to make necessary cultural adaptations of the translation, and to test its reliability in a sample of adult Serbian patients.

Methods

The instrument

The oral health-related quality of life ⁻OHQoL-UK(W) questionnaire is a 16-item questionnaire, and each item asks about opinion of patients about "effect" of oral health on certain aspect of quality of life and "impact" or extent of this effect [4, 5]. The items are rated on a scale from 1 to 9 (1 = extreme bad effect, 9 = extreme good effect). There are no items with reversed scoring within the scale, and total score is calculated by simple summation of scores on individual items, ranging from 16 to 144.

OHQoL-UK(W) translation

Translation and cultural adaptation of the OHQoL-UK(W) questionnaire was made according to the International Society for Pharmacoeconomics and Outcomes Research (ISPOR) guidelines [9]. Permission for translation of the OHQoL-UK(W) questionnaire from English into Serbian was granted by the authors of the original scale (Drs. R. Bedi and C. McGrath) [3–5]. The original scale was first translated into Serbian by two independent translators, authors of this article. The final Serbian version was derived from combination of the two independent translations at the meeting of the study investigators. The Serbian version was then translated back into English by Ron Strauss, native English speaker and also fluent speaker of Serbian, citizen of USA and Real Estate Agent, who had not read the original English version of the OHQoL-UK(W).

Back-translation in English was then compared with original English version by the study investigators, and the final Serbian version of the OHQoL-UK(W) was agreed at a new meeting of the investigators. The final OHQoL-UK(W) translation was then tested on 8 local dentistry patients (at the Oral health primary care facility in Kragujevac, Serbia) for clarity and comprehension. A few minor changes (only punctuation) were made after this preliminary administration and the final Serbian version of the OHQoL-UK(W) was prepared for reliability testing. The whole process of translation was also in accordance with recommendations by Streiner and Norman [10].

Patients

Final Serbian version of the OHQoL-UK(W) was tested for reliability on patients of the Oral health primary care facility in Kragujevac, Serbia, on one occasion, between November 1, 2015 and November 1, 2016. The sample was composed of 250 participants (167 females, 83 males; average age 37.3 ± 17.6 years), as it was minimum number to achieve sufficient statistical power, and it was consecutive, i.e. all patients who visited the facility and satisfied inclusion and exclusion criteria were included. The inclusion criteria were: being in a need of a dental intervention (treatment of dental caries), preserved cognitive capacity and sufficient literacy. The exclusion criteria were age below 18 or above 75 years [11] and diagnosis of a major mental disease (major depression, schizophrenia or bipolar disorder).

All of the included participants (250) agreed to fill in the questionnaire. Besides the OHQoL-UK(W) scale, the patients were offered to estimate their oral health on the visual analogue scale (VAS), 10 cm long, with marked millimeters, from 1 to 100. At the same time, values of their decay-missing-filled teeth (DMFT) index was recorded by dentists. The study was approved by the Ethics Committee of the Oral health primary care facility in Kragujevac, Serbia, including the written informed consent forms.

Reliability testing

Reliability of the Serbian translation of the OHQoL-UK(W) was tested by two methods. Firstly, internal consistency was determined through calculation of Cronbach's alpha for the questionnaire as a whole. Secondly, the questionnaire was divided by split-half method to two parts with the same number of questions (8 each), and Cronbach's alpha for each of the parts was calculated. Using the alphas for both parts, number of questions in each part and average correlation between questions in both parts of the original questionnaire, the Spearman-Brown coefficient for the questionnaire as a whole was calculated by the Spearman-Brown "prediction" formula [10].

Factorial analysis

Factorial analysis was used to reveal whether certain phenomenon (in this case quality of life) has only one or more facets (domains). Confirmatory factorial analysis of the Serbian translation of the OHQoL-UK(W) was made by the principal components method [12]. First, suitability of the questionnaire and sample for factorial analysis was tested by Kai-

ser-Meyer-Olkin measure of sampling adequacy and by the Bartlett's test of sphericity. Then, the factors were extracted at first without rotation, with conditions that Eigenvalues had to be greater than 1, and using Scree-plot (the extracted factors were above the "elbow" of the graph). Second, referent axes were rotated orthogonally, by the Varimax method, and another extraction of the factors was made, using the same criteria as for the non-rotated solution. Extracted factors were than compared with the factors of the original OHQoL-UK(W) scale, and named accordingly.

Validity

Criterion validity of the Serbian translation of the OH-QoL-UK(W) was tested by correlation of its total scores with total scores of the same study participants on VAS and with the DMFT index values. The scores and index values were correlated by the Spearman's method, since they did not follow normal distribution. All calculations were made in the Statistical Program for Social Sciences (SPSS), version 18.

Results

Characteristics of the participants are presented in the Table 1.

Reliability

Results of the OHQoL Serbian translation among participants showed high internal consistency, with Cronbach's

alpha being 0.947. When the OHQoL-UK(W) scale was divided by the split-half method to two parts, with the same number of questions, Cronbach's alphas were 0.950 and 0.868, for the both parts, respectively; the value of the Spearman-Brown coefficient for the OHQoL-UK(W) as a whole calculated from the split-half method by the Spearman-Brown "prediction" formula was 0.918. The mean total score (± standard deviation) of the scale was 109.4 ± 25.2. Translated questions to Serbian, mean values and standard deviations of responses for each question, as well as skewness and kurtosis of distributions, are shown in the Table 2.

Factorial analysis

The Kaiser-Meyer-Olkin test confirmed sampling adequacy with its value of 0.958 and the Bartlett's test of sphericity was highly significant ($\chi^2 = 4,174.508$; df = 120; $p = 0.000$). The orthogonal rotation could not be performed, because only one factor was extracted in the first place (with loading of 10.847, which explains 67.8% of variance). Our results confirmed the factor analysis of the original scale, where only one factor was extracted, too [1].

Validity

The total score of the OHQoL-UK(W) correlated significantly with the VAS score (Spearman's correlation coefficient 0.221, $p = 0.000$), and with the value of DMFT index (Spearman's correlation coefficient -0.372, $p = 0.000$) (see Table 1 for absolute values of VAS score and DMFT index).

Table 1

Characteristics of the study participants (n = 250)

Parameter	Value
Age (years), mean ± standard deviation	37.3 ± 17.6
median (max–min)	29.0 (74–18)
Male/female, n (%)	83/167 (33.2/66.8)
Having at least one chronic, non-contagious, systemic disease, n (%)	
yes/no	49/201 (19.6/80.4)
Having allergy of any kind, n (%)	
yes/no	34/216 (13.6/86.4)
Smoking cigarettes, n (%)	
yes/no	70/180 (28.0/72.0)
Drinking alcohol every day, n (%)	
yes/no	8/242 (3.2/96.8)
Had major surgery in the past, n (%)	
yes/no	80/170 (32.0/68.0)
DMFT index, mean ± standard deviation	13.1 ± 7.1
median (max–min)	12 (28–1)
VAS score, mean ± standard deviation	51.9 ± 35.8
median (max–min)	51 (100–0)

n (%) – number (%) of participants; DMFT – decay-missing-filled teeth; VAS – visual analogue scale.

Table 2

Descriptive statistics for each of the translated items of the OHQoL-UK(W)

Item	Mean	Standard deviation	Skewness	Kurtosis
Eating (Kakav uticaj ima stanje Vaše usne duplje na to kako se hranite i uživate u hrani?)	6.90	1.890	-0.817	0.390
Appearance (Kakav uticaj ima stanje Vaše usne duplje na Vaš izgled?)	6.84	1.890	-0.616	-0.219
Speech (Kakav uticaj ima stanje Vaše usne duplje na Vaš govor?)	7.08	1.791	-0.586	-0.202
General health (Kakav uticaj ima stanje Vaše usne duplje na Vaše opšte zdravstveno stanje?)	6.95	1.792	-0.628	0.160
Sleep (Kakav uticaj ima stanje Vaše usne duplje na Vašu sposobnost da se opustite i spavate?)	6.80	1.882	-0.498	-0.263
Social life (Kakav uticaj ima stanje Vaše usne duplje na Vaš društveni život?)	6.98	1.765	-0.408	-0.598
Romantic relationship (Kakav uticaj ima stanje Vaše usne duplje na Vaše ljubavne veze?)	6.97	1.783	-0.277	-0.910
Smiling (Kakav uticaj ima stanje Vaše usne duplje na Vaš osmeh i smejanje?)	6.98	2.020	-0.786	-0.065
Self-confidence (Kakav uticaj ima stanje Vaše usne duplje na Vaše samopuzdanje?)	6.96	1.884	-0.512	-0.611
Worry (Kakav uticaj ima stanje Vaše usne duplje na Vašu bezbrižnost (nedostatak zabrinutosti?)	6.63	1.916	-0.344	-0.493
Mood (Kakav uticaj ima stanje Vaše usne duplje na Vaše raspoloženje?)	6.76	1.932	-0.458	-0.434
Work (Kakav uticaj ima stanje Vaše usne duplje na Vaš posao ili sposobnost obavljanja svakodnevnih poslova?)	6.61	1.781	0.017	-0.945
Finance (Kakav uticaj ima stanje Vaše usne duplje na Vaše prihode?)	6.50	1.842	-0.069	-0.564
Personality (Kakav uticaj ima stanje Vaše usne duplje na Vašu ličnost?)	6.70	1.763	-0.238	-0.465
Comfort (Kakav uticaj ima stanje Vaše usne duplje na Vašu udobnost?)	6.59	1.878	-0.406	-0.175
Breath (Kakav uticaj ima stanje Vaše usne duplje na Vaš zadah?)	7.01	4.401	7.280	68.962

OHQoL-UK(W) – Oral health-related quality of life questionnaire.

Discussion

The concept of the OHQoL-UK(W) scale is based on assumption that oral health affects quality of life, and it was indeed shown in studies where large proportion of respondents perceived oral health as important predictor of their quality of life [13]. Positive influence of good oral health on quality of life is especially present in younger, more educated persons who more frequently visit their dentists [14, 15]. This effect was captured in our sample, too, since it consisted of whole spectrum of participants in regard to education and age.

While kurtosis for majority of the OHQoL-UK(W) items was within the acceptable range for normal distribution, responses of the participants were significantly skewed to the left, i.e. majority of the participants tended to score higher on the scale from 1 to 9 (mostly about 7). Responses to the item about influence of breath on quality of life were skewed the most, and they peaked much above the responses to other items. Similar phenomenon was observed in Serbian population of elderly patients with another instrument for measuring health-related quality of life (Geriatric Oral Health Assessment Index) [16], probably reflecting cultural specificities in Serbia, where patients are not that demanding when oral health is in question, i.e. their estimate is over-optimistic. Concerns about oral health and periodontal condition are below average in Serbian patients, as compared to patients from other countries [17], which could explain why their estimate regarding own oral health-related quality of life was unrealistically high.

Although the OHQoL-UK(W) has questions that aim to capture physical, social and psychological aspects of quality of life separately, it actually measures one phenomenon (as confirmed by factor analysis) because these aspects of oral health-related quality of life are interconnected and dependent one on another. Oral cavity is not only essential for feeding, but it is an instrument of interpersonal and social communication, so it is not surprising that all aspects of quality of life are simultaneously affected by the oral health status [18, 19].

Recent systematic review found 18 different instruments for measuring oral health-related quality of life, and the best psychometric properties were demonstrated for the Early Childhood Oral Health Impact Scale and Child Perceptions Questionnaire 11–14 [20]. Specific instruments showed worse properties than instruments generic for oral health in total. Our translated questionnaire is generic, and it showed high reliability and validity, within the range of other generic instruments. However, its responsiveness (temporal stability) was not measured, and better interpretation of scores (eg. estimating the minimal important difference) remains to be explored in future studies.

Conclusion

Our study showed that Serbian translation of the OHQoL-UK(W) is as reliable as the original instrument in English, since it has very similar degree of internal consistency, and correlates satisfactorily with the VAS score and in-

versely with the DMFT index. Also, there is only one factor which is composed of all items of the Serbian translation of the scale, which corresponds to the factorial structure of the original scale (also only one factor). Therefore, Serbian translation of the OHQoL-UK(W) is reliable instrument for measuring oral health-related quality of life in adult dentistry patients, which could be of great help in clinical practice when dentists evaluate effects of therapy and prepare future treatment plans.

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Comparative clinical evaluation of the therapeutic effects of low-level laser and hyaluronic acid on *gingivitis catarrhalis* in children

Komparativna klinička evaluacija terapijskih efekata lasera male snage i hijaluronske kiseline na kataralni gingivitis kod dece

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Abstract

Background/Aim. *Gingivitis catarrhalis* is the most common disease of the oral mucosa in children, representing an inflammation of the gingiva of an exudative nature. The aim of this study was to evaluate the effectiveness of low-level laser therapy and hyaluronic acid therapy on *gingivitis catarrhalis* in children using the appropriate clinical parameters. **Methods.** The study involved 100 children with permanent dentition in whom *gingivitis catarrhalis* had been diagnosed. The examinees were divided into two groups: the group I consisting of patients with gingival inflammation (50 examinees) in whom the therapy with hyaluronic acid was applied after the removal of soft and hard dental deposits, and the group II consisting of patients with gingival inflammation (50 examinees) in whom low-level laser therapy was applied after the removal of soft and hard dental deposits. Clinical evaluation of the therapeutic effects of low-level laser and hyaluronic acid on *gingivitis catarrhalis* was performed using

the appropriate indices: the Greene-Vermillion Plaque Index (PI), Muhlemann bleeding index (BI), and Community Periodontal Index of Treatment Needs (CPITN). **Results.** Using the Student's *t*-test for dependent samples, a statistically significant difference was obtained ($p < 0.001$) between the PI, BI, and CPITN indices before and after the therapy in both examined groups. Moreover, the CPITN index after the therapy in the group II was statistically significantly lower ($p < 0.05$) than that obtained in the group I. **Conclusion**. The results demonstrated an exceptional effect of hyaluronic acid and low-level laser therapy, supplementing basic therapy, in the treatment of catarrhal gingivitis in children. Somewhat better results were achieved with the combination of basic therapy and low-level laser.

Key words:
adolescent; gingivitis; hyaluronic acid; low-level laser therapy; oral health; periodontal index; treatment outcome.

Apstrakt

Uvod/Cilj. Kataralni gingivitis je najčešće oboljenje oralne sluzokože kod dece i predstavlja inflamaciju gingive eksudativne prirode. Cilj rada je bio da se kliničkim parametrima oceni efikasnost lasera male snage i hijaluronske kiseline na kataralni gingivitis kod dece. **Metode.** Ispitivanjem je obuhvaćeno 100 dece sa stalnom denticijom, kojima je dijagnostikovan *gingivitis catarrhalis*. Isptitanici su podeljeni u dve grupe: I grupu su činili pacijenti sa inflamiranom gingivom (*gingivitis catarrhalis* – 50 ispitanika), kojima je nakon uklanjanja mekih i čvrstih naslaga primenjena terapija aplikovanjem hijaluronske kiseline; II grupu su činili pacijenti sa inflamiranom gingivom (*gingivitis catarrhalis* – 50 ispitanika), kod kojih je nakon uklanjanja mekih i čvrstih naslaga primenjena terapija laserom male snage. Klinička procena terapijskih efekata lasera male snage i hijaluronske kiseline na *gingivitis catarrhalis* vršena je uz pomoć odgovarajućih in-

deksa: plak indeks po Greene-Vermillion-u (PI), Muhlemannov indeks krvarenja (IKR) i *Community Periodontal Index of Treatment Needs* (CPITN). **Rezultati.** Studentovim *t*-testom zavisnih uzoraka dobijena je statistički značajna razlika ($p < 0,001$) između PI, IKR i CPITN indeksa pre i PI, IKR, i CPITN indeksa posle terapije u obe ispitivane grupe. Takođe CPITN indeks posle terapije ispitanika II grupe bio je statistički značajno niži ($p < 0,05$) u odnosu na ispitanike I grupe. **Zaključak.** Dobijeni rezultati pokazuju izuzetno dobar efekat hijaluronske kiseline i lasera male snage uz baznu terapiju u lečenju kataralnih gingivitisa kod dece. Nesto bolji rezultati dobijeni su u kombinaciji bazične terapije i lasera male snage.

Ključne reči:
adolescenti; gingivitis; hijaluronska kiselina; laser male snage; usta, zdravlje; periodontalni indeks; lečenje, ishod.

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Introduction

Gingivitis catarrhalis is the most common disease of the oral mucosa in children, representing an inflammation of the gingiva of an exudative nature. It occurs as the consequence of gingival tissue reaction to the stimuli produced by local factors. *Gingivitis catarrhalis* is characterized by bleeding from the gingiva upon provocation, and the intensity of bleeding is proportional to the severity of gingival inflammation.

The treatment of *gingivitis catarrhalis* involves primarily the standard (basic) therapy – removal of any causal agents and motivation and education of children to maintain adequate oral hygiene. The removal of causal agents involves the removal of any agents directly or indirectly involved in the onset of the disease. These are, above all, local factors, such as dental deposits (dental biofilm and calculus), then iatrogenic factors, caries, bad habits, and some dietary factors. Clinical improvements are directly related to the reduction or removal of subgingival biofilm [1, 2].

Gingivitis catarrhalis is most commonly caused by the bacteria present in the dental plaque. These bacteria produce some specific enzymes (proteinases and hyaluronidases) which destroy the structure of the connective tissue (above all, collagen types I and IV). Furthermore, they tend to depolymerize the structure of hyaluronic acid and thus damage the tissue of the tooth supporting structure. In further course of the disease, additional pathological changes usually appear, which, if left untreated, can ultimately lead to the loss of teeth.

The use of hyaluronic acid is a fundamentally new biological approach in dentistry in the prevention and treatment of lesions and inflammatory changes in the oral cavity. The substance has also been studied as a metabolite or inflammation marker present in the gingival fluid, and also as an important factor involved in growth, development and regeneration of tissue [3, 4].

The beneficial effects of laser light in the therapy of gingivitis have also been a focus of attention. The first ruby laser was developed by Maiman in 1960. Soon after that, its possible use in dentistry was recognized. The interest in the development of this technology in all disciplines of dentistry has been on the rise ever since. The fact that the use of low-power laser is entirely painless, noninvasive and without any adverse effects is especially important in that regard [5–7]. Exceptionally good results are achieved with the use of low-level laser as an adjuvant to standard, basic therapy, in the treatment of periodontal inflammations [8, 9].

Nowadays, it is a well known fact that the rays of low-level laser light can have both primary (photochemical, photoelectric, and photoenergetic) and secondary effects (stimulation of the cell metabolism and microcirculation), with the resultant therapeutic laser light effects, such as analgetic, biostimulative, antiinflammatory, and antiedematous effects [10–13].

The aim of this study was to evaluate, using clinical parameters, the effectiveness of low-level laser and hyaluronidase in *gingivitis catarrhalis* in children.

Methods

The study involved 100 children with permanent dentition (aged 13–17 years) diagnosed with catarrhal gingivitis.

Their gender representation was balanced. The examinees were divided into two groups. The group I consisted of the patients with gingival inflammation (*gingivitis catarrhalis* – 50 examinees) in whom the therapy with hyaluronic acid was applied. Hyaluronic acid was administered by gently rubbing in the gel into the inflamed gingiva daily for a week. The group II included the patients with gingival inflammation (*gingivitis catarrhalis* – 50 examinees) in whom, after the removal of soft and hard dental deposits, the therapy with low-level laser was applied using the Scorpion-dental-Optima laser in 5 daily sessions (with 635 nm wavelength, initial power of 25 mV, and a 120 s exposure).

Clinical evaluation of the therapeutic effects of low-level laser and hyaluronic acid on *gingivitis catarrhalis* was performed using the appropriate indices. The following indices were determined for all patients, both before and after the therapy: Greene-Vermillion Plaque Index (PI), Muhlemann Bleeding Index (BI), and Community Periodontal Index of Treatment Needs (CPITN).

The study was approved by the Ethical Committee of the Faculty of Medicine, University of Niš (in accordance with the World Medical Association Declaration of Helsinki).

The examined parameters were represented with mean values and standard deviations (SD). The coefficient of variation was determined as the measure of homogeneity of the examined samples in relation to the examined parameters. The Student's *t*-test of independent samples was used to test statistically significant differences between the mean values of these two groups. The entry and tabular representation of results were done using the MS Office Excel, and calculations were performed using the SPSS ver. 15.0 software package.

Results

The values of PI, BI and CPITN were shown in Table 1. Student's *t*-test of independent samples detected a statistically significant difference ($p < 0.001$) between PI, BI and CPITN indices before and after therapy in both studied groups. Further, the CPITN value after the therapy in the group II was statistically significantly lower ($p < 0.05$) compared to that in the group I of examinees.

Discussion

Inflammation of the gingiva is common in children. Early diagnosis and treatment are very important, since if left untreated, the inflammation may involve other periodontal tissues and the process becomes irreversible. A complex etiopathogenesis of the disease which involve periodontal tissues, developing in a complex anatomical substratum, makes any monitoring of its course very difficult. The pathological processes involving the tissue of periodontium begin without any external manifestation, and initial reactions can not be at all detected. The stage of the disease is of key importance regarding the necessary treatment and prognosis. Each case of gingivitis has to be treated, so that the disease is prevented to progress and involve deeper periodontal tissues and, as a result, irreversible changes are avoided. Newer and more effective treatment tools and methods are therefore sought for.

Table 1

Mean values of dental indices in the studies grups before and after the therapy

Dental indices	Before terapy		After terapy	
	mean ± SD	CV	mean ± SD	CV
PI				
group I	1.68 ± 0.47	28.05	0.00 ± 0.00*	
group II	1.82 ± 0.39	31.32	0.00 ± 0.00*	
BI				
group I	1.74 ± 0.44	25.46	0.16 ± 0.37*	231.46
group II	1.00 ± 0.61	32.34	0.08 ± 0.27*	342.56
CPITN				
group I	1.50 ± 0.51	33.67	0.24 ± 0.43*	179.76
group II	1.60 ± 0.49	30.93	0.08 ± 0.27*†	342.56

Group I – patients treated with hyaluronic acid; Group II – patients treated with low-level laser (both groups had equal number of examinees, 50 each).
PI – Plaque Index; BI – Mulhemann Bleeding Index; CPITN – Community Periodontal Index of Treatment Needs;
SD – standard deviation; CV – coefficient of variation.
*$p < 0.001$ vs. before the therapy; †$p < 0.05$ vs. group I.

In the group I of examinees, in addition to the usual, basic therapy of chronic gingivitis, hyaluronic acid was topically applied. The obtained posttreatment values of PI, BI and CPITN demonstrated that hyaluronic acid, owing to its antiinflammatory, antiinfective, antiedematous and regenerative actions help in the healing of chronic gingivitis in children. Hyaluronic acid is a natural biological substance in the gingival connective tissue [14, 15]. In chronic gingivites, under the action of bacterial enzymes (hyaluronidase), hyaluronic acid is decomposed. As a result, the structure of the gingival tissue is lost, with a resultant increased exchange of fluids between the tissue and the vascular system and consequential edema creation. Increased capillary permeability enables bacteria and their toxins to penetrate the tissue more easily, which further intensifies inflammation. Applied to the inflamed gingival tissue, hyaluronic acid exerts its antiinflammatory, antiedematous and antiproliferative effects [4, 16, 17]. The results of this study corroborate other findings that topical application of hyaluronic acid to gingival tissue, in the form of a gel or spray, is able to reduce bleeding and inflammation of the gingiva [16, 18]. It can be applied daily without any adverse effects [19]. A significant clinical improvement after the treatment of gingivitis with hyaluronic acid, manifested among other things as reduced gingival bleeding, has been reported by other authors as well [20–22].

In the group II of examinees, low-level laser therapy supplemented basic therapy. The obtained posttreatment values of PI, BI and CPITN showed that low-level laser, thanks to its antiinflammatory, antiedematous and biostimulation effects, was able to help in the healing of chronic gingivitis in children. Various researchers have reported that low-level laser therapy supplementing basic therapy is able to reduce gingival inflammation and that it can be successfully used in the therapy of gingivitis and parodontopathy [8, 23–25]. The results of some investigations have shown that laser therapy exerts analgetic effects only [26]. However, in recent decades, laser therapy has also been attributed with significant antiin-flammatory properties. Low-level laser light reduces inflammation and produces clinically apparent antiinflammatory and antiedematous effects [27, 28]. Laser light provokes increased tissue regeneration. The action of low-level laser reduces blood vessel permeability and suppresses exudative processes, which in further course reduces gingival edema. Moreover, blood vessel permeability is normalized. By their biostimulation effect, low-level lasers increase cellular growth and proliferation, and induce changes in the circulation of lymph and blood (leading to a better blood supply and facilitated tissue drainage). Inflammatory response can be normalized or reduced by photochemical effects of laser radiation [29]. The similar was observed in this study too, where the applied basic therapy supplemented by low-level laser produced a significant downgrading of inflammation, as documented by the appropriate indices.

If it is not diagnosed timely, catarrhal gingivitis progresses and the pathological process involves other periodontal tissues, resulting in parodontopathy and subsequent loss of teeth. That is why it is important to intensify health education activities and prevent the onset and development of catarrhal gingivitis with all the available prevention and prophylactic measures and tests to establish the risk of the disease. This is essential bearing in mind that periodontal diseases can be the risk factor in the onset and development of other consecutive diseases, such as, for instance, cardiovascular, renal, and skin diseases.

Conclusion

Current dental health care, observed from the point of view of new technological advancements, is able to offer much more in the resolution of various dental problems than it has been in the relatively recent past. The results we obtained showed an exceptional effect of hyaluronic acid and low-level laser in the treatment of catarrhal gingivitis in children. Slightly better results were obtained with the combination of basic (standard) therapy and low-level laser.

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Smoking, alcohol consumption and human papillomavirus infection as risk factors for oral cavity and oropharyngeal tumors in Serbia – A pilot study

Pušenje, alkohol i humani papiloma virus kao faktori rizika od razvoja oralnih i orofaringealnih tumora u Srbiji – pilot studija

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Abstract

Background/Aim. The oral cavity and oropharyngeal cancers are among the most common cancers worldwide with the multifactorial etiology. The aim of this study was to determine the major risk factors among patients with oral cavity and oropharyngeal tumors in Serbia. **Methods.** A total of 63 patients with biopsy proven malignant (33 patients) or benign (30 patients) oral cavity or oropharyngeal lesions were included in this study. The data about gender, age, smoking habits and alcohol consumption were obtained from the routine medical files. The detection and genotyping of human papillomavirus (HPV) was done in paraffin embedded tissue samples using *in situ* hybridization. **Results.** Malignant lesions were more frequent in men, smokers and patients who consume alcohol with a statistically significant difference compared to the patients with benign lesions. The prevalence of HPV infection was higher in patients with malignant lesions compared to patients with benign lesions, but without statistically significant difference. High risk genotypes were detected only in patients with malignant lesions of tonsils and base tongue cancer, while low risk types were demonstrated in patients with benign lesions with a highly statistically significant difference. **Conclusion.** The results point to the significant association of tobacco smoking, alcohol consumption and high risk HPV genotypes as risk factors for oral cavity and oropharyngeal carcinomas in Serbian patients.

Key words:
alcohol drinking; carcinoma, squamous cell; human papillomavirus; mouth neoplasms; pharyngeal neoplasms; risk factors; serbia; smoking.

Apstrakt

Uvod/Cilj. Karcinomi usne duplje i orofaringealne regije su među najčešćim malignitetima u svetu. Cilj ove studije bio je da utvrdi faktore rizika od pojave oralnih i orofaringealnih tumora kod bolesnika u Srbiji. **Metode.** Studijom su bila obuhvaćena 63 bolesnika sa patohistološkom potvrdom malignih lezija (33 bolesnika) i benignih lezija (30 bolesnika) u usnoj duplji/orofaringealnoj regiji. Uvidom u medicinsku dokumentaciju dobijeni su podaci o demografskim karakteristikama (pol i starost) i navikama bolesnika (pušenje i konzumacija alkohola). Tehnikom *in situ* hibridizacije identifikovan je humani papilomavirus (HPV) u tkivima tumora oralne i orofaringealne regije, fiksiranim u formalinu i ukalupljenim u parafin. **Rezultati.** Maligne lezije su bile statistički značajno češće kod muškaraca koji su konzumirali alkohol i duvan. Infekcija HPV bila je češća kod bolesnika sa malignim lezijama u odnosu na bolesnike sa benignim lezijama, ali bez statistički značajne razlike. Visokoonkogeni tipovi 16/18 otkriveni su samo kod bolesnika sa malignim lezijama i to tonzila i baze jezika, dok su niskoonkogeni tipovi 6/11 identifikovani kod bolesnika sa benignim lezijama. **Zaključak.** Rezultati ove pilot studije ukazuju na povezanost pušenja, konzumacije alkohola i visokoonkogenih tipova HPV sa razvojem karcinoma usne duplje i orofaringealne regije kod bolesnika u Srbiji.

Ključne reči:
alkohol, pijenje; karcinom skvamoznih ćelija; papillomavirus, humani; usta, neoplazme; farinks, neoplazme; faktori rizika; srbija, pušenje.

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Introduction

Tumors of the oral cavity and oropharynx are among the most common tumors worldwide, with an estimated 529,500 incident cases and 292,300 deaths from oropharyngeal cancer in 2012, accounting for about 3.8% of all cancer cases and 3.6% of cancer deaths. In 2012, the estimated age-standardized rate of oral cavity cancer was relatively large (2.7 per 100,000 for both sexes combined; 3.7 in men and 1.8 in women), with substantial differences by sex, age, and region. The estimated age-standardized rate of oropharyngeal cancer was 1.4 per 100,000 for both sexes combined (2.3 for men and 0.5 for women), where the countries with high Human Development Index (HDI) scores had the highest proportional incidence of oropharyngeal cancer of both men and women [1].

The estimated age-standardized rate of oral cavity and oropharyngeal cancer for both sexes in Serbia was 11.7 per 100,000 in 2012, with the higher incidence in men (18.8 for men and 5.2 for women) [2].

Squamous cell carcinoma (SCC) is the most common epithelial malignancy in oral cavity and oropharynx. More than 90% of cases and over 50% of the cancers are often preceded by potentially malignant disorders such as leukoplakia, oral lichen planus and submucous fibrosis [3].

Etiology of oral cavity and oropharyngeal cancer is considered to be a multifactorial process where environmental factors, viral infections and genetic alterations interact and induce malignant cell transformation [3].

Numerous studies demonstrated that the major risk factors for the development of oral cavity and oropharyngeal cancers are tobacco smoking, alcohol consumption and human papilloma virus (HPV) infections, where tobacco smoking and alcohol consumption have synergistic effects with a nearly sevenfold increase of risk [1, 4, 5].

Tobacco smoke condensate contains substances that act as both initiators and promoters of carcinogenesis. The risk of cancer development from smoking is significant up to approximately five years after quitting. Alcohol is well documented risk factor for oral and oropharyngeal cancers. Animal studies have shown that ethanol promotes 4-NQO-induced oral carcinogenesis [4]. The oncogenic potential of high-risk HPV genotypes is very well documented for anal-genital carcinomas. High-risk HPV genotypes (16, 18, 31, 33, etc.) transform the epithelial cells in the way that their proteins, E6 and E7 gene products, inhibit the function of tumor suppressor genes, thus inactivating the cellular proteins p53 and Rb [6]. Different studies suggest that HPV may be associated with the development of oral and oropharyngeal cancers with the similar molecular mechanisms. It is estimated that 25%–35% of oral and oropharyngeal cancers are infected with HPV. High-risk genotypes 16 and 18 seem to be the most important viruses responsible for carcinogenesis and can be found in premalignant and malignant lesions of the oral cavity in up to 80% of cases [4].

The aim of this study was to determine contribution of tobacco smoking, alcohol consumption and prevalence and genotype distribution of HPV as tumor risk factors among patients with oral cavity and oropharyngeal tumors in Serbia.

Methods

Patients data

From January 2005 to January 2006, a total of 63 patients of both sexes with biopsy proven malignant or benign oral cavity or oropharyngeal lesions were treated at the Clinic of Otorhinolaryngology and Maxillofacial Surgery, Clinical Center of Serbia in Belgrade. The study group of patients with malignant lesions included 33 patients where the majority of patients had tonsils cancer (13 patients) or tongue cancer (9 patients). The control group of patients with benign lesions included 30 patients with tonsil hypertrophy and mucosa hypertrophy or fibroepithelial polyps localized in the oral cavity or oropharyngeal region, where the majority of patients (10 patients) were with miscellaneous benign lesions localized at buccal mucosa, retromolar trigonum and gingiva (Table 1).

The data about gender, age, smoking habits and alcohol consumption were obtained from the routine medical files. Patients, aged from 20 to 79 years (mean age 54.7 ± 4.6), were classified into three groups in relation to age: patients from 20 to 39 years of age, 40–59 and from 60 to 79 years. In relation to alcohol consumption, patients were divided into the following groups: every day, occasionally and no consumption. According to tobacco smoking, patients were classified as smokers and nonsmokers.

Samples for HPV detection and typing

The sample preparation and HPV detection and typing were carried out in the Virology Department, Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, Serbia. Paraffin embedded tissue samples of malignant or benign oral cavity or oropharyngeal lesions were cut into 4 μm–6 μm paraffin sections and collected on treated glass slides. The prepared samples were used for the HPV DNA detection and typing.

Table 1

Localization of malignant and benign oral cavity and oropharyngeal tumors

Lesions	Tumor localization					
	tongue	floor of mouth	tonsil	soft palate	miscellaneous*	total
Malignant	9	3	13	4	4	33
Benign	8	0	8	4	10	30
Total	17	3	21	8	14	63

*localizations on buccal mucosa, retromolar trigonum and gingiva were classified as "miscellaneous".

HPV detection and typing

HPV detection was performed using HPV DNA Screening – REMBRANDT *in situ* hybridization kit (Kreatech Diagnostics, Amsterdam, Netherlands), according to the manufacturer's instructions. The presence of colored hybrids of HPV DNA and probes in the cells under the light microscope was considered to be positive for HPV.

After that, HPV positive samples were typed using HPV DNA typing – REMBRANDT *in situ* hybridization kit with 16/18, 31/33/35 and 6/11 HPV probes (Kreatech Diagnostics, Amsterdam, Netherlands), according to the manufacturer's instructions. The presence of colored hybrids of HPV DNA and type specific probes in the cells under the light microscope was considered to be positive for HPV types.

Statistical analysis

Data were put in the spreadsheet package EXCEL for Windows XP and statistical analysis was performed with SPSS ver. 20.0 using Fisher's exact test. Differences being with $p < 0.05$ were considered to be significant.

Results

The majority of patients in groups with malignant and benign lesions were aged 40–59 years. Malignant lesions were more common in men than in women with statistically significant difference between patients with malignant and benign lesions (Table 2).

Regarding social habits, the majority of patients with malignant lesions were smokers, with statistically significant difference between examined groups. The alcohol consumption was more frequent in patients with malignant changes as compared to patients with benign lesions, with statistically significant difference (Table 2).

The prevalence of HPV infection was higher in the group of patients with malignant lesion as compared to patients with benign lesion, but there were no statistically significant difference between these two groups (Table 2). High-risk genotypes 16/18 were detected only in patients with malignant lesions, while low-risk types 6/11 were demonstrated in patients with benign lesions with a highly statistically significant difference. In one patient with malignant lesion, multiple infection with high-risk 16/18 and low-risk HPV genotypes 6/11 was detected (Table 2).

All HPV positive patients with malignant lesions were men, smokers, 40–59 years of age. Out of 5 HPV positive patients, 2 patients reported occasional alcohol consumption (Table 3). According to the localization of malignant lesions, 80% of positive HPV results were found in tonsil cancers and 20% in tongue cancers (Table 3). Out of all SCC specimens of tonsils, 30.76% were positive to HPV (4/13) and 11.11% samples collected from the tongue were positive to HPV infection (1/9). Multiple infections with high-risk and low-risk HPV genotypes were detected in patient with tonsil cancer.

In patients with benign lesions, HPV was detected in smokers, 40–59 years of age with occasional consumption of alcohol. One HPV positive result was detected in man and one in female. All HPV positive samples were obtained from benign lesions of tongue (Table 3).

Table 2
Risk factors in patients with malignant and benign oral cavity and oropharyngeal tumor lesions

Risk factors	Lesions		*p*
	malignant	benign	
Age (years)			
20–39	0	4 (13.3)	
40–59	21 (63.6)	20 (66.7)	0.06
60–79	12 (36.4)	6 (20)	
Gender			
male	29 (87.87)	10 (33.33)	0.001
female	4 (12.13)	20 (66.66)	
Smoking			
yes	31 (93.9)	16 (53.3)	0.001
no	2 (6.1)	14 (46.7)	
Alcohol consumption			
every day	9 (27.7)	0	
occasional	14 (42.4)	12 (40)	0.001
no	10 (30.3)	18 (60)	
HPV detection			
positive	5 (15.15)	2 (6.66)	0.429
negative	28 (84.85)	28 (93.34)	
HPV types			
16/18	4 (80)	0	
16/18 + 6/11	1 (20)	0	0.047
6/11	0	2 (100)	

Note: Results are given as number (%) of patients.

Due to the small number of HPV positive patients with malignant and benign lesions, the statistical analysis for the association of HPV and social habits was not performed.

Discussion

Oral cavity and oropharyngeal carcinomas are primarily diseases of older age, occurring most frequently in patients older than age 45. Epidemiological studies over last 20 years have shown a steady increase in the incidence of these cancers in younger adults (in age 18–45 years) [7]. This study cannot confirm this data since all of the patients with malignant lesions were over 40 years of age. However, many oral and oropharyngeal cancers present at a late stage of disease due to the delay in diagnosis. The delay in younger patients could be longer as cancer is not suspected in this age [8].

Numerous epidemiological studies demonstrated higher incidence of oral cavity and oropharyngeal cancers in men compared to women [1, 9]. This is consistent with the results of our study, where the majority of patients with malignant lesions were men (87.87%), with the statistically significant difference.

Table 3

The frequency of human papillomavirus (HPV) according to gender, age and social habits of patients with malignant and benign oral cavity and oropharyngeal tumor lesions

Parameter	Malignant lesions		Benign lesions	
	HPV positive	HPV negative	HPV positive	HPV negative
Gender				
male	5 (15.15)	24 (72.73)	1 (3.33)	9 (30)
female	0 (0)	4 (12.12)	1 (3.33)	19 (63.33)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Age				
20–39	0 (0)	0 (0)	0 (0)	4 (13.33)
40–59	5 (15.15)	16 (48.49)	2 (6.66)	18 (60)
60–79	0 (0)	12 (36.36)	0 (0)	6 (20)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Alcohol consumption				
no	3 (9.09)	7 (21.21)	0 (0)	18 (60)
occasionally	2 (6.06)	12 (36.36)	2 (6.66)	10 (33.33)
every day	0 (0)	9 (27.27)	0 (0)	0 (0)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Smoking				
yes	5 (15.15)	26 (78.79)	2 (6.66)	14 (46.66)
no	0 (0)	2 (6.06)	0 (0)	14 (46.66)
Total	5 (15.15)	28 (84.85)	2 (6.66)	28 (93.33)
Tumor site				
tonsils	4 (12.12)	9 (27.27)	0 (0)	8 (26.66)
tongue	1 (3.03)	8 (24.24)	2 (6.66)	6 (20)
soft palate	0 (0)	4 (12.12)	0 (0)	4 (13.33)
floor of mouth	0 (0)	3 (9.09)	–	–
miscellaneous	0 (0)	4 (12.12)	0 (0)	10 (33.33)
Total	5 (15.15)	28 (84.85)	0 (0)	28 (93.33)

Note: Results are given as number (%) of patients.

It is generally accepted that etiology of oral cavity and oropharyngeal carcinoma is multifactorial and the most common risk factors of these malignant diseases include tobacco smoking, alcohol consumption and HPV infection [1, 4]. It has been demonstrated that tobacco and alcohol are traditional risk factors for oral and oropharyngeal cancers in adults, regardless of age. Individuals who smoke more than 20 cigarettes a day and consume more than 100 g of alcohol per day are believed to be at increased risk for oral and oropharyngeal epithelial dysplasia. In addition, alcohol has been found to be an independent risk factor for these cancers among non-smokers, as well as tobacco smoking in non-drinkers. Moreover, both factors together seem to enhance the carcinogenic effect [4, 7]. This is supported in this study as almost all of the patients with malignant lesions were smokers (93.9%) and 69.1% of them consumed alcohol, which was significantly different from patients with benign lesions. The results of this study are similar to previously reports from our country [10, 11]. The majority of these reports examined the correlation of genetic and epigenetic markers and above mentioned risk factors in oral and oropharyngeal cancers [11–13].

It is also demonstrated that benign lesions of oral and oropharyngeal regions are associated with tobacco smoking and alcohol consumption. However, the risk is lower compared to malignant lesions [14]. This is supported by results of this study, where around 50% of patients with benign lesions were smokers with occasional consumption of alcohol.

Current literature data shows that at any given time approximately 7% of the population has a prevalent oral/oropharyngeal HPV infection. Most of these HPV infections do not progress to cancer and are usually cleared by the immune system. It has been suggested that the delayed clearance of oral/oropharyngeal HPV infection may be a risk factor for development of oral cavity and oropharyngeal cancers [15].

A detection rate of HPV DNA in oral cavity and oropharyngeal cancers ranges from 25%–35%, with the dominance of high risk genotypes 16 and 18 [3]. There have been numerous publications studying HPV presence in oral cavity and oropharyngeal tumors with the variability in detection rates of 0%–100%. This variability may be due to the multiple anatomical sites encompassed, the use of various detection techniques [*in situ* hybridization, southern blot hybridization, polymerase chain reaction (PCR)] and different sampling methods such as biopsies, scrapes, oral rinses, brushes [16]. Furthermore, numerous studies demonstrated that the locations of HPV positive oral cavity and oropharyngeal carcinoma are tonsils, the base of the tongue and oropharyngeal harbor [17].

The results of this study showed higher detection rate of HPV using *in situ* hybridization method in the group of patients with malignant lesion compared to patients with a benign lesions (15.15% vs. 6.66%), but this was not statistically significant difference. *In situ* hybridization, which is used in this study, is a highly specific method that protects tissue morphology, but the method with lower sensitivity relating to PCR [18]. In the study of Kozomara et al. [11], HPV detection rate using PCR was 64% in the tissues of tongue and floor of mouth cancers. The low percentage of HPV positivity in our pilot study may be due to the small cohort of patients, the analysis of multiple anatomic sites and limitations of *in situ* hybridization method. However in the study of Popovic et al. [19], HPV detection rate using PCR was 10% in the tissues of oral cancers.

According to the localization, in this study HPV was detected only in patients with tonsils and base tongue carcinomas, which is consistent with the previous findings [20]. In addition, the detection rate of high-risk genotypes 16 and 18 in our study was consistent with previous studies [21], where these types have been detected only in patients with malignant lesions (80%), while low risk types 6/11 were demonstrated in patients with benign lesions, with a highly statistically significant difference between these two groups.

Literature data favors the fact that negative HPV oral cavity and oropharyngeal carcinomas develop in senior patients (in the seventh decade of life), with predomination of males, whose main risk factors for cancer occurrence are smoking, alcohol consumption, bad oral hygiene and a vitamin-poor diet. HPV positive carcinomas are more likely to occur in men of younger age, which is explained by the change of sexual habits [9, 22]. This is consistent with the findings in our study, where all HPV positive patients with malignant and benign lesions were aged 40–59 years.

The majority of studies have shown that patients with HPV positive oral cavity and oropharyngeal cancers are less likely to have a history of tobacco exposure and alcohol consumption [15, 23]. In this study, the consistence was found for alcohol consumption, but not for tobacco smoking because all HPV positive patients with malignant lesions were smokers. Similar results were found for all HPV positive patients with benign lesions.

To the best of our knowledge, this is the first study which examined the association of risk factors both in malignant and benign lesions of oral and oropharyngeal region.

Conclusion

The obtained results of this study showed a significant association of tobacco smoking, alcohol consumption and HPV infection with oral cavity and oropharyngeal tumors in Serbian patients. These results as well as those of future studies with a larger cohort, would possibly provide more detailed information about the major risk factors for oral cavity and oropharyngeal tumors and may contribute to their prevention in Serbia.

Acknowledgement

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Unruptured distal anterior cerebral artery mirror aneurysms associated with ruptured middle cerebral artery aneurysm: A case report

Nerupturisane distalne identične bilateralne aneurizme prednjih moždanih arterija udružene sa rupturisanom aneurizmom srednje moždane arterije

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Abstract

Introduction. Distal anterior cerebral artery (DACA) aneurysms, also known as pericallosal aneurysms are rare, while aneurysms in mirror position are extremely rare. These aneurysms have high tendency for rupture (PHASES score is always > 4). In more than a half of the patients with the DACA aneurysm rupture, imaging reveals intracerebral hematoma which is a predictor of poor outcome. **Case report.** A 49-year-old female patient was treated endovascularly in other institution, due to middle cerebral artery aneurysm (MCA) rupture, when the two small bilateral aneurysms at the distal segments of anterior cerebral artery (ACA) were revealed, left one measuring 4.5 mm and the right one measuring 6 mm in size, with the aneurysmal neck width of 3 mm and 4 mm, respectively. The decision was made by the interventional neuroradiologist only to treat the bleeding MCA aneurysm immediately. The patient was referred to our department six months later, and it was decided to perform microsurgical occlusion of the remaining DACA aneurysms. Unilateral interhemispheric approach was chosen to reach the distal ACAs and aneurysms at pericallosal-callosomarginal junction were clipped and completely excluded from the circulation. **Conclusion.** Management of DACA aneurysms is a surgical challenge, even for experienced neurosurgeons. It is controversial whether these should be surgically clipped or coiled endovascularly, especially in cases like this one when a same-stage, endovascular coiling might look like a perfect approach. Surgical treatment should be prompt due to their tendency to early rupture. Careful evaluation for multiplicity is mandatory.

Key words:
aneurysm, ruptured; anterior cerebral artery; endovascular procedures; intracranial aneurysm; microsurgery; middle cerebral artery; neurosurgical procedures; treatment outcome.

Apstrakt

Uvod. Aneurizme distalnog segmenta prednje moždane arterije [*distal anterior cerebral artery* (DACA)], takođe poznate kao perikalozna arterija, retke su, dok su bilateralne aneurizme u identičnoj poziciji ekstremno retke. Te aneurizme imaju veliku tendenciju ka rupturi (PHASES skor je uvek > 4). U više od polovine bolesnika sa rupturom DACA aneurizme formira se intracerebralni hematom, koji je prediktor lošeg ishoda lečenja. **Prikaz bolesnika.** Bolesnica, stara 49 godina je, zbog rupture aneurizme na srednjemoždanoj arteriji [*middle cerebral artery* (MCA)], prethodno lečena endovaskularnom procedurom u drugoj ustanovi, kada su dijagnostikovane i dve male simetrične aneurizme na DACA obostrano. Dimenzija leve aneurizme bila je 4,5 mm, a desne 6 mm, dok su širine vrata bile 3 mm, odnosno 4 mm. Tada je interventni radiolog doneo odluku da leči samo krvareću aneurizmu na MCA. Bolesnica je upućena u našu ustanovu 6 meseci kasnije i doneta je odluka da se sprovede mikrohirurško lečenje aneurizmi na DACA. Uz pomoć unilateralnog interhemisferičnog pristupa i mikrohirurške tehnike obe simetrične aneurizme na kalozo-kalozomarginalnom spoju isključene su iz cirkulacije. **Zaključak.** Lečenje DACA aneurizmi je hirurški izazov, čak i za iskusne neurohirurge. I dalje postoji kontroverza u vezi izbora modaliteta lečenja – mikrohirurgija ili endovaskularna procedura, pogotovu u slučajevima kada se *coiling* u istom aktu sa udruženim aneurizmama čini kao odličan

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izbor. Zbog tendencije ka ranoj rupturi tih aneurizmi, mikrohirurško lečenje ne treba odlagati. Obavezna je provera postojanja udruženih aneurizmi.

Introduction

Distal anterior cerebral artery (DACA) aneurysms, also known as pericallosal aneurysms are rare, and account for approximately 2%–9% of all ruptured intracranial aneurysms [1–4]. Studies have previously shown association of these aneurysms with multiple intracranial aneurysms disease, with multiple aneurysms presence in 55% of cases [4–6]. Several smaller series of DACA aneurysms indicated the frequency of bilateral aneurysms in 10%–20% of cases [1, 7], while mirror positioned DACA aneurysms are extremely rare [8–10]. DACA aneurysms are frequently associated with congenital anomalies and anatomic variations of DACA [8], although, there are reports of patients with DACA mirror aneurysms without any other vascular variation [9].

Typically, DACA aneurysms are small in size, with a wide neck, and with branches originating from the neck or fundus of the aneurysm [11]. The pericallosal-callosomarginal bifurcation is the most common location of DACA aneurysms [12, 13]. These aneurysms have high tendency for rupture (PHASES score is always > 4) [6, 14, 15]. In most of ruptures (67%–90%), DACA aneurysms were less than 7 mm in diameter [6, 16]. In more than a half of patients with the DACA aneurysm rupture, imaging reveals intracerebral hematoma (ICH), which is much more frequent then in other ruptured aneurysms (53%–73% vs. 26%) [16, 17]. Treatment options available include endovascular coiling, surgical clipping or by-pass surgery, which is the treatment of choice only in complex cases [4, 17–20].

We presented a case of surgically treated unruptured mirror aneurysms of DACA, accidentally seen during previous endovascular treatment after middle cerebral artery (MCA) aneurysm rupture.

Case report

Six months before admission to our department, a 49-year-old female patient was treated endovascularly in other institution due to MCA aneurysm rupture manifested with subarachnoid hemorrhage. Digital subtraction angiography (DSA), performed in the course, confirmed the existence of bilobular right MCA aneurysm, and also revealed two small bilateral aneurysms at the distal segments of anterior cerebral artery (ACA), left one measuring 4.5 mm and the right one measuring 6 mm in size, with the aneurysmal neck width of 3 mm and 4 mm, respectively, without other vascular malformations revealed (Figure 1).

The decision was made by the interventional neuroradiologist only to treat the bleeding MCA aneurysm immediately, while both ACA aneurysms were deemed unsuitable for endovascular treatment at the given moment. The postprocedural period passed without any complications. Follow-up multislice computed tomography (MSCT) angiography confirmed the existence of bilateral aneurysms on DACA segments one more time, as well as complete occlusion of the right MCA aneurysm (Figure 2).
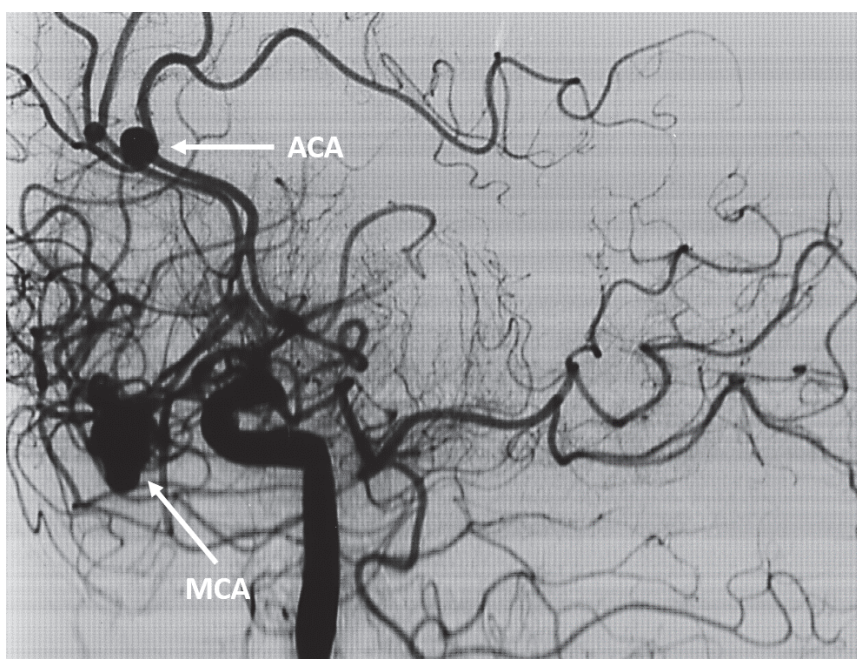

Fig. 1 – Digital subtraction angiography examination revealed middle cerebral artery bilobular aneurysm associated with two small bilateral aneurysms on the distal anterior cerebral artery segments.
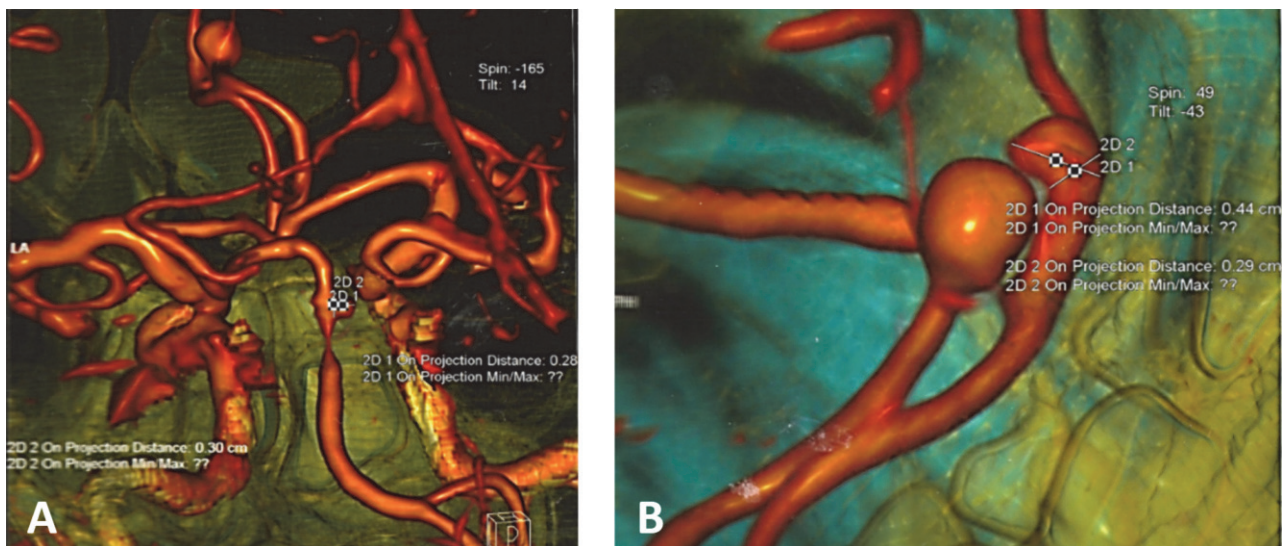
Fig. 2 – Multislice computed tomography angiography before the surgical procedure showed mirror anterior cerebral arteries aneurysms: A) anterior view; B) lateral magnified view with the smaller aneurysm measurement.

The patient was referred to our department six months later, and it was decided to perform microsurgical occlusion of the remaining ACA aneurysms. Unilateral interhemispheric approach was chosen to reach the distal ACAs. Retraction brain injury was prevented by evacuation of about 15 mL of cerebrospinal fluid by lumbar puncture preoperatively. Proximal pericallosal ACA segments were identified and then bilateral aneurysms at pericallosal-callosomarginal junction. Both aneurysms were clipped and excluded from the circulation completely, also major draining veins were preserved.

Postoperative course went well and the patient was discharged from our Department on the seventh postoperative day without any neurological deficit. Two months after the surgery, follow-up MSCT angiography revealed that all three aneurysms were completely excluded from circulation.

Discussion

Management of DACA aneurysms is a surgical challenge, therefore it is controversial whether these should be surgically clipped or coiled endovascularly, especially in cases when these are incidentally seen during the endovascular procedure for other aneurysm embolization, when a same-stage, endovascular coiling might look like a perfect approach to occlude mirror DACA aneurysms.

Although endovascular coiling is less invasive, and considered less harmful for the patient, it is associated with significantly higher periprocedural rupture [18] and procedure-related morbidity [21] than other circle of Willis aneurysms. Surgical clipping results, on the other hand, are same or slightly better than for aneurysms at other locations [16].

DACA aneurysms are still treated with microsurgical clipping more often than endovascular coiling due to their distal location and morphologic features, nevertheless surgical clipping remains demanding. Moreover, because of their rare occurrence, neurosurgeons often have the lack of experience in surgical treatment of these aneurysms [5, 7, 11, 16].

Non-experienced surgeons are avoiding to operate due to location of the DACA aneurysms in the narrow interhemispheric space [22], difficulties in establishing proximal control, and the high frequency of wide-necked and sclerotic aneurysms in this location, in particular those involving the origin of the branching arteries [4, 21].

Regarding the aneurysm size, only a few cases of a large and giant DACA aneurysms have been reported [5, 6, 14]. Average diameter at the moment of the rupture according to Gherasim et al. [19] was 5.5 mm vs. 9 mm compared with all other intracranial aneurysms which can be explained due to the lack of resistant arachnoid membranes at the level of the pericallosal cisterns. In our case, aneurysms at the distal segments of ACA, were measuring 4.5 mm on the left, and the right one measuring 6 mm in size, therefore demanding prompt surgical treatment.

According to meta-analysis of Petr et al. [23], aneurysm recurrence occurred in 3% after surgery and in 19.1% after endovascular treatment, although, in this series, there were no significant differences in procedure-related morbidity and mortality. The most important factor affecting the mortality and morbidity is the presence of associated aneurysms [5]. One stage surgery with unilateral craniotomy is suggested for bilateral DACA and mirror aneurysms, which is relatively straightforward due to their proximity [4], but also in cases when DACA aneurysms were associated with aneurysms at different location to reduce the morbidity and mortality [24, 25].

Initial haemorrhagic event related to DACA aneurysm rupture in more than a half of patients is ICH, which is considered to increase the risk of poor (lethal) outcome [26, 27]. The high incidence of ICHs, higher than for aneurysms elsewhere, is obviously related to the narrow pericallosal cistern and the dense attachments to the adjacent brain surface [26, 28]. Intraventricular hemorrhage is a little less frequent, appearing in 25%−30% of the patients [28]. This fact is also supporting our decision to proceed with surgical clipping, due to the possibility of immediate management of the intraoperative/intraprocedural rupture. Also, the

risk of ischemic event is better handled, due to better intraoperative overview and handling of the small branches originating from the aneurysm dome.

Bearing in mind the tendency for rupture regardless of small aneurysm size, high incidence of intracerebral hemorrhage, and a relatively high risk of aneurysm recurrence after endovascular treatment at this location [11, 16, 23, 26], we believe that both interventional radiologists and our decision for subsequent early microsurgical treatment was justified.

All patients with DACA aneurysms should be carefully evaluated with DSA or MSCT angiography for the presence of additional aneurysms due to the tendency for multiplicity [5,26]. Even when DACA aneurysms were revealed during an endovascular procedure, surgical treatment should be undertaken as soon as possible [21, 26]. More than one aneurysm should not be treated in the same procedure. They should be aggressively treated even if they are very small because of their tendency to early rupture [15].

Conclusion

Successful surgical management of DACA aneurysms mostly depends on understanding of their unique microsurgical anatomy and the surgeon's experience, as well as careful preparation and examination of the patient.

Sufficient brain relaxation, accurate localization of the aneurysm, early identification of the proximal ACA segment, and preservation of the major draining veins remain necessary for a safe surgery.

Acknowledgement

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Suppurative gastritis in a HIV-positive patient: A case report

Supurativni gastritis kod HIV pozitivnog bolesnika

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Abstract

Introduction. Suppurative gastritis (SG) is a rare disease characterized by a bacterial infection of the stomach wall. This condition has high mortality rate, especially in patients with predisposing factors such as alcoholism, immunodeficiency and previous endoscopic gastric procedures. **Case report**. A 41 year old male was hospitalized with epigastric pain, fever and vomiting. The symptoms started a few days after esophagogastroduodenoscopy (EGD). His personal medical history included periodical excessive alcohol consumption. Based on initial blood tests the patient was diagnosed with sepsis and was promptly started a treatment with antibiotics. In the first few days of hospitalization there was an improvement in inflammation marker levels, but the patient was still febrile and with the referred epigastric pain. A computed tomography scan showed marked thickening of the gastric wall and EGD revealed deep ulcers in the stomach with fibrinopurulent exudate. Histological examination of gastric biopsies showed necrosis and abscesses. Blood cultures were positive for *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* with subsequent change in antibiotics. The repeated blood tests showed leucopenia and the patient tested positive for human immunodeficiency virus (HIV). A second EGD showed pus in the stomach, with a gastric aspirate culture positive for *Enterococcus spp*. The treatment was modified and a third EGD showed healed gastric mucosa confirmed by histopathological evaluation. **Conclusion.** Taking in consideration the high mortality rate of SG, it is necessary to make an early diagnosis and start the treatment against specific pathogens, since it can be crucial for a better outcome of this clinical condition.

Key words:
gastritis; hiv infections; diagnosis; anti-bacterial agents; treatment outcome.

Apstrakt

Uvod. Supurativni gastritis (SG) je retko oboljenje koje karakteriše bakterijska infekcija želudačnog zida. Ovo stanje prati visoka stopa mortaliteta, naročito kod bolesnika sa predisponirajućim faktorima kao što su alkoholizam, imunodeficijencija i prethodne endoskopske procedure u želucu. **Prikaz bolesnika**. Muškarac starosti 41 godinu je hospitalizovan zbog epigastričnog bola, febrilnosti i povraćanja. Simptomi su počeli nekoliko dana nakon ezofagogastroduodenoskopije (EGD). U ličnoj anamnezi naveo je periodično ekscesivno konzumiranje alkohola. Na osnovu prvobitnih laboratorijskih nalaza, bolesniku je postavljena dijagnoza sepse i započeta antibiotska terapija. Prvih nekoliko dana hospitalizacije došlo je do poboljšanja markera inflamacije, ali su kod bolesnika perzistirali febrilnost i epigastrični bol. Kompjuterizovanom tomografijom zabeleženo je zadebljanje zida želuca, a tokom EGD viđene su duboke ulceracije i fibrinopurulentni eksudat u želucu. Histološkim pregledom biopsija želuca uočeno je prisustvo nekroze i apscesa. Hemokulture su bile pozitivne na *Stenotrophomonas maltophilia* i *Pseudomonas aeruginosa*, nakon čega je korigovana antibiotska terapija. U ponovljenim laboratorijskim nalazima zabeležena je leukopenija, a test na virus humane imunodeficijencije (HIV) je bio pozitivan. Kontrolnom EGD viđeno je prisustvo gnoja u želucu, a kulturom gastričnog aspirata izolovan je *Enterococcus spp*. Terapija je korigovana i poslednjom EGD viđena je zaceljena sluznica želuca, što je i potvrđeno patohistološkim pregledom. **Zaključak.** Uzimajući u obzir visoku stopu mortaliteta SG, neophodno je rano postaviti dijagnozu i započeti lečenje protiv specifičnog uzročnika, što može biti presudno za bolji ishod ovog kliničkog stanja.

Ključne reči:
gastritis; infekcija, hiv; dijagnoza; antibiotici; lečenje, ishod.

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Introduction

Suppurative gastritis (SG), also known as phlegmonous gastritis, is a rare disease characterized by a bacterial infection of the stomach wall [1–3]. It is usually related to *Strepotococcus* infection [4]. This condition has high mortality rate, around 27%, especially in patients with predisposing factors such as alcoholism, immunodeficiency and previous endoscopic gastric procedures [1, 5]. Early diagnosis and rapid antibiotic treatment, with or without surgery, are pivotal for the survival of patients with SG [1, 3].

Herein, we report the first case, to our knowledge, of SG caused by *Enterococcus spp.* in a human immunodeficiency virus (HIV) patient.

Case report

A 41 year old male presented at the Emergency Department of Clinical Center of Vojvodina in Novi Sad (Serbia), with a two days history of severe epigastric pain, fever and vomiting. These symptoms started a few days after the execution of esophagogastroduodenoscopy (EGD) following the eradication treatment for *Helicobacter pylori* gastritis. His personal medical history included periodical excessive alcohol consumption and multiple fractures after a car accident.

Laboratory test results on hospital admission revealed increased inflammatory markers – C reactive protein 285.9 mg/L, procalcitonin 40.76 ng/mL, increased levels of blood urea nitrogen (14.2 mmol/L) and serum creatinine (323 μmol/L) and thrombocytopenia (119 × 10^9/mL). Based on these results the patient was initially diagnosed with sepsis and was promptly started a treatment with ceftriaxone, ciprofloxacin and metronidazole. An abdominal computed tomography (CT) scan showed marked thickening of the gastric wall, splenomegaly and enlarged mesenteric lymph nodes. In the first few days of hospitalization, we registered an improvement in inflammation marker levels, but the patient was still febrile and with the referred epigastric pain. However, the blood cultures were negative.
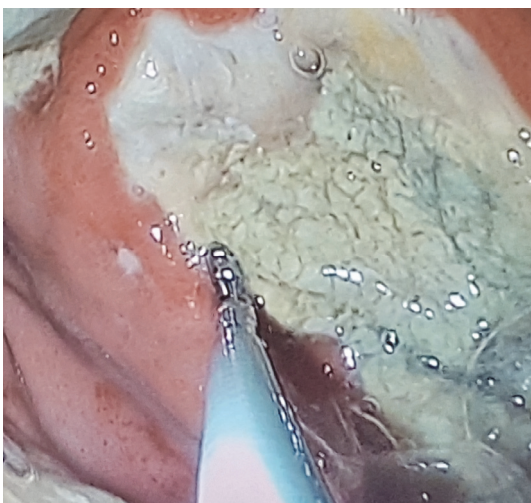
Due to the CT finding, EGD was performed and it revealed multiple deep ulcers (diameter 3.5 cm) in the gastric corpus with fibrinopurulent exudate (Figure 1). Consequently, the patient was switched to total parental nutrition. New blood cultures were positive for *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, with subsequent change in antibiotic treatment with amoxicillin and gentamycin. Histological examination of gastric biopsies showed necrotic detritus on the surface of gastric mucosa with coagulation necrosis and abscesses (Figure 2).
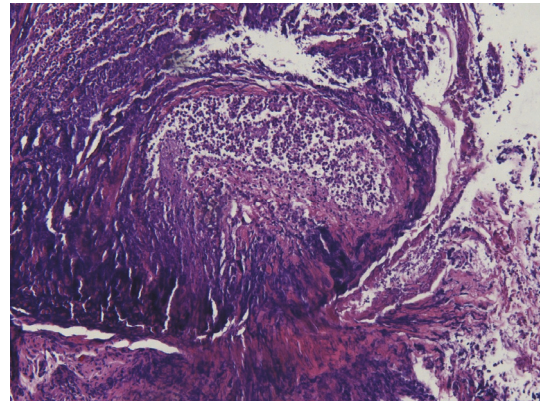
Fig. 2 – Histological examinations of gastric biopsies showed necrotic detritus on the surface of gastric mucosa with coagulation necrosis and abscesses.

Blood tests, repeated in the third week after admission, showed leucopenia, and in suspicion of immunodeficiency the patient was screened for HIV and the test came back positive. In the fifth week of illness, a second EGD was performed and showed signs of inflammation and pus in the stomach (Figure 3), with a gastric aspirate culture positive for *Enterococcus spp.* In accordance with antibiotic resistance, the treatment was modified with vancomycin, and after two weeks, a third EGD showed healed gastric mucosa confirmed by histopathological evaluation (Figure 4).

Fig. 1 – Esofagogastroduodenoscopy revealed multiple deep ulcus (diametar 3.5 cm) in the gastric corpus with fibrinopurulent exudate.

Fig. 3 – A second esophagogastroduodenoscopy in the fifth week of illness was perfomed and showed signs of inflammation and pus in the stomach.

Fig. 4 – A third esophagogastroduodenoscopy showed healed gastric mucosa confirmed by histopathological evaluation.

Discussion

SG is an exceedingly rare diagnosis [6]. It is more common in patients with gastrointestinal comorbidities that cause mucosal injury such as chronic gastritis, gastric cancer or ulcer [5]. Medical history might reveal esophagectomy, gastric biopsies and othe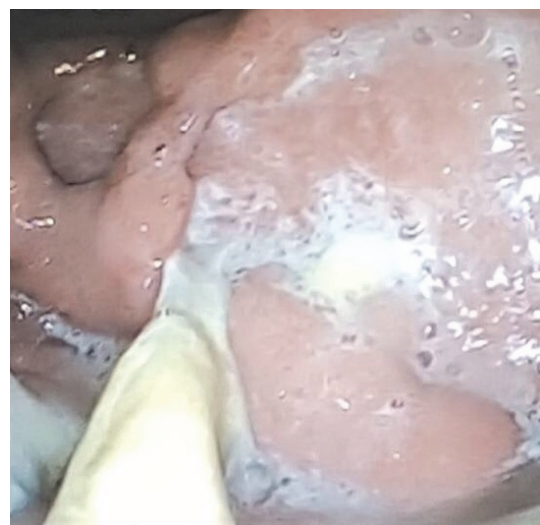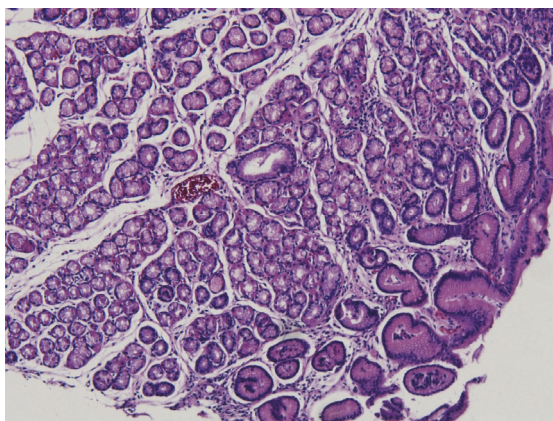r invasive procedures performed in weeks preceding the initiation of symptoms [1]. Around half of the patients diagnosed with purulent gastritis have immunosuppression factors [1]. It is also closely related to septicemia [5].

Gastric ulcers in patients with HIV infection are most commonly associated with opportunistic infections (*Mycobacterium avium*, cytomegalovirus or herpes simplex virus) or opportunistic tumors. So far, the only known case of purulent gastritis in a HIV patient was related to a patient with Kaposi sarcoma [7]. Pathogens causing SG can be identified from gastric tissue and fluid cultures [2]. The most frequent pathogen is *Streptococcus spp.* (especially *S. pyogenes*) in about 57%–70% of cases [1, 3, 4]. Other identified pathogens include *Staphylococcus spp*, *Escherichia coli*, *Haemophilus influenzae,* as well as *Proteus* and *Clostridium spp* [1, 3, 4]. Polymicrobial infection is described in around 17% of cases [1]. In our case two bacteria were identified in the blood cultures, and *Enterococcus spp.* was isolated from the gastric aspirate.

The usual clinical presentation of SG consists of severe and acute epigastric pain, fever and vomiting [5]. As these symptoms are nonspecific, SG is often misdiagnosed as more common conditions such as perforated peptic ulcer and other causes of acute abdomen [5].

SG can be initially diagnosed by EGD, abdominal CT or endoscopic ultrasound (EUS), while the deffinitive diagnosis is acquired by histological examination and culture of the gastric biopsies [5]. Typical signs of SG during EGD include erythema and edema of the gastric folds with fibrinopurulent exudate [1]. CT scan and EUS may show thickening of the gastric wall [1, 3].

Although SG predominantly involves the submucosa of the stomach, the inflammation may progress and involve all layers [3]. Histopathologically, the submucosa is thickened due to infiltration by neutrophil granulocytes and plasma cells [3, 5]. In advanced cases, possible histological findings include necrosis, abscess formation, intramural hemorrhage and thrombosis of the submucosal blood vessels [5]. Differential diagnosis of SG commonly includes superinfected malignancy, gastric lymphoma, gastrointestinal stromal tumor, tuberculosis [1, 6].

Optimal treatment for SG consists of antibiotics with surgery reserved for refractory and complicated cases [1]. The histological description was a key factor in our case because it provided prompt diagnosis and early treatment.

Conclusion

Taking in consideration the high mortality rate of SG, it is necessary to make an early diagnosis and start the treatment against specific pathogens, since it can be crucial for a better outcome of this clinical condition.

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Prenatal ultrasonographic manifestations of partial trisomy 12q(12q24.2→qter) and partial monosomy 2q (2q37.3→qter)

Prenatalne ultrazvučne manifestacije parcijalne trizomije 12q (12q24.2→qter) i parcijalne monozomije 2q (2q37.3→qter)

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Hospital, Friedrich Schiller University, [†]Institute of Human Genetic, Jena, Germany;
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Abstract

Introduction. Partial trisomy of chromosome 12 long arm is rare condition with significant clinical impact and is usually diagnosed postnatally. **Case report.** We present prenatal sonographic findings and molecular cytogenetic characterization of partial trisomy 12q and partial monosomy 2q in two consecutive pregnancies of a healthy non-consanguineous couple. A 35-year-old pregnant woman G3P1A1 was referred to genetic counseling due to sonographic anomalies detected in the fetus. First trimester ultrasound examination revealed hyperechogenic focus in the left cardiac ventricle, single umbilical artery, hyperechogenic bowel and unilateral clubfoot with knee joint ankylosis. Previous pregnancy of the couple was terminated at 26th gestation weeks due to multiple fetal anomalies: bilateral ventriculomegaly, corpus callosum hypoplasia, single umbilical artery and clubfoot. In G3P1A1, amniocentesis was performed and cytogenetic analyses revealed a derivative chromosome 2. Subsequent cytogenetic analyses of parental lymphocytes showed that paternal karyotype was normal, while maternal karyotype showed a der(2). Metaphase fluorescence *in situ* hybridization (FISH) studies demonstrated partial trisomy 12q24.2→12qter and partial monosomy 2q37.3→2qter in the fetus, resulting from an unbalanced segregation of a maternal balanced translocation t(2;12)(q37.3;q24.2). To date, this is the first such prenatally detected case. Literature search revealed three more cases of prenatally detected partial trisomy 12q and anomalies described were consistent with ones detected in present case. Our findings contribute to further clinical delineation of partial trisomy 12q. **Conclusion.** Prenatal detection of single umbilical artery, clubfoot, arthogryposis and ventriculomegaly should alert suspicion to chromosome 12q aberrations.

Key words:
pregnancy; ultrasonography prenatal; chromosome 2, monosomy 2q; chromosome 12, trisomy 12q.

Apstrakt

Uvod/Cilj. Parcijalna trizomija dugog kraka hromozoma 12 predstavlja retku hromozomsku aberaciju koja ima značajnu kliničku sliku i najčešće se dijagnostikuje postnatalno. **Prikaz bolesnika.** Prikazali smo prenatalnu ultrazvučnu sliku i molekularnu citogenetičku karakterizaciju parcijalne trizomije 12q i parcijalne monozomije 2q u dve uzastopne trudnoće kod zdravog para koji nije u srodstvu. Trudnica stara 35 godina je tokom svoje treće trudnoće upućena u genetičko savetovalište zbog ultrazvučno viđenih anomalija ploda. Na ultrazvučnom pregledu tokom prvog trimestra trudnoće uočen je hiperehogeni fokus u levoj komori srca, jedna pupčana arterija, hiperehogena creva i iskrivljeno stopalo sa ankilozom kolena. Prethodna trudnoća ovog para prekinuta je u 26. nedelji gestacije zbog multiplih anomalija ploda: obostrane ventrikulomegalije, hipoplazije žuljevitog tela, jedne pupčane arterije i iskrivljenog stopala. Amniocenteza urađena tokom treće trudnoće pokazala je prisustvo derivatnog hromozoma 2. Citogenetička analiza roditeljskog kariotipa iz limfocita periferne krvi pokazala je da je očev kariotip normalan, dok je kod majke bio prisutan derivatni hromozom 2. Metodom metafazne fluorescentne *in situ* hibridizadije (FISH) potvrđena je parcijalna trizomija 12q24.2→12qter i parcijalna monozomija 2q37.3→2qter kod fetusa kao posledica nebalansirane segregacije maternalnih hromozoma. Do danas, ovo je prvi ovakav slučaj dijagnostikovan prenatalno. Prema literaturnim podacima, u tri do sada objavljena slučaja parcijalne trizomije 12q opisane su anomalije koje su u skladu sa anomalijama uočenim kod fetusa iz ovde prikazanog slučaja. Naša studija doprinosi daljoj kliničkoj karakterizaciji parcijalne trizomije 12q.

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Zaključak. Prenatalno uočena jedna pupčana arterija, iskrivljeno stopalo, artrogripoza i ventrikulomegalija treba da ukažu na moguće postojanje aberacije dugog kraka hromozoma 12.

Introduction

Unbalanced chromosomal aberrations are rare findings at prenatal diagnosis but they have a significant clinical impact. Partial monosomy 2q and partial trisomy 12q is rarely described in literature. So far, only three cases with partial trisomy 12q24.2 have been seen prenatally [1–3]. We present case on prenatal ultrasound findings in two consecutive pregnancies of a women carrier of balanced translocation t(2;12)(q37.3;q24.2). Since the fetuses were affected with similar pattern of congenital anomalies our findings contribute to further clinical delineation of partial trisomy 12q.

Case report

A 35-year-old pregnant woman was referred for genetic counseling in her third pregnancy due to sonographic anomalies detected in fetus. The women and her partner were healthy and non-consanguineous Caucasians. The first pregnancy of the couple resulted in birth of a healthy boy. In the second pregnancy, first trimester biochemical screening for chromosomal abnormalities showed increased risk for T21 (1:71). Amniocentesis was performed and karyotype was normal (46,XY, banding level not available) when checked by a local community hospital. Level II ultrasound examination at 22nd week of gestation (w.o.g.) revealed several abnormalities in fetus: bilateral ventriculomegaly, single umbilical artery and bilateral clubfoot. Fetal brain magnetic resonance imaging (MRI) scan was performed and it confirmed presence of moderate symmetrical bilateral ventriculomegaly with hypoplasia of corpus callosum rostral part.

Pregnancy was terminated at 26 w.o.g. due to multiple fetal anomalies on parents request.

During third pregnancy, fetal ultrasound examination at 14th + 5 w.o.g. showed presence of hyperechogenic focus in the left cardiac ventricle, single umbilical artery, hyperechogenic bowel and unilateral clubfoot with knee joint ankylosis (Figure 1). The fetal biometry was appropriate for gestational age. First trimester biochemical screening was below cut-off for trisomies 13, 18 and 21. At 18th w.o.g., level II ultrasound examination revealed borderline dilatation of lateral ventricles and confirmed previous sonographic findings (Figure 1). Second trimester biochemical screening showed high risk for trisomy 21 (1:86).The pregnancy was terminated at 22nd w.o.g. on parents' request.

Amniocentesis was performed and cytogenetic analyses applying G-banding techniques (550 bands) revealed a derivative chromosome 2 in male fetus. Subsequent cytogenetic analyses of parental lymphocytes showed that paternal karyotype was normal, while maternal karyotype also showed a der(2). Metaphase fluorescence *in situ* hybridization (FISH) studies demonstrated partial trisomy 12q (12q24.2→qter) and partial monosomy 2q (2q37.3→qter) in the fetus, resulting from an unbalanced segregation of a maternal balanced translocation t(2;12)(q37.3;q24.2) (Figure 2). Fluorescence *in situ* hybridization using a subtelomeric probe for chromosome 2qter (Abbott, Vysis) and whole chromosome paints for chromosomes 2 and 12 (home made probes) confirmed the findings (Figure 2).

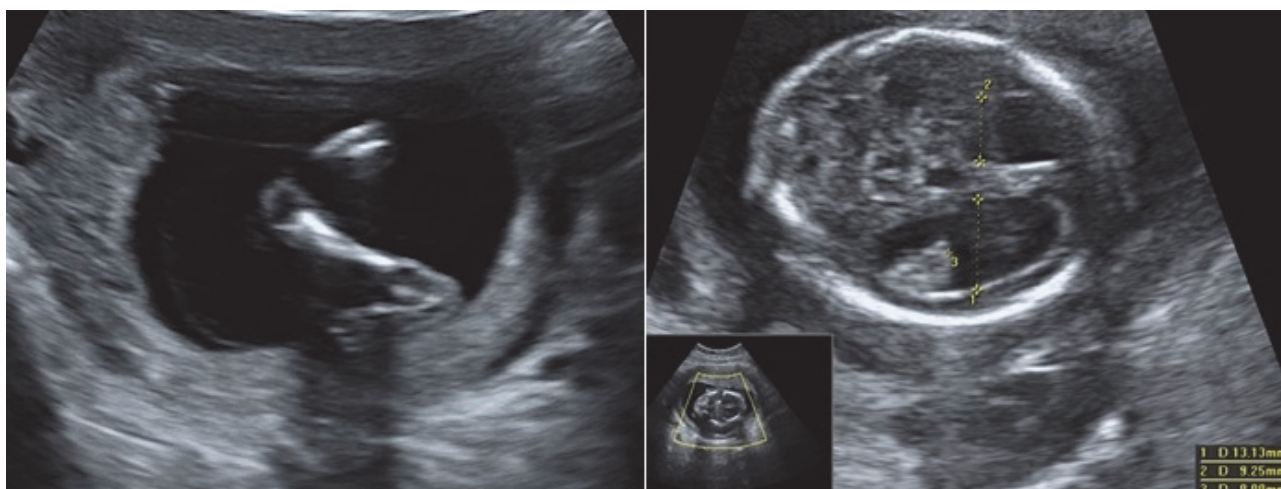The study follows the principles of Declaration of Helsinki.

Fig. 1 – Ultrasound image showing: A) clubfoot, and B) ventriculomegaly.
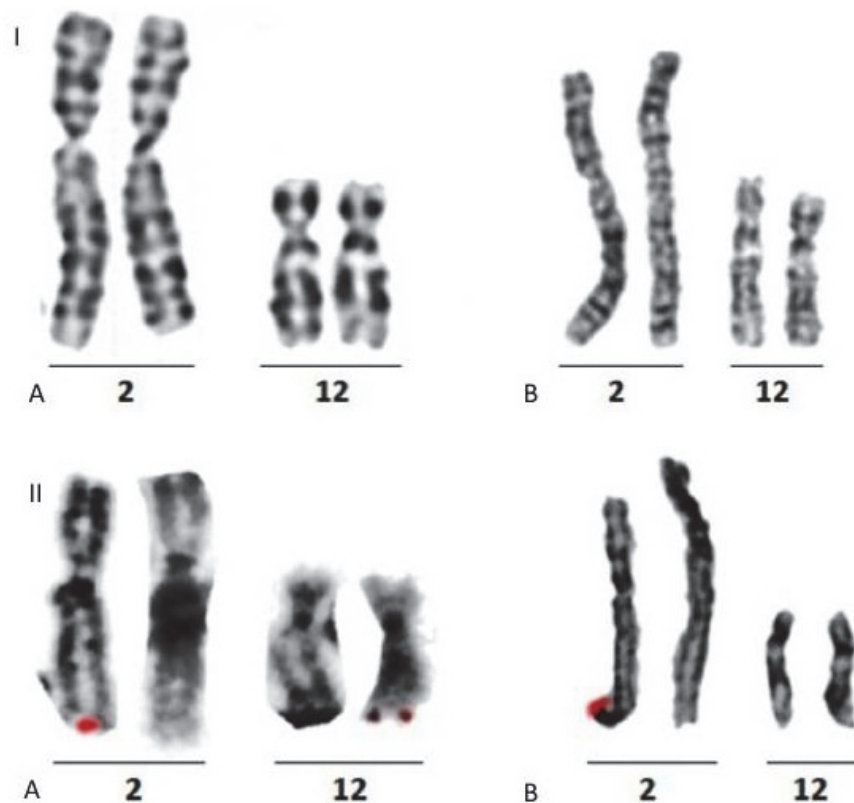
Fig. 2 – Part I: Maternal chromosomes showing balanced 2;12 translocation (A). Fetal chromosomes showing der (2) (B); Part II: fluorescence *in situ* hibridization (FISH) results of balanced and unbalanced situation as present in mother (A), and fetus (B), respectively. Only FISH results for subtelomeric probe 12qter (subtel12qter) are depicted.

Discussion

To date, this is the first case report of early prenatal manifestations of partial trisomy 12q24.2-12qter and partial monosomy 2q37.3-2qter. Although karyotype of the first fetus was reported as normal, based on pattern of detected anomalies in the fetus as well as fact that mother is a balanced translocation carrier, we suggest that it also carried der(2), but that due to relatively small size of translocated segments it escaped detection. Occurrence of almost identical fetal abnormalities in two consecutive pregnancies described here (single umbilical artery, clubfoot and bilateral borderline ventriculomegaly), further contributes to the efforts to establish the partial trisomy 12q as a clinically recognizable syndrome. As the imbalance on chromosome 2 is according to FISH only in the range of 2–3Mb, literature search was concentrated on partial trisomy 12q only. The latter retrieved three more cases with prenatally diagnosed duplication of similar 12q segment. Peng et al. [3] described male fetus who had partial trisomy 12q21.2-12qter with prenatal sonographic findings of thick nuchal fold, pericardial effusion, arthrogryposis, single umbilical artery, micropenis and ventriculomegaly. Chen et al. [1] reported a case of partial duplication 12q24.32-12qter in a male fetus with microcephaly, cerebellar hypoplasia, borderline ventriculomegaly, micrognathia, ventricular septal defect (VSD) and rocker-bottom feet. Third case of partial duplication 12q24.21-12qter presented prenatally was described with single umbilical artery,

micrognathia, ventriculomegaly, thick nuchal fold and coarctation of the aorta [2]. Although chromosomes involved in rearrangements, as well breakpoints on chromosome 12 differ among described cases, it is plausible to assume that central nervous system (CNS) malformations (ventriculomegaly, corpus callosum hypoplasia/agenesis, cerebellar hypoplasia), foot deformity and absence of one umbilical artery can comprise a basis of prenatal manifestations for 12q duplication syndrome. Also, in several cases of postnataly diagnosed duplication 12q corpus callosum anomalies (hypoplasia, partial agenesis) and foot malformations have been described [4,5]. Phenotypic contribution of monosomy 2q37.3 cannot be excluded in our cases, but based on literature review of case reports with pure duplications involving 12q24 region we believe that described anomalies are more consistent with partial trisomy 12q [6–9].

Conclusion

Additional studies are needed in order to make more precise genotype-phenotype correlations, but prenatal detection of single umbilical artery, clubfoot, arthogryposis and ventriculomegaly should alert suspicion to chromosome 12q aberrations.

Acknowledgement

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Listeria monocytogenes multifocal cerebritis in an immunocompetent adult

Listeria monocytogenes multifokalni cerebritis kod imunokompetentnog bolesnika

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Abstract

Introduction. Multifocal cerebritis is a rare and severe disease and just a several cases caused by *Listeria monocytogenes* were described in the literature. **Case report.** A 64 year old man was admitted to the hospital with disturbed consciousness (Glasgow Coma Scale score: 9) after being febrile for 16 days with history of fever, headache and middle ear pain. He did not have any other comorbidities neither he was immunocompromised. Penicillin allergy was noted for him. On neurologic exam, meningeal or focal neurologic signs were not evident, but computed tomography (CT) brain scan with contrast injection showed 3 hypodense zones in the occipital and 1 in the right temporal lobe. Laboratory findings in blood and cerebrospinal fluid (CSF) were indicative for the infectious nature of changes in the endocranium (multifocal cerebritis). Initial therapy was the combination of cefotaxime, amikacin and metronidazole, but after the isolation of *L. monocytogenes* from CSF and blood culture, therapy was switched to co-trimoxazole. Recovery of consciousness with establisment of alert state occurred after 6 days of co-trimoxazole administration. Total therapy took 36 days. During that period all clinical and laboratory parameters normalized. The patient was discharged as recovered, with sequelas of amnesia and slurring of speech. **Conclusion.** In the treatment of multifocal cerebritis caused by *L. monocytogenes,* adequate choice and long-term therapy with antibiotics are necessary. The drug of choice is ampicillin but in the case of allergy to it, co-trimoxazole is a good replacement.

Key words:

meningitis, listeria; listeriosis; anti-infective agents; drug combinations; tomography; trimetoprim, sulfamethoxazole drug combination.

Apstrakt

Uvod. Multifokalni cerebritis koji uzrokuje *Listeria monocytogenes* je retko i teško oboljenje koje je u literaturi opisano samo u nekoliko slučajeva. **Prikaz bolesnika.** Bolesnik star 64 godine primljen je u bolnicu poremećene svesti (*Glasgow Coma Scale* skor: 9) nakon 16 dana prethodne febrilnosti, glavobolje i bola u desnom uvu. Nije imao drugih prethodnih bolesti, niti je bio imunokompromitovan. Dobijen je podatak o alergiji na penicilin. Pri neurološkom pregledu nisu evidentirani meningealni znaci i fokalni neurološki poremećaji, a snimak endokranijuma kompjuterizovanom tomografijom sa kontrastom pokazao je tri hipodenzne zone u okcipitalnom i jednu u desnom temporalnom lobusu. Laboratorijski nalazi u krvi i cerebrospinalnoj tečnosti upućivali su na infektivnu prirodu promena u endokranijumu (multifokalni cerebritis). Incijalna terapija bila je kombinacija cefotaksima, amikacina i metronidazola, a nakon izolacije *L. monocytogenes* u kulturi cerebrospinalne tečnosti i hemokulturi, terapija je zamenjena ko-trimoksazolom. Oporavak stanja svesti sa uspostavljanjem budno-svesnog stanja nastupio je nakon šest dana od primene ko-trimoksazola. Ukupno trajanje terapije ko-trimoksazolom iznosilo je 36 dana. U tom periodu normalizovali su se svi klinički i laboratorijski parametri. Bolesnik je otpušten kao oporavljen, sa sekvelama amnezije i usporenog govora. **Zaključak.** U lečenju multifokalnog cerebritisa uzrokovanog *L. monocytogenes* neophodan je adekvatan izbor i dugotrajna primena antibiotske terapije. Lek izbora je ampicilin, ali u slučaju alergije na njega, ko-trimoksazol predstavlja dobru zamenu.

Ključne reči:

meningitis, listeria; infekcija, listerija; antibiotici, kombinovani; kotrimoksazol; tomografija

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Introduction

Listeria monocytogenes is an important bacterial agent which affects patients with immunosuppression. The most common manifestation of listeria infection involving the central nervous system (CNS) is meningoencephalitis; other less common manifestations include rhomboencephalitis, *ie* brainstem encephalitis (encephalitis of the pons and medulla), and cerebritis with abscess in the absence of meningitis [1]. Brain abscess, recorded in about 1% of affected by this bacterium [1] is a focal form of the infection which usually begins as cerebritis. Multifocal cerebritis is a rare and severe disease and just a several cases caused by *L. monocytogenes* were described in the literature [2–5].

Listeriosis can be a deadly disease: when CNS is involved, fatality rate is 36% [6]; for neurolisteriosis in blood-culture positive patients, mortality is significantly higher [7]. Even when the listeria neuroinfection resolves, sequelae can persist, ranging from neurologic to psychiatric. Psychiatric sequelae can be episodic attacks of stupor or semi-stupor, psycho-organic syndrome, and loss of intellectual abilities with difficulty in concentration and a generalised apathy [8–10].

L. monocytogenes is sensitive to a wide range of antibiotics, but resistant to third generation cephalosporins, usually given as the first line antibiotics when bacterial infections of CNS are suspected. That is why the role of microbiology laboratory is to warn the clinicians about the specificities of the antimicrobial susceptibility of the pathogen as soon as possible, thus increasing the possibility of patient's survival and better recovery. We present the case of the *L. monocytogenes* multifocal cerebritis treated with co-trimoxazole, without surgical intervention.

Case report

A 64 year old man was admitted to the Intensive Care Unit of the Clinic for Infectious and Tropical Diseases of the Clinical Center of Serbia in Belgrade, with disturbed consciousness (Glasgow Coma Scale score: 9) with 16-day history of mild fever (38 °C), headache and infection of the right middle ear, although the discharge from the ear could not be obtained. He did not take antibiotics. His medical history before that was unremarkable, with no immunosuppressive diseases or alcoholism. Penicillin allergy was noted for him. Upon physical examination, he had a body temperature of 38.2 °C and the meningeal signs were not present. Neurologic examination did not show any focal signs, but the computed tomography (CT) brain scan with contrast injection showed three hypodense zones in the occipital and one in the right temporal lobe (Figure 1). Because of febrile condition and disturbed consciousness, neuroinfection was highly suspected (multifocal cerebritis) and decision of lumbar puncture was made. Obtained cerebrospinal fluid (CSF) was opalescent, containing 520 cellular elements, 80:20 ratio of polymorphonuclear leucocytes and lymphocytes, glucose 0.7 mmol/L (blood glucose 5.7 mmol/L), proteins 1.61 g/L, CRP 9 mg/L. Laboratory data from blood showed the following results: erythrocyte sedimentation rate 70 mm/h, neutro-

phils 21.4×10^9, fibrinogen: 6.9 g/L, CRP: 45 mg/L. CSF and blood samples were immediately sent for culture. Initial empirical antimicrobial therapy for multifocal cerebritis with cefotaxime, amikacin and metronidazole was prescribed.
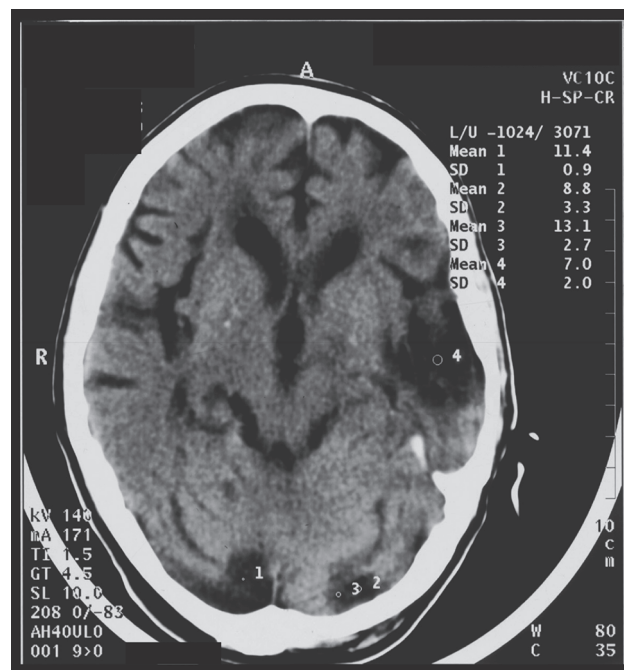

Fig. 1 – Patient's computed tomography (CT) brain scan on presentation; 4 foci visible marked 1, 2, 3 and 4.

The first results from the laboratory revealed rare neutrophils in direct smear of CSF. The next day sparse gray colonies grew on blood agar and they appeared as Gram positive rods on Gram stain, catalase positive and capable of esculin hydrolysis. API *Listeria* system (bioMerieux, Marcy-l'Etoile, France) was set up immediately: the next day it revealed *L. monocytogenes*, code: 6510. The same agent grew from blood culture. The strain was susceptible to penicillin, ampicillin, gentamicin, vancomycin, meropenem, erythromycin, rifampicin and co-trimoxazole when tested by disc diffusion method. On the third day of the patient's stay at the hospital, the antibiotic therapy was switched to co-trimoxazole (20 mg/kg based on trimethoprim component, divided in 4 doses, in a total daily dose of 1,600 mg).

The improvement occurred after 6 days, when his mental status has improved and gradually it became normal (Glasgow Coma Scale score: 15); headache disappeared, as well as fever and pain in the middle ear. Therapy with co-trimoxazole took 36 days in total. At the end of the therapy all parameters of inflammation were in normal range: in blood – erythrocyte sedimentation rate was 18 mm/h, leukocites 6.7×10^7, fibrinogen 3.7 g/L, CRP 6 mg/L, and in CSF – 3 cellular elements (lymphocites), glucose 2.9 mmol/L (blood glucose 5.2 mmol/L), proteins 0.49 g/L, CRP < 0.5 mg/L. Control bacterial cultures of blood and CSF were sterile. The patient refused control CT imaging because of the fear of irradiation. After getting satisfactory results, he was discharged, but sequelae persisted. They were of psychic nature – he complained of mild amnesia and slurring of speech.

Discussion

Listeriosis is often associated with certain serious illnesses, namely haematological malignancies or cirrhoses or other immunosuppressive comorbidities [7], presenting as opportunistic infection in diseases where cellular immunity is already impaired. In presenting case, the only disorder the patient suffered from before he lost consciousness was middle ear infection, fever and headache of the duration of 16 days. The data lead to the presumption that middle ear infection can be the source, but we could not find any report about *L. monocytogenes* as a causative agent of that infection in adults, so the theory of contiguous focus of infection from that part of the body, although frequently proven for brain abscess of other etiologies [11, 12], most likely could not be in this case.

The second route of infection like cerebritis is intraaxonal. *L. monocytogenes* can invade the brain tissue by migrating along cranial nerves: V, VII, IX, X, and XII, all of them innervating the oropharynx [13]. It can be speculated that in the present case the bacterium gained entrance to cranial nerves from oropharynx, spread within them and consequently invaded the brain tissue since it was capable of retrograde intra-axonal migration [13]. The ability of *L. monocytogenes* to invade cells, including endothelium of cerebral capillaries in CNS, may favored its spread from CNS to the rest of the body [14], the reason why microorganisms were recovered from blood culture, in addition to the culture of CSF. Corroboration of that thesis is experiment with a rat model of brain abscess caused by *L. monocytogenes* when infectious agent was uniformly present in the circulation of infected rats despite the intracisternal route of infection [14]. Bacteremia is an important feature in human cases of cerebritis due to *L. monocytogenes* [12], while generally in brain abscesses, the report yield of blood cultures is modest, 14%–50% [15].

The third pathogenetic mechanism of the infection in the present case lies in fact that *Listeria* typically enters the body through the gastrointestinal tract, after ingestion the contaminated food. This is the most probable mechanism of development of multifocal cerebritis in the present case. In infected hosts, the bacteria colonize the gut, cross the intestinal wall at Peyer's patches to invade the mesenteric lymph nodes and *via* the lymphatic circulation access the blood. Bacteria are continuously removed from blood by the reticuloendothelial system, but once they become sequestrated in the liver and spleen, they multiply in intracellular sites, including resident macrophages and hepatocytes. Early recruitment of polymorphonuclear cells lead to hepatocyte lysis, creating necrotic foci and thereby bacterial release in the circulation. This causes prolonged septicaemia, thus exposing the brain to infection [16]. Bone marrow has a key function in that process: a specific subset of its monocytes, marked Ly-6ChighCD11bpos, are recruited to transport *Listeria* from the bone marrow to bloodstream and from there into the brain [17].

L. monocytogenes gains access to the brain parenchyma via the cerebral capillary endothelium, a single layer of specialized human brain microvascular endothelial cells characterized by tight junctions. *L. monocytogenes*-infected monocytes can penetrate these endothelial cells via the middle cerebral artery resulting in cerebritis and, subsequently, brain abscess formation [1]. It seems that in the pathogenesis of neuroinfection caused by *L. monocytogenes*, persistent bacteremia is necessary [18] and it has been confirmed by the studies of Cone et al. [1] or Dee and Lorber [12], who reviewed three and eight cases of multiple cerebral abscess, respectively. In all of them, the etiologic agent was isolated from blood culture, like in the present case report, and in our patient it was probably manifested by mild fever and headache.

Fever, altered sensorium and headache are the most common symptoms of CNS listeriosis, but 42% of patients do not have meningeal signs on admission. Compared with patients with acute meningitis due to other bacterial pathogens, patients with *Listeria* infection had a significantly lower incidence of meningeal signs, and so it was with our patient. Lumbar puncture was performed on the admission because the patient had disturbance of consciousness, fiver and laboratory findings which implied neuroinfection.

Patients with brain abscess, encephalitis, or rhombencephalitis should be treated for at least 6 weeks and this is the reason for duration of therapy of 36 days. The combination of ampicillin with gentamicin is generally recommended as a first-line therapy for the treatment of listeriosis in humans [19, 20]. Studies *in vitro* or on animal models show the higher activity of penicillin antibiotics (ampicillin or amoxicillin) or combination of penicillin and aminoglycoside antibiotic, or quinolones [14, 21] than co-trimoxazole, although the last antibiotic penetrates the cell wall well and has bactericidal activity. In cases of penicillin hypersensitivity, cotrimoxazole is the treatment of choice.

There are scarce data in the literature about the usage of co-trimoxazole in invasive human listeria infections, but according to some case reports it seems to have a good effect [22–25]. A retrospective study of 22 cases of listeria meningoencephalitis even demonstrated superiority of that antibiotic combined with ampicillin over gentamicin with ampicillin [26]. Cephalosporins have limited activity against listeria. Vancomycin has poor penetration into the central nervous system due to its hydrophilic nature and high molecular weigh [27]. Although meropenem has better *in vitro* activity then ampicillin, clinical data are not conclusive and failure after treatment was suspected on the basis of case-reports [28].

This report is a confirmation of the efficacy of cotrimoxazole in the conservative treatment of severe disease such as listeria multifocal cerebritis with bacteremia. Amnesia and slurring of speech can appear insignificant in the absence of more severe psychiatric syndromes and highly lethal disease.

Conclusion

Multifocal cerebritis due to *L. monocytogenes* in immunocompetent patients is rare diseases. Co-trimoxasole as somewhat neglected antibiotic showed good efficiency as alternative choice in the patient allergic to penicillin. This case showed that a severe CNS infection can be cured by sufficiently long therapy with co-trimoxasole.

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Vignettes on the Ervin G Erdös's visit to Yugoslavia

Kratke priče o poseti Ervina G. Erdosa Jugoslaviji

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This article includes my personal reminiscences on the great American scientist Ervin G. Erdös (1922–2019) who was born in Budapest. He got his MD in Munich in 1952, and worked there, as a research fellow with Eugen Werle who discovered kallikrein [1]. When Erdös came to the USA, he continued to work on peptides and peptidases at the universities of Pittsburgh, Oklahoma City, Dallas and Chicago. His discoveries on enzymes that generate and inactivate various biologically active peptides contributed to our understanding of the renin-angiotensin and kallikrein-kinin systems [2, 3]. I collaborated with him during the period of almost half century, and I often worked in Dr. Erdös's research laboratories in Oklahoma City, Dallas, and Chicago. He visited me in Sarajevo, as a Fulbright Visiting Professor [4]. Among other research findings, we discovered angiotensin-converting enzyme (ACE) in the retina, that opened up a new research direction for many scientists interested in ocular diseases [5, 6]. We shared research interests through visits across the Atlantic between the former Yugoslavia and the United States.

In the following very short stories, the American Professor Ervin G. Erdös is presented as an American Professor, and the author of this article as his host companion.

One day in the nineteen seventy-six, an American Professor arrived at the Zagreb airport. He had been invited by a colleague to visit the University of Sarajevo. On this particular day, he and several other passengers passed through passport control without anyone noticing. All airport employees were distracted by watching their national team compete in a soccer match on TV.

The Professor stayed in Sarajevo for three weeks as a Fulbright Visiting Professor, where he was a consultant and lecturer at the medical school. His hosts had arranged for him to visit Split, Sombor, and Belgrade afterwards, to show off the beauty of their country. During these side trips, he had many interesting encounters that he would later relate to his colleagues back home in Dallas and Chicago. He especially liked to retell the story on the police chief in Split, Sombor market, visit to charda, and peasant household in Ravno Selo. Some of his stories are presented in the following vignettes.

The Professor and his host companion from Sarajevo drove toward Split and did some sightseeing in Dalmatia. An unexpected problem arose in Split when the reception desk clerk at his hotel noticed that the Professor's passport lacked a seal with the date of his entry into the country. A summons to the police department (the SUP) followed. His host companion immediately called a colleague from Split to come, and these two fellows accompanied the Professor to the SUP. The head of the city police read to them aloud an extract from the Law about foreigners. The companion translated in English the statement that a person who enters the country illegally must leave within 24 hours. "But I arrived in Zagreb on a practically empty plain," the Professor said. "And I with other passengers passed through passport control without anyone noticing because all the employees at that time were watching the Yugoslav national team compete in a soccer match." The head of the SUP digested this information with little effect. The young man from Split then spoke up. "Look here; this is a distinguished scientist with a worldwide reputation. He is a man of integrity, and I would personally guarantee for him as a citizen of split." Again, this plea fell upon deaf ears. The man from Split argued passionately, but the head of the SUP was resolute. The host companion could see

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that they were at an impasse-there seemed to be no hope of a positive resolution. Then he had an idea, and he remarked: "After visiting Split, the Professor is invited to Sombor to stay for two days in Tito's villa. He will lecture there to a select audience of doctors and scientists from the Vojvodina Province."

As the host companion had hoped, his reference to the Yugoslavian president had an immediate impact. The SUP head started to apologize to the Professor for having kept him for so long, and offered drinks all around. Although they politely declined the drinks, the Professor was much amused by that sudden burst of hospitality. The truth was that the University of Sarajevo had arranged for him to stay in a beautiful villa owned by the municipality of Sombor. This property originally belonged to Tito's Deputy President, Aleksandar Ranković, and when he lost his position, the villa was retained for use by distinguished guests.

The Professor stayed two nights in Sombor. As promised, he addressed a group of local doctors and selected professors from the Medical School in Novi Sad. As in other places in Yugoslavia, he visited the local market where all the country folk gathered to sell and buy their produce. There was much material for his camera in the markets, especially since that particular Friday was a big market day in Sombor. As usual, the market scene was an impressive medley of people, products, and languages and cultures. In addition to the dialects of Serbo-Croatian, Hungarian, Ukrainian, other languages could be heard. Even before daybreak, more than a hundred people from the neighboring villages began to arrive with their goods, disturbing the quiet of the still sleeping little town. The din of cars and vans, complete with the rattle of wagons and the cries of people. The pre-dawn commotion soon gave way to the bustle of citizens appearing by the dozens to purchase the various local goods on display, ranging from the fresh country produce to small farm animals. There were manufactured goods available and supplied by artisans and factories from different parts of Yugoslavia, Hungary, Romania, Ukraine, and Italy. Sombor Cheese is one of the best known homemade products of the region. This delicate cheese is made from whole milk and is sold in small vats weighing 5–10 lbs. There is a widely circulated story that Sir Winston Churchill was so fond of the Sombor Cheese, that a package of cheese was regularly forwarded to him.

One evening, the Professor was invited by his host companion to drive to Apatin for dinner. A half hour's drive took the Professor and some friends to a charming charda on the Danube. The name charda comes from the Hungarian language; it refers to any one of the small, pleasant restaurants located along the river featuring good local food and drink and the music of Tsigani, or Gypsy, musicians. The guests could also participate in folk dances, such as the czardash. This dance from the Carpathian basin originated in the homeland of the legendary Count Dracula, who became known in the literature as a vampire. (Vampire is one of the very few words by which, according to W.W. Skeat's Etymological dictionary, the Serbian language has enriched the English language.)

That night the charda served fish paprikash, roasted carp, homemade noodles, and Žilavka, the famous wine from the Mostar region. The headwaiter invited the Professor and his guests to see how the paprikash was prepared. At least twenty small kettles were suspended on chains over an open fire. The chief cook pointed out the one in which their stew was cooking. Meanwhile, a dozen Gypsy musicians played Serbian, Hungarian, and Gypsy songs on violins, tamburitzas, and contrabasses. As the meal was ending, the orchestra leader approached the Professor's table to play and sing according to particular requests. It was clear that they regarded his visit as a great honor.

After his stay in Sombor, the Professor and his host companion stopped in Ravno Selo to visit his host guides's relatives where he could see a typical home in the village of Vojvodina. The Professor was amazed not only by their tidy house with its large, well-ordered garden, a tractor, and other farm equipment, but also by the fact that they had a telephone in the house. His reaction to see the telephone was not surprising, because even as late as more than 40 years ago, a telephone was a rarity in many parts of the world, including villages of Europe.

On Tuesday, November 12th, 2019 Dr. Erd's called me. He invited me for a visit and at the very end, added: "Rajko, despite my bad condition, on some days, I feel quite well, and I would like to go with you out to a café." I knew that his poor health was not good enough for that, so I decided to visit him instead. I would make him some Turkish coffee, just like we often did when he visited my laboratory in Sarajevo as a Fulbright Visiting Professor [7].

The next day Ervin went to the hospital for a scheduled appointment, but unfortunately, he did not return home. His condition suddenly worsened, and he died two days later, on Sunday, 17 November 2019. He was 97 years old.

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C O R R I G E N D A

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2. In the Case Report by **_Zorica Gajinov, Tatjana Roš, Milana Ivkov-Simić, Branislava Gajić, Sonja Prčić, Milan Matić_**: „Tick-borne lymphadernopthy acquired in Serbia – report of two cases (Ubodom krpelja izazvana limfadenopatija – porikaz dve bolesnice zaražene u Srbiji). **Vojnosanit Pregl 2018; 75(11): 1134–1137. (https://doi.org.10.2298/VSP161223035G),**

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Literatura

U radu literatura se citira kao superskript, a popisuje rednim brojevima pod kojima se citat pojavljuje u tekstu. Navode se svi autori, ali ako broj prelazi šest, navodi se prvih šest i *et al*. Svi podaci o citiranoj literaturi moraju biti tačni. Literatura se u celini citira na engleskom jeziku, a iza naslova se navodi jezik članka u zagradi. Ne prihvata se citiranje apstrakata, sekundarnih publikacija, usmenih saopštenja, neobjavljenih radova, službenih i poverljivih dokumenata. Radovi koji su prihvaćeni za štampu, ali još nisu objavljeni, navode se uz dodatak „u štampi". Rukopisi koji su predati, ali još nisu prihvaćeni za štampu, u tekstu se citiraju kao „neobjavljeni podaci" (u zagradi). Podaci sa *Interneta* citiraju se uz navođenje datuma pristupa tim podacima.

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Tabele

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

Ilustracije

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

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

Skraćenice i akronimi

Skraćenice i akronimi u rukopisu treba da budu korišćeni na sledeći način: definisati skraćenice i akronime pri njihovom prvom pojavljivanju u tekstu i koristiti ih konzistentno kroz čitav tekst, tabele i slike; koristiti ih samo za termine koji se pominju više od tri puta u tekstu; da bi se olakšalo čitaocu, skraćenice i aktinome treba štedljivo koristiti.

Abecedni popis svih skraćenica i akronima sa objašnjenjima treba dostaviti pri predaji rukopisa.

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