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CONTENTS / SADRŽAJ

Howard Taylor Ricketts (February 9, 1871–May 3, 1910), left, and Stanislaus Josef Mathias von Prowazek (November 12, 1875–February 17, 1915), right, renowned scientists who investigated the causes of many infectious diseases, including epidemic typhus (lat. *Typhus exanthematicus*). The cause of this disease, the bacterium *Rickettsia prowazekii*, was named after them.

During the First World War, an epidemic of typhus broke out in Serbia. You can read about how the *Politika* daily newspaper reported on the epidemic, on pages 789–793.

Hauard Tejlor Rikets (9. februar, 1871–3. maj, 1910), levo, i Stanislav Jozef Matijas fon Provazek (12. novembr, 1875–17. februar, 1915), desno, poznati su naučnici koji su istraživali uzroke mnogih zaraznih bolesti uključujući i pegavi tifus (lat. *Tiphus exanthematicus*). Uzročnik ove bolesti, bakterija *Rickettsia prowazekii*, nazvana je po njima.

Tokom Prvog svetskog rata, u Srbiji je izbila epidemija pegavog tifusa. Kako je dnevni list *Politika* izveštavao o toj epidemiji, možete pročitati na str. 789–793.

ORIGINAL ARTICLES

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Is it appropriate when the Heart Team changes the decision regarding the modality of myocardial revascularization?

Da li je u redu kada kardiohirurški konzilijum promeni odluku o načinu revaskularizacije miokarda?

Stefan Veljković*, Maja Milošević*, Miodrag Ostojić*†, Srdjan Bošković*†, Aleksandra Nikolić*†, Milovan Bojić*‡, Petar Otašević*†

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Abstract

Background/Aim. Decision-making by the Heart Team is an established way of making appropriate decisions regarding the management of patients with coronary artery disease. In clinical practice, it is not infrequent to see changes in decisions made by different Heart Teams. However, clinical implications regarding changes in the Heart Team decisions are not clear. The aim of this study was to determine clinical implications of change in the Heart Team decision in patients in whom surgical myocardial revascularization was advised first but consequently changed to percutaneous coronary intervention (PCI). **Methods.** We retrospectively analyzed data for 1,501 patients admitted to a single tertiary care high-volume center for coronary artery bypass grafting (CABG). In all patients, decisions were made by the Heart Team prior to admission. Upon admission, decisions were reevaluated by another Heart Team. The decision regarding the mode of revascularization was changed in 73 (4.86%) of patients. Propensity matching was made with patients from the same population who underwent CABG. Patients in both groups were followed for major adverse cardiac events (MACE) and total mortality for 12 months. **Results.** PCI and CABG groups were balanced with respect to demographic and clinical characteristics. All patients had two- and three vessel disease, with similar incidence of left main stenosis (26% in the PCI group and 30.10% in the CABG group). EuroSCORE II was similar between the groups (2.48 ± 2.38 vs. 2.36 ± 2.92). During the follow-up period, a total of 5 (6.80%) MACE in the PCI group and 12 (5.80%) MACE in the CABG group were observed (log rank 0.096, $p = 0.757$). A total of 6 (8.20%) patients died in the PCI group, and 15 (7.30%) patients died in the CABG group (log rank 0.067, $p = 0.796$). **Conclusion.** Our data indicate that patients in whom CABG was advised first but consequently changed to PCI have a prognosis similar to CABG patients over 12 months after the index procedure.

Key words:
cardiologists; coronary disease; decision making; mortality; myocardial revascularization; percutaneous coronary intervention; treatment outcome.

Apstrakt

Uvod/Cilj. Odlučivanje od strane kardiohirurškog konzilijuma je uspostavljen način donošenja odgovarajućih odluka koje se tiču zbrinjavanja bolesnika sa oboljenjem koronarnih arterija. U kliničkoj praksi nisu retkost promene u odlukama različitih kardiohirurških konzilijuma. Međutim, kliničke implikacije u vezi sa promenama odluka kardiohirurških konzilijuma nisu jasne. Cilj rada je bio da se utvrde kliničke implikacije promene u odluci kardiohirurškog konzilijuma kod bolesnika kojima je prvo preporučena hirurška revaskularizacija miokarda, ali je ta odluka posledično promenjena u perkutanu koronarnu intervenciju (PKI). **Metode.** Retrospektivno su analizirani podaci za 1 501 bolesnika koji su bili primljeni u jedan centar visokog obima tercijarne nege za koronarni arterijski bajpas grafting (KABG). Kod svih bolesnika odluke su bile donete od strane kardiohirurškog konzilijuma pre prijema. Posle prijema, odluke su ponovo procenjivane od strane drugog kardiohiruškog konzilijuma. Odluka o načinu revaskularizacije promenjena je kod 73 (4,86%) bolesnika. Urađeno je usklađivanje skora podudarnja sa bolesnicima iz iste populacije koji su podvrgnuti KABG. Bolesnici u obe grupe praćeni su zbog velikih neželjenih kardiovaskularnih događaja (VNKVD) i ukupnog mortaliteta tokom 12 meseci. **Rezultati.** Grupe PKI i KABG bile su uravnotežene u odnosu na demografske i kliničke karakteristike. Svi bolesnici su imali dvosudovnu ili trosudovnu koronarnu bolest, sa sličnom učestalošću stenoze glavnog stabla (26% u PKI i 30,10% u KABG grupi). EuroSCORE II je bio sličan između grupa (2,48 ± 2,38 vs. 2,36 ± 2,92). Tokom perioda praćenja primećeno

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je ukupno 5 (6,80%) VNKVD u PKI grupi i 12 (5,80%) VNKVD u KABG grupi (log rank 0,096, p = 0,757). Ukupno 6 (8,20%) bolesnika umrlo je u grupi PKI, a 15 (7,30%) je umrlo u KABG grupi (log rank 0,067, p = 0,796). **Zaključak.** Naši podaci ukazuju na to da bolesnici kojima je prvi put savetovan KABG, ali je odluka posledično promenjena na PKI imaju sličnu prognozu kao i bolesnici sa KABG, 12 meseci nakon indeksne procedure.

Ključne reči:
kardiolozi; koronarna bolest; odlučivanje; mortalitet; miokard, revaskularizacija; perkutana koronarna intervencija; lečenje, ishod.

Introduction

There are two different modalities of myocardial revascularization: coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI) [1]. Clinical practice and trials have shown that neither CABG nor PCI can individually provide the solution for the entire spectrum of patients with coronary artery disease. Therefore, the decision regarding myocardial revascularization must be made for each patient individually, based on the estimated surgical mortality, the anatomical complexity of coronary artery disease and the possibility of complete revascularization [2]. Decision making is particularly difficult in patients with left main stenosis and multivessel coronary disease, as well as in patients with numerous risk factors and comorbidities, including anatomic risk factors (chronic total occlusion and high-risk bifurcation stenosis) [3].

Previous studies indicate that a lower rate of major adverse cardiovascular events (MACE), as well as the possibility of complete revascularization, are the most important advantages of CABG over PCI. Therefore, in previous decades, CABG was considered a standard of care for patients with left main (LM) stenosis, as well as multivessel coronary disease [4]. On the other hand, these data were obtained before the advent of drug-eluting stents (DES), and therefore may not be applicable in the current era. By slowly releasing antiproliferative and antimitotic agents to the arterial wall, DESs have dramatically reduced the incidence of restenosis and improved clinical outcomes in these patients (reducing rates of recurrent angina and repeat revascularizations and improving the quality of life) [5, 6]. Also, when risk factors for postoperative morbidity and mortality are considered, the decision regarding modality of revascularization can shift from CABG towards PCI [7]. Patients with numerous comorbidities are more suitable for PCI, mostly because of the shorter hospitalization period, faster recovery, and less frequent postprocedural stroke [7, 8].

The SYNTAX study was the first randomized clinical trial to compare clinical outcomes after surgical and percutaneous myocardial revascularization in patients with LM stenosis and/or multivessel coronary disease [9]. The SYNTAX study introduced SYNTAX score as a unique tool that predicts the outcome after myocardial revascularization based on the anatomical complexity of coronary artery disease.

Guidelines on myocardial revascularization suggest a balanced, multidisciplinary decision-making process by the Heart Team, consisting of cardiac surgeons, interventional cardiologists, and attending cardiologists [2, 10]. The Heart Team should meet on a regular basis to analyze and interpret the available diagnostic evidence, determine the need for myocardial revascularization, and assess the long-term safety and efficacy of the percutaneous and surgical revascularization. Interdisciplinary institutional protocols should be developed for common case scenarios. On the other hand, patients with complex coronary disease and multiple comorbidities require an individual approach, with additional input from other specialties when needed [2]. In these patients, decision making is not easy, and it is often the case that by reexamining the documentation and the patients' preferences the Heart Team changes the primary decision.

Therefore, the aim of this study was to determine clinical outcomes in patients in whom surgical myocardial revascularization was advised first, but consequently changed to PCI.

Methods

Patients

We retrospectively analyzed data for 1,501 patients admitted to Dedinje Cardiovascular Institute, Belgrade (Serbia) for CABG. In all patients, the decision regarding myocardial revascularization was made by the Heart Team prior to admission. Upon admission, the initial decision made by the Heart Team was reevaluated by another Heart Team. The decision regarding revascularization modality was changed from CABG to PCI in 73 (4.86%) of patients. The reasons for the change of the Heart Team's decision were: reevaluation of coronary anatomy in favor of PCI in 48 patients, high surgical risk in 24 patients, and the patient's preference in 1 patient.

The patients were followed during a 12-month period after the index procedure for major adverse cardiovascular and cerebrovascular events (MACCE) and total mortality. MACCE included nonfatal cerebrovascular insult, nonfatal myocardial infarction, and cardiovascular causes of death. Total mortality was a composite of cardiovascular and noncardiovascular causes of death. The data regarding MACCE and total mortality were collected on control exams one, six, and twelve months after the index procedure, as well as by phone calls to patients and/or their relatives.

The aim of the study was to compare the difference in the occurrence of MACCE and total mortality between the group of patients who underwent CABG and the group of patients in whom revascularization modality was changed to PCI, as well as to compare total mortality between these two groups.

For each patient the following data were collected: age, gender, hypertension (HTA), smoking, diabetes mellitus (DM), prior myocardial infarction (MI), presence of chronic obstructive pulmonary disease (COPD), and presence of peripheral vascular disease (PVD). For each patient left ventricle ejection fraction (LVEF) and creatinine clearance (CrCl) were calculated. CrCl was calculated by the Cockcroft-Gault equation, using an online calculator (https://www.mdcalc.com/creatinine-clearance-cockcroft-gault-equation). LVEF was evaluated by transthoracic echocardiographic examination (TTE) using the Vivid® 9 ultrasound machine (GE Healthcare; Wausheka, Wisconsin, USA), based on the Simpson method.

We used SYNTAX score and SYNTAX score II, which were taken out from guidelines in 2018, in order to analyze the anatomical complexity of coronary artery disease, in addition to clinical parameters. SYNTAX score predicts the outcome after myocardial revascularization based on the anatomical complexity of coronary artery disease [11]. SYNTAX score II is a tool that improves decision-making between CABG and PCI by combining anatomical and clinical variables. By providing accurate assessment of mortality after myocardial revascularization, SXNTAX score II identifies patients for whom either CABG or PCI had a more favorable long-term outcome, and patients for whom long-term outcomes between CABG and PCI were similar [12]. SYNTAX score and SYNTAX score II were calculated using online calculators (http://www.syntaxscore.com/). SYNTAX score and SYNTAX score II were calculated only in the PCI group because we did not have access to coronary angiograms of the patients in the CABG group. Based on the SYNTAX score value, the patients who underwent PCI were divided into three groups: group I: 0–22; group II: 23–32; group III: ≥ 33. Based on the SYNTAX score II recommendation for revascularization modality, those patients were also divided into three groups: CABG only, CABG or PCI, PCI only.

EuroSCORE II was used to calculate perioperative risk for all patients. EuroSCORE II is a prediction model which estimates perioperative mortality for patients undergoing cardiac surgery [13]. The data used to calculate EuroSCORE II were obtained from the Dedinje Cardiovascular Institute patient database. EuroSCORE II was calculated using an online calculator (http://www.euroscore.org/calc.html).

Heart Team

The Heart Team discusses optimal treatment modalities for all patients admitted to Dedinje Cardiovascular Institute. Heart Teams consist of cardiac surgeons, clinical cardiologists, and interventional cardiologists. Our Heart Team consists of 10 cardiac surgeons, 10 interventional cardiologists, and 4 clinical cardiologists. By analyzing coronary angiograms, echocardiographic findings, functional test findings, as well as the clinical parameters, the Heart Team makes a decision regarding the optimal treatment modality for each patient. If myocardial revascularization is preferable, the Heart Team makes a decision regarding the revascularization modality. The Heart Team makes approximately 8,600 decisions annually.

PCI procedure

PCI was performed by one of 10 interventional cardiologists. As an arterial approach for the PCI procedure, the right radial artery or the right femoral artery were used. Standard PCI protocol was used in all patients: initially, optimal dose of unfractionated heparin (UFH) [60–100 international units (IU) per kilogram (kg)] was administered, following implantation of optimal size DES. Hemostasis was performed by a transradial bracelet (in case of transradial approach) or by manual compression (in case of transfemoral access). At hospital discharge, patients who had undergone the PCI procedure dual antiplatelet therapy for at least 6 months, as well as other therapy according to the guidelines [2].

In order to compare 73 patients in whom the indication was changed from CABG to PCI (the PCI group), with 1,501 patients in whom surgical revascularization was performed, we used propensity score matching (PSM). The matching score is the probability that a unit with certain characteristics will be assigned to the treatment group (as opposed to the control group). PSM creates the participants' treatment ratios (the treatment and control groups). A “matched” set consists of at least one participant in the treatment group and one in the control group having a similar matching score.

In a group of 73 patients in whom the indication was changed from CABG to PCI (the PCI group), we selected clinical and demographic characteristics which we considered to be the best descriptors of the patient population. Afterwards, logistic regression was performed in the whole group of patients, based on these characteristics, in order to calculate propensity scores. Once the propensity scores were calculated, we performed the nearest neighbor matching with replacement, in order to compare one patient in the PCI group to more than one patient with similar clinical and demographic characteristics in the CABG group. Using PSM, a total of 206 patients, in whom surgical revascularization was performed, were identified (the CABG group).

The data analyzed in the study are presented as absolute numbers, percentages, or as mean value ± standard deviation (SD). The paired samples *t*-test was applied in order to compare subgroups for continuous variables. Differences in the incidence of MACCE and total mortality between the two groups were analyzed by using the Kaplan-Meier analysis. The value of $p < 0.05$ was considered a statistically significant difference. Statistical data analysis was performed by using the IBM SPSS Statistics (version 25).

Results

Clinical and demographic characteristics

Regarding the clinical and demographic characteristics, there was no statistically significant difference between the patients in the two groups, PCI and CABG ($p > 0.05$). In both

groups patients were dominantly male (69.90% vs. 72.80%). The average age in the PCI group was 66.18 ± 8.56 years, while in the CABG group it was 65.60 ± 5.05 years. The most common risk factor in both groups was hypertension (98.60% in the PCI group and 92.70% in the CABG group). 43.80% of the patients in the PCI group and 43.20% of the patients in the CABG group had DM. Clinical and demographic characteristics of patients are presented in Table 1.

Table 1

Clinical and demographic characteristics of patients who underwent percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG) in relation to the revascularization modality

Variable	PCI group (n = 73)	CABG group (n = 206)	*p*
Age (years), $\bar{x}$ ± SD	66.18 ± 8.564	65.6 ± 5.05	0.49
Male, n (%)	51 (69.9)	150 (72.8)	0.63
DM, n (%)	32 (43.8)	89 (43.2)	0.93
HTA, n (%)	72 (98.6)	191 (92.7)	0.06
Smoking, n (%)	42 (57.5)	97 (47.1)	0.13
COPD, n (%)	6 (8.2)	12 (5.8)	0.47
PVD, n (%)	21 (28.8)	43 (20.9)	0.17
MI, n (%)	43 (58.9)	117 (56.8)	0.76
LM, n (%)	19 (26)	62 (30.1)	0.51
LVEF, $\bar{x}$ ± SD	43.01 ± 13.14	44.32 ± 12.04	0.44
CrCl, $\bar{x}$ ± SD	77.66 ± 31.11	79.49 ± 15.39	0.52
ES II, $\bar{x}$ ± SD	2.48 ± 2.38	2.36 ± 2.92	0.76

$\bar{x}$ – mean value; SD – standard deviation; DM – diabetes mellitus; HTA – hypertension; COPD – chronic obstructive pulmonary disease; PVD – peripheral vascular disease; MI – myocardial infarction; LM – left main; LVEF – left ventricular ejection fraction; CrCl – creatinine clearance; ES II – EuroSCORE II.

EuroSCORE II

The mean value of EuroSCORE II in the PCI group was 2.48 ± 2.38%. Compared to the CABG group, where the mean value was 2.36 ± 2.92%, there was no statistically significant difference ($p = 0.764$).

SYNTAX score and SYNTAX score II

As for the SYNTAX score in the PCI group, we identified 34 patients in the group I (46.60%), 25 patients in the group II (34.20%) and 14 patients in the group III (19.20%). Regarding the recommendation for the revascularization modality based on the SYNTAX score II, 27 patients (37%) had a recommendation for "CABG only", 45 patients (61.6%) had a recommendation for "CABG or PCI", and 1 patient (1.40%) had a recommendation for "PCI only".

Procedure characteristics

In the PCI group, in most cases (43.80%), one coronary artery was treated. Two coronary arteries were treated in 32.90% of patients, while three coronary arteries were treated in 5.50% of patients. The PCI procedure of the LM coronary artery was performed in 17.80% of patients. In the CABG group, surgical myocardial revascularization with triple coronary artery bypass grafting was performed in 59.20% of patients, while quadruple CABG was performed in 28.60% of patients. Surgical myocardial revascularization with double CABG was performed in 10.20% of patients, while 1% of patients underwent surgical revascularization with single CABG, and the same percentage with quintuple CABG.

Follow-up

The follow-up period was 12 months, and included 100% of patients in both groups. In the PCI group, 5 MACCEs (6.80%) were observed, of which all 5 were due to cardiovascular causes of death. Neither nonfatal MI nor nonfatal stroke was observed. In the CABG group, 12 MACCEs (5.80%) were observed, of which all 12 were due to cardiovascular causes of death, while nonfatal MI and nonfatal stroke were not detected. Total mortality in the PCI group was 6 (8.20%), of which 5 deaths were due to cardiovascular causes, while one death was due to an accident (a fall from a window). In the CABG group, total mortality was 15 (7.30%), with cardiovascular deaths in 12 patients, while death due to respiratory failure occurred in 2 patients, and 1 death was caused by lung carcinoma. Kaplan-Meir analysis showed no statistically significant difference in MACCE rates (Log-Rank p value = 0.757)

(Figure 1) and total mortality (Log-Rank *p* value = 0.796) (Figure 2) between the PCI and CABG groups.

(2.48% in the PCI group and 2.36% in the CABG group). As for anatomical complexity of coronary artery disease, the

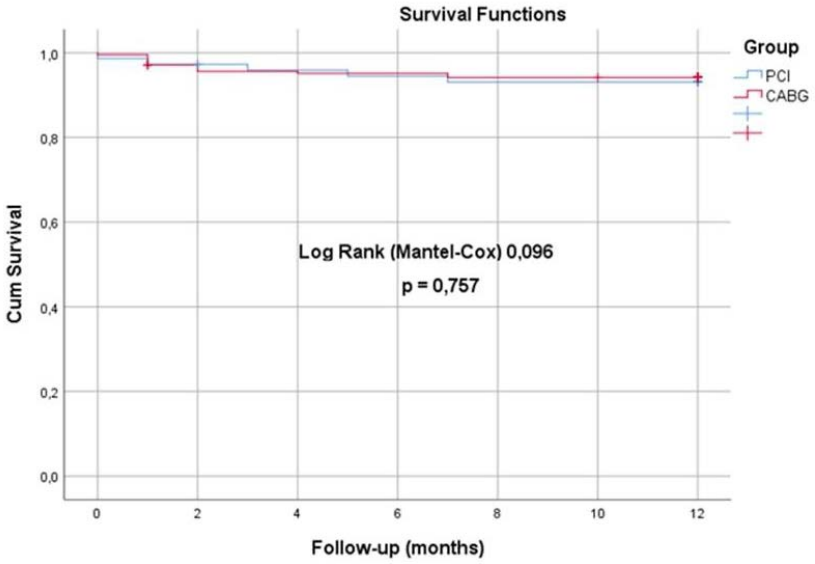

Fig. 1 – Kaplan-Meir analysis of major adverse cardiovascular and cerebrovascular events (MACCE) in the percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) groups.

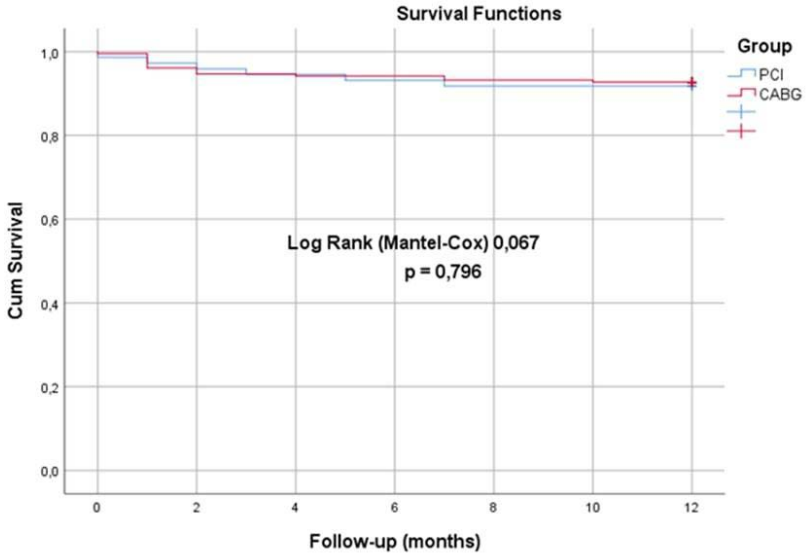

Fig. 2 – Kaplan-Meir analysis of total mortality in the percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) groups.

Discussion

Our results indicate that during a one-year follow-up period there was no statistically significant difference in overall mortality and incidence of MACCE between patients who underwent CABG and those who were reassigned to PCI.

The present study included mostly patients with less complex coronary artery disease. The average perioperative risk, as described by EuroSCORE II, was relatively low majority of patients in the PCI group had low SYNTAX score (46.60% of patients). Observing SYNTAX score II, most patients who underwent PCI were suitable for both PCI and CABG. Clinically and anatomically uncomplicated coronary artery disease, as well as similar patient characteristics in both groups, are possible explanations for a relatively good outcome and low incidence of adverse events, which did not significantly differ in the examined groups.

Interestingly, in the group of patients who underwent surgical revascularization but were not included in PSM, a

total of 87 MACCEs (7.12%) were observed, of which 28 (2.29%) were due to cardiovascular causes of death, while 32 (2.62%) patients had nonfatal myocardial infarction, and 27 (2.21%) patients had nonfatal stroke. Total mortality in this group was 50 (4.09%), of which 28 deaths were due to cardiovascular causes and 22 deaths were due to noncardiovascular causes.

Our results are comparable to the results of the ARTS II study, which compared safety and efficacy of drug-eluting stents in patients with *de novo* multivessel coronary artery disease with historical controls that underwent surgical revascularization [14]. Similarly to our study, during a one-year follow-up period, the ARTS II study showed that there was no statistically significant difference in overall mortality and incidence of MACCEs between these two groups. On the other hand, after two and three years, following a comparatively greater number of additional MACCEs in the DES group, the overall MACCE rate was insignificantly higher in the DES group. This was mainly caused by relatively higher rates of reintervention in the DES group compared to the CABG group, as a result of late stent thrombosis. Results showed that 32% of adverse events occurred due to late stent thrombosis, mostly after two- and three-year follow-up periods.

The SYNTAX trial was designed to assess the optimal revascularization strategy between percutaneous coronary intervention and coronary artery bypass grafting, for patients with left main and/or three-vessel coronary disease. A five-year follow-up of these patients has shown that the outcome is significantly affected by the complexity of coronary artery disease [15]. In patients with a low SYNTAX score (0–22), total mortality and MACCE rates did not significantly differ between the treated groups. On the other hand, the difference in total mortality and the MACCE rates was observed only in the patients with intermediate (23–32) and high (≥ 33) SYNTAX scores after the third year [16], mostly due to stent thrombosis and MI. These results suggest that CABG is a standard of care for patients with complex lesions (high or intermediate SYNTAX score), while patients with less complex coronary disease (SYNTAX score ≤ 22) can safely and efficiently be treated with PCI. In our study, almost half of the patients (n = 34) in the PCI group had a low SYNTAX score, which can possibly explain relatively good outcomes and low rates of both endpoints.

The outcome after myocardial revascularization depends not only on anatomical and clinical complexity of coronary artery disease, but also on comorbidities and risk factors, including DM. In our study, 32 (48.3%) patients in the PCI group and 89 (43.2%) patients in the CABG group had DM. The FREEDOM trial compared outcomes after PCI and CABG in high-risk diabetic patients with multivessel coronary disease [17]. All the treated patients had numerous comorbidities, including hypertension, hyperlipidemia, and diabetes, and nearly half of the patients had intermediate SYNTAX score (23–32). The optimal revascularization modality in these patients is a common subject of discussion. This trial showed that in the patients with a low SYNTAX score, there is no difference in incidence of both endpoints between the PCI group and the CABG group. This difference occurs in the patients with intermediate and high SYNTAX scores, with significantly lower incidence of both endpoints in the CABG group, similarly to our study.

In our study, the average perioperative risk was relatively low (the average EuroSCORE II was 2.48% in the PCI group and 2.36% in the CABG group). The Heart Team's decision was changed in 24 (32.88%) of patients due to a high perioperative risk. The average EuroSCORE II in these patients was 3.72%. In high risk patients, decision-making by the Heart Team is difficult, and the optimal revascularization modality is often the subject of discussion. As a result, most of the trials comparing PCI and CABG exclude this group of patients. The AWESOME study compared long-term survival between PCI and CABG groups in patients with medically refractory ischemia and an increased risk of adverse outcomes after CABG [18]. Results of this study have shown that there is no statistically significant difference in survival between the two treated groups, suggesting that PCI is a safe alternative for CABG in patients with estimated high perioperative mortality.

Our results, as well as the results from previously mentioned randomized clinical trials, indicate that safety and efficacy of PCI is comparable to surgical revascularization during a one-year follow-up. Higher rates of total mortality and MACCEs in the PCI group are observed after 2–3 years, predominantly as a result of late stent thrombosis and MI. However, in patients with less complex coronary disease (a low SYNTAX score), as well as in patients with a high operative risk, PCI with DES implantation is a safe and efficient alternative to CABG.

Limitations of the study

This study has several limitations: (1) The SYNTAX score was not calculated for the patients who underwent coronary artery bypass grafting, because we did not have access to all their coronary angiograms, which made it difficult to compare these two groups of patients based on the anatomical complexity of coronary artery disease; (2) a small number of patients from a single center; and (3) a relatively short follow-up period that might not provide data about long-term outcomes. However, we believe that these limitations did not have major effects on the results of the study.

Conclusion

Our study showed that it appears that during a one-year follow-up period there is no statistically significant difference in overall mortality and MACCEs between selected patients who underwent surgical revascularization and those in whom surgical myocardial revascularization was firstly advised but consequently changed to PCI.

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The increasing doses of methotrexate pharmacokinetics after intravenous administration in rats – model selection

Farmakokinetika rastućih doza metotreksata nakon intravenske primene kod pacova – odabir modela

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Abstract

Background/Aim. Methotrexate (MTX) plays a significant role in the treatment of various diseases, but the toxicity remains the main issue of its use, especially when administered in high doses. Considering altered pharmacokinetics of MTX as a factor strongly implicated in the large interpatient variability and unexpected toxicity in certain patients, the accurate description of MTX pharmacokinetic behaviour of both low and high doses is of the utmost importance. Therefore, the objective of this study was to determine the pharmacokinetics of MTX after intravenous (*iv*) administration in ascending doses of 5, 40, 80 and 160 mg/kg in rats and to select the appropriate mathematical model describing MTX pharmacokinetics. **Methods.** Plasma concentrations of MTX were measured using the liquid chromatography - mass spectrometry (LC/MS) method. Pharmacokinetic parameters were calculated by non-compartmental and two-compartmental integer-order analyses. **Results.** MTX showed linear pharmacokinetics following *iv* administration up to the dose of 80 mg/kg. The administration of a high dose of MTX (160 mg/kg) resulted in the similar pharmacokinetic behaviour as when applied in the twice lower dose (80 mg/kg), which can be explained by dose-dependent changes in the expression of solute carrier (SLC) and ATP binding cassette (ABC) transport proteins and intracellular metabolism. Furthermore, the classical two-compartment model could not explain the pharmacokinetics of MTX in a small percentage of experimental animals, which opens up new strategies for the use of fractional order pharmacokinetic models in MTX therapy optimisation. **Conclusion.** These results of pharmacokinetic analyses may be helpful in adjusting the dosage regimen of MTX, but the application of novel pharmacokinetic models, such as those based on fractional calculus, is still needed in the process of MTX therapy optimisation.

Key words:
methotrexate; drugs, dose-response relationship; models, biological; treatment, outcome; rats.

Apstrakt

Uvod/Cilj. Metotreksat (MTX) ima značajnu ulogu u lečenju različitih bolesti, ali toksičnost predstavlja glavni ograničavajući faktor njegove primene, naročito kada se primenjuje u visokim dozama. Imajući u vidu izmenjenu farmakokinetiku MTX, kao faktora koji je snažno povezan sa značajnom varijabilnošću kliničkog odgovora i neočekivanom toksičnošću kod određenih bolesnika, tačan opis farmakokinetičkog ponašanja MTX primenjenog u niskim i visokim dozama je od izuzetnog značaja. Stoga je cilj ove studije bio da se odredi farmakokinetika MTX nakon intravenske (*iv*) primene u rastućim dozama od 5, 40, 80 i 160 mg/kg kod pacova i da se odabere odgovarajući matematički model koji dobro opisuje farmakokinetiku ovog leka. **Metode.** Koncentracije MTX u plazmi su merene korišćenjem tečne hromatografije kuplovane sa masenom spektrometrijom (LC/MS). Farmakokinetički parametri su izračunati pomoću neprostornih i dvoprostornih celobrojnih matematičkih analiza. **Rezultati.** MTX je pokazao linearnu farmakokinetiku koja prati *iv* primenjene doze do 80 mg/kg. Davanje visoke doze MTX (160 mg/kg) rezultiralo je sličnim farmakokinetičkim ponašanjem kao kada se primenjuje u dvostruko nižoj dozi (80 mg/kg), što se može objasniti dozno-zavisnim promenama u ekspresiji SLC i ABC transportnih proteina i intracelularnom metabolizmu ovog leka. Osim toga, klasični model sa dva kompartmana nije mogao da objasni farmakokinetiku MTX kod malog procenta eksperimentalnih životinja, što otvara nove mogućnosti za korišćenje frakcionih farma

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kokinetičkih modela u optimizaciji MTX terapije. **Zaključak.** Dobijeni rezultati farmakokinetičkih analiza na životinjama mogu biti korisni u prilagođavanju režima doziranja MTX, ali je primena novih farmakokinetičkih modela, poput onih baziranih na frakcionom računu, kao i određivanje farmakokinetičkog ponašanja MTX kod različitih bolesnika, neophodno u procesu pune optimizacije terapije ovim lekom.

Ključne reči:
metotreksat; lekovi, odnos doza-reakcija; modeli, biološki; lečenje, ishod; pacovi.

Introduction

Methotrexate (MTX), formerly known as amethopterin, is an antifolate and antimetabolite drug, a chemical analogue of folic acid, differing from folic acid only in the substitution of an amino for a hydroxyl group at the N4-position of the pteridine ring and in the addition of a methyl group at the N-10 position. These structural differences confer high affinity for dihydrofolate reductase (DHFR), leading to the strong inhibition of this enzyme [1].

MTX was first administered to children with acute lymphoblastic leukemia (ALL) in 1948 and it became the first drug that induced remission, which resulted in Food and rug administration (FDA) approval in 1953. Nowadays, it has been used in high doses to treat several malignancies including pediatric ALL, choriocarcinoma, osteosarcoma, non-Hodgkin lymphoma, etc. Despite numerous advances in cancer chemotherapy, it still remains a mainstay of therapy since its discovery 70 years ago [2]. Furthermore, MTX has been used, alone or in combination, in low doses, for the treatment of autoimmune diseases such as rheumatoid arthritis, polyarthritis, ankylosing spondylitis, psoriasis, systemic scleroderma, Crohn's disease, inflammatory myopathies and systemic lupus erythematosus [3]. MTX was also demonstrated to be the effective treatment for early unruptured ectopic pregnancy with several treatment regimens available, without adversely affecting ovarian reserve or subsequent fertility [4].

MTX plays a significant role in the treatment of various diseases, but the toxicity remains the main issue of its use, especially when administered in high doses. The main adverse effects include myelosuppression, renal insufficiency, mucositis and neurotoxicity. The adequate management of intoxication by MTX is of the utmost interest since prompt actions can reverse the damage and save the patient's life [5]. Most minor and major toxic effects induced by MTX are associated with the folate depletion. However, two different actions of MTX, one in low (rheumatologic) doses and the other in high (oncologic) doses, should be emphasized, with distinct toxicity profiles as well. While adverse effects following low doses of MTX are minor, usually controlled with symptomatic treatment or with folic acid supplementation, serious adverse effects following high doses of MTX may require leucovorin (folinic acid) rescue [6, 7].

MTX has a narrow therapeutic range, i.e. the range between minimal effective and toxic concentrations, and therefore either non-effectiveness and/or toxicity may occur after MTX administration [8]. High-dose MTX, defined as a dose higher than 500 mg/m^2, used to treat a range of adult and childhood cancers, is safely administered to most patients, but it can cause serious, life-threatening adverse effects. MTX must be thus dosed correctly and monitored appropriately. Therapeutic drug monitoring is a standard practice for guidelines related to leucovorin rescue, especially when high-dose MTX infusions are applied in patients with impaired MTX clearance or other risks related to prolonged cytotoxic concentrations, such as kidney or liver damage [9, 10].

Besides toxicity, the major issue in MTX dosing represent inter- and intrapatient variability as well. It was shown that the standard fixed MTX dose can produce up to a 7-fold spread in the range of drug concentrations in different patients [11]. High-dose MTX can undoubtedly reduce tumour recurrence and prolong disease-free survival, but the pharmacokinetics of the drug shows large interpatient variability and contributes to the unexpected toxicity in some patients. Several factors responsible for clinical response variability observed among patients treated with MTX have been described [12, 13]. Metabolic enzyme and transporter gene polymorphisms may be one of the most significant factors, which have been in a research focus in recent years and which can provide further support for the study of MTX treatment individualization [14].

Considering the narrow therapeutic range of MTX and the numerous factors implicated in clinical response profile, there have been developed several strategies for the therapy optimisation. The most widely used strategy used to optimise patients' MTX clinical response profile includes therapeutic drug monitoring [9]. Besides toxicity, unexpected adverse effects of MTX such as low cellular uptake, uncontrolled drug release, lack of specificity in both cellular and systemic level, drug resistance, difficulties in biological tracing, opened up new strategies in developing new advanced hybrid drug formulations based on drug delivery systems with improved pharmacokinetic properties [15].

Considering altered pharmacokinetics of MTX as a contributing factor to its serious toxic effects, much effort has been put in revealing mechanisms of MTX pharmacokinetic behaviour that may lead to the optimised drug therapy in patients at high risk. Several studies on high-dose MTX pharmacokinetics in children with ALL have been performed and conventional compartmental or non-compartmental pharmacokinetic models were not able to completely describe pharmacokinetic behaviour in some patients [16].

Based on the above-mentioned facts, the purpose of our study was to determine the pharmacokinetics of MTX after *iv* administration at 5, 40, 80 and 160 mg/kg doses in rats. Although information is available regarding the pharmacokinetics of MTX after the *iv* administration in different single doses in rats, there are no data regarding the pharmacokinetics and linearity in ascending doses. Furthermore, the suitability of two-compartment model to de-

scribe experimentally obtained concentration values was evaluated and compared to the results of non-compartmental pharmacokinetic analysis.

Methods

Chemicals

LC-grade solvents acetonitrile and water were obtained from Fisher Scientific Chemical (Loughborough, England); ammonium formate was from Fluka analytical (Munich, Germany); aminopterin was from Sigma-Aldrich company (St. Louis, USA); methotrexate was purchased from Pfizer (New York, USA).

Laboratory animals and experimental procedures

Male Wistar rats weighing 250–270 g (obtained from the Military Medical Academy, Belgrade, Serbia) were used for the experiments. Animals were housed in UniProtect air-flow cabinet (Ehret GmbH, Emmendingen, Germany) and standard plexiglass cages at a constant 22 C ±1°C room temperature, 55% ± 1.5% humidity and with standard circadian rhythm (12 h day/night cycle). They were allowed free access to tap water and standard pelleted laboratory rodent feed (Veterinary Institute Subotica, Serbia) during the whole experiment. The experimental procedures were conducted in accordance with the European Directive (2010/63/EU) for animal experiments and they were reviewed and approved by Ethics Committee for Protection and Welfare of Experimental Animals at the University of Novi Sad, Serbia.

The rats were randomly allocated to four groups, each of which consisted of 5 animals. All animals were anaesthetised with urethane (1,250 mg/kg *ip*) and had their right external jugular vein cannulated. MTX solutions were prepared by dissolving the drug in isotonic saline with 0.1M NaOH to concentrations of 5, 40, 80, and 160 mg/mL MTX, thus allowing the administration of equal volumes to all rats. MTX doses of 5, 40, 80, and 160 mg/kg were administered as bolus injections through a central venous catheter. Heparinised venous blood samples of 200 μL were drawn from tail vein prior to drug administration and subsequently 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480 minutes after MTX administration. Haematocrit samples were drawn from the tail vein at the same time points and the plasma was obtained after centrifugation. All animals were hydrated with 3 mL/kg/h of saline. Plasma samples were kept at -80°C prior to further analyses.

Analytical assays

Liquid chromatography-mass spectrometry (LC/MS analysis)

Liquid chromatography was performed on a Thermo Finnigan Surveyor HPLC System (Thermo Fisher Scientific Inc, Waltham United States) consisting of a quaternary MS pump and autosampler. Chromatographic separation was performed on LC column Agilent Eclipse Plus C_{18} 5μm with dimensions 2,1 x 150 mm (Agilent Technologies Inc, Santa Clara, USA) with ZORBAX Eclipse Plus-C_{18} precolumn (Agilent Technologies Inc, Santa Clara, USA), on room temperature. Isocratic elution was utilised with flow rate 400 μL/min of 40% acetonitrile as a mobile phase B. Mobile phase A consisted of ammonium formate 2.5 mM in 0.04% triethylamine in water: acetonitrile 90/10 v/v. Injection volume was 10 μL. MS detection was carried out on Thermo Scientific™ LCQ Fleet™ ion trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham United States). Electrospray ionisation (ESI) source of instrument was operated in the negative mode with the following settings capillary voltage, -24 kV and capillary temperature, 350 °C.

Sample preparation

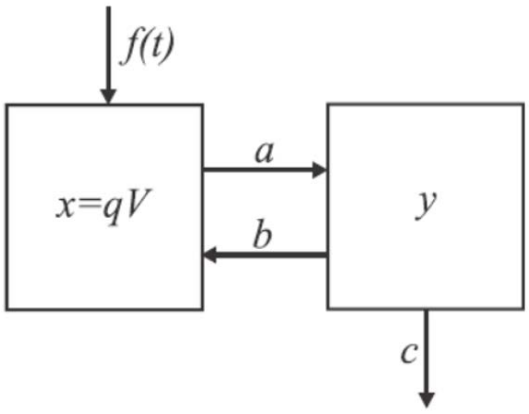
In 20 μL of rat plasma sample, 20 μL of internal standard – aminopterin was added. Samples were prepared utilizing simple precipitation process, consisting of the addition of 40 μL of acetonitrile. After that, vortexing samples were centrifuged for 6 min at 10000 × g. The clear supernatant was transferred to a sample vial and placed in the autosampler at 10°C until analysis.

Pharmacokinetic calculations

Plasma concentration-time curves of MTX in each animal were drawn and pharmacokinetic variables of MTX were determined using non-compartmental model analysis in PKSolver software [17]. MTX plasma concentration-time data were analysed using a non-compartmental model. Plasma half-life ($t_{1/2}$) was calculated from the elimination rate constant, k. Total area under the plasma concentration-time curve (AUC) was calculated by the trapezoidal method and extrapolated to infinity. The mean residence time (MRT) was calculated from the AUC and area under the moment curve (AUMC).

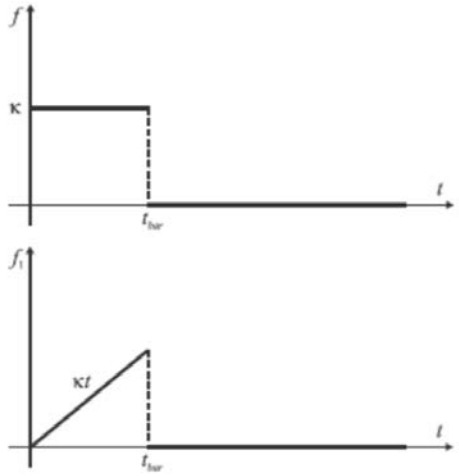
Two-compartmental integer-order pharmacokinetics analysis was performed in Mathematica software, release 11.0.1.0, with standard routines for interpolation, numerical integration, and the least squares method used in system identification procedure.

Pharmacokinetic two-compartment model:



Input function:

$$f(t) = \kappa(u(t) - u(t - t_{bar}))$$



q(t) [µmol/L] – concentration of MTX in blood plasma.

Pharmacokinetic model equations for the two-compartment model:

$$\frac{dq}{dt} = \frac{1}{V} f(t) - aq(t) + \frac{b}{V} y(t),$$

$$\frac{dy}{dt} = aq(t)V - (b + c)y(t)$$

Initial conditions: $q(0) = 0, y(0) = 0.$

a, b, c, V and t_{bar} are unknown parameters.

Statistical analysis

All pharmacokinetic parameters were calculated for each animal and the data presented as arithmetic mean ± standard deviation (SD). Statistical differences in the pharmacokinetic parameters among dose groups were determined using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test and using Student's independent samples *t*-test. Statistical analysis was performed by using IBM SPSS software 23.0 (Chicago, USA). The differences were considered significant if $p < 0.05$.

Results

Mean plasma concentration-time profiles obtained for MTX administered in ascending doses (5 mg/kg, 40 mg/kg, 80 mg/kg and 160 mg/kg) in male Wistar rats are shown in Figure 1. Plasma concentrations were measured using LC/MS method at 12 time points in the period of 8 hours. In the first 30 minutes (first 4 time points), there were statistically significant differences among all 4 investigated groups. In 45th and 60th minute of the pharmacokinetic analysis, concentration-time curves of animal groups receiving 80 mg/kg and 160 mg/kg started to overlap and there were no significant differences ($p = 0.61$ and $p = 0.63$ for 45th and 60th minute, respectively). These two curves representing pharmacokinetic behaviour of MTX in doses of 80 mg/kg and 160 mg/kg remained similar until the end of analysis (480 minutes). From 90th minute, statistically significant differences were not present anymore also between the groups receiving 40 mg/kg and 80 mg/kg ($p = 0.15$). In the 120th

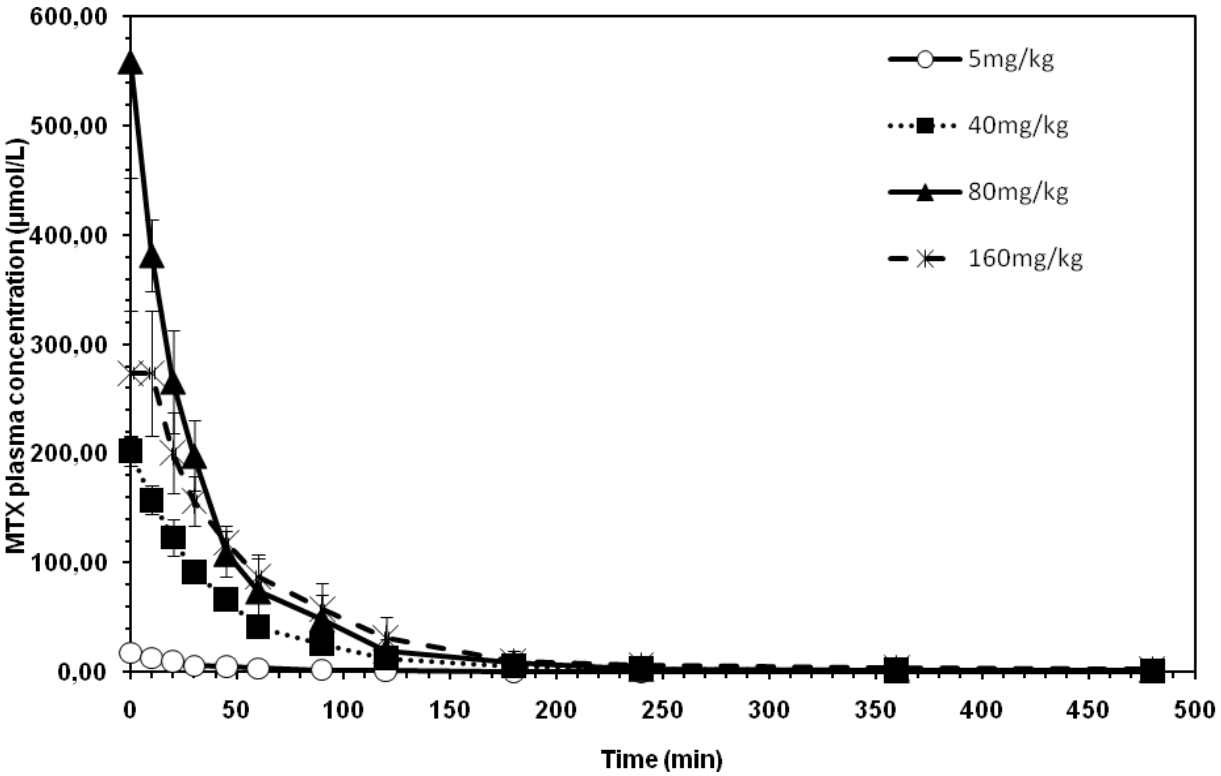


Fig. 1 – Mean plasma concentration-time profiles of methotrexate (MTX) after the *iv* administration in ascending doses (5 mg/kg, 40 mg/kg, 80 mg/kg, 160 mg/kg) to rats (n = 5).

minute, all 4 plasma concentration-time curves were overlapped without statistically significant differences, except between animal groups receiving 5 mg/kg and 160 mg/kg (p = 0.002). From 180th minute, the pharmacokinetic profiles for all 4 investigated groups were similar, without statistically significant differences.

Pharmacokinetic parameters for different doses of MTX (calculated using non-compartmental and two-compartmental integer-orfigder pharmacokinetic models) are summarized in Tables 1 and 2, respectively. Using non-compartmental pharmacokinetic analysis, it was demonstrated that the AUCs, both calculated to the last time point and extrapolated to infinite time, were directly proportional to the doses, in a dose range 5–80 mg/kg. On the contrary, the administration of MTX dose of 160 mg/kg resulted in the similar AUC value as when administered in a dose of 80 mg/kg. In addition, the values of drug clearance were in the range 0.0016–0.0029 L/min for the dose range 5–80 mg/kg, while that value was 0.0043 when MTX was administered in the dose of 160 mg/kg. The volume of distribution of MTX was two-fold higher in animals receiving 160 mg/kg (0.722 L) in comparison to those receiving 80 mg/kg (0.358 L). The elimination rate constant remained similar in all investigated MTX doses. The results of two-compartmental pharmacokinetic analysis were similar, particularly in terms of AUC values, i.e. values reflecting the actual body exposure to a drug after the administration of a dose of the drug (Table 2).

Discussion

Considering altered pharmacokinetics of MTX as a factor strongly implicated in the large interpatient variability and unexpected toxicity in certain patients, the accurate description of MTX pharmacokinetic behaviour of both low and high doses is of the utmost importance. Therefore, the aim of the present study was to determine the pharmacokinetics of MTX after *iv* administration in ascending doses of 5, 40, 80 and 160 mg/kg in rats and to select the appropriate mathematical model describing MTX pharmacokinetics.

MTX pharmacokinetics has been reported in the literature for both healthy individuals and patients suffering from haematological malignancies, rheumatoid arthritis, Crohn's disease, etc [18–21]. However, numerous factors contributing to the variability of MTX pharmacokinetics have been identified and therefore accurate models describing MTX pharmacokinetics are needed to provide optimal therapy for different patients.

Table 1

Pharmacokinetic (PK) parameters for methotrexate (MTX) after a single bolus *iv* injection in rats calculated by using non-compartmental analysis

PK parameter		Groups			
		5 mg/kg mean ± SD	40 mg/kg mean ± SD	80 mg/kg mean ± SD	160 mg/kg mean ± SD
K_e	(1/min)	0.014 ± 0.003	0.009 ± 0.004	0.010 ± 0.009	0.011 ± 0.006
$t_{1/2}$	(min)	50.79 ± 11.19	95.44 ± 58.95	156.56 ± 126.50	113.02 ± 124.53
C_0	(μmol/L)	17.65 ± 4.15	202.28 ± 13.27	559.43 ± 107.33	375.16 ± 88.21
AUC_{0-t}	(μmol/L*min)	706.0 ± 204.8	8950.6 ± 777.4	17519.5 ± 4240.2	17006.6 ± 3765.1
$AUC_{0-\infty}$	(μmol/L*min)	750.6 ± 211.1	9151.2 ± 785.0	18801.5 ± 3672.5	17468.1 ± 3760.6
MRT	(min)	65.00 ± 14.49	64.92 ± 10.66	57.04 ± 27.37	76.93 ± 26.93
Vd	(L)	0.207 ± 0.028	0.261 ± 0.140	0.358 ± 0.270	0.722 ± 0.873
Vd/m	(L/kg)	1.088 ± 0.121	1.288 ± 0.698	2.179 ± 1.721	3.375 ± 3.802
CL	(L/min)	0.0029 ± 0.0007	0.0020 ± 0.0003	0.0016 ± 0.0003	0.0043 ± 0.0010

K_e – elimination rate constant; $t_{1/2}$ – drug half-life; C_0 – plasma drug concentration at time 0; AUC_{0-t} – area under the curve from time 0 to the last measurable concentration; $AUC_{0-\infty}$ – area under the curve from time 0 extrapolated to infinite time; MRT – mean residence time; V_d – Volume of distribution; V_d/m – Volume of distribution per kg; CL – clearance.

Table 2

Pharmacokinetic (PK) parameters for methotrexate (MTX) after a single bolus *iv* injection in rats calculated by using two-compartmental model

PK parameter		Groups			
		5 mg/kg mean ± SD	40 mg/kg mean ± SD	80 mg/kg mean ± SD	160 mg/kg mean ± SD
a	(1/s)	0.116 ± 0.010	0.294 ± 0.127	3.245 ± 2.202	11.255 ± 2.314
b	(1/s)	0.316 ± 0.439	0.0757 ± 0.008	0.818 ± 0.442	0.588 ± 0.096
c	(1/s)	0.132 ± 0.178	0.028 ± 0.005	0.042 ± 0.007	0.023 ± 0.005
k	(μmol/min)	11.17 ± 5.08	77.11 ± 37.78	88.49 ± 14.91	143.96 ± 41.87
t_{bar}	(min)	0.240 ± 0.168	0.276 ± 0.149	0.337 ± 0.066	0.537 ± 0.153
Vd	(L)	0.090 ± 0.042	0.026 ± 0.009	0.012 ± 0.002	0.012 ± 0.002
Vd/m	(L/kg)	0.462 ± 0.176	0.136 ± 0.062	0.073 ± 0.014	0.056 ± 0.014
Cmax	(μmol/L)	26.24 ± 11.08	702.94 ± 261.58	1600.36 ± 123.76	1367.37 ± 412.11
AUC	(μmol/L*min)	711.8 ± 216.7	9340.3 ± 585.7	16296.0 ± 3654.3	15402.6 ± 2700.4

a, b, c, d, k, t_{bar} – mathematical model parameters; Vd – Volume of distribution; Vd/m – Volume of distribution per kg; Cmax – maximal plasma drug concentration; AUC – area under the curve.

After absorption or intravenous administration, MTX is mainly converted in the liver to the major active metabolite of MTX, 7-hydroxymethotrexate. To a lesser extent, MTX is metabolized in the intestine to pteroate (2.4-diamino-N10-methylpteroic acid, DAMPA) and glutamic acid. However, most of the administered dose is found unchanged in urine (60–90%). MTX can also be taken up mainly by solute carriers (SLCs) in erythrocytes, where it undergoes polyglutamation. MTX polyglutamates are obtained by the equilibrium between two enzymes, folylpolyglutamate synthetase and gamma-glutamyl hydrolase. Depending on the number of glutamic acid residues, MTX might be retained inside the cells or transported outside the cells by efflux transporters, mainly by adenosine triphosphate (ATP) binding cassette (ABC) transporters [9, 22]. Therapeutic efficacy is dependent on the formation of MTX polyglutamates, as it keeps intracellular pool of the drug and enhances its affinity towards various target enzymes [2].

The results of our study demonstrated that MTX exerted linear pharmacokinetics following *iv* administration of 5, 40 and 80 mg/kg doses, since the AUC was directly proportional to the dose. On the other hand, the administration of a high dose of MTX (160 mg/kg) unexpectedly resulted in the similar AUC value as when administered in a twice lower dose (80 mg/kg). AUC values reflect the actual body exposure to a drug after the administration of a dose of the drug, and are inversely proportional to the drug clearance. Actually, clearance is the only factor determining the average drug concentration after the *iv* injection of a given dose. The individual factors that can impact clearance include the intrinsic functions of liver or kidneys and blood flow to these organs.

Nonlinear pharmacokinetics has been determined after *iv* administration of MTX in a dose range 0.31–31 mg/kg in rats. Tissue-specific, very slowly decreasing terminal plateau phase was observed in liver, kidneys, bone marrow and stomach after MTX administration in studied doses, which was explained by its strong binding to dihydrofolate reductase (DHFR) [23]. Furthermore, it was shown that the increasing dose of MTX from 50 to 100 mg/kg administered as *iv* infusion in rats did not modify MTX pharmacokinetic parameters, except for a 1.7-fold increase of AUC in plasma and a 3.8-fold increase of AUC in tumour extracellular fluid, which resulted in a 2.3-fold increase in penetration [24].

As it can be observed in Table 1, the values of the drug clearance were in the range 0.0016–0.0029 L/min for the dose range 5–80 mg/kg, while that value was 0.0043 when MTX was administered in the dose of 160 mg/kg. The calculated pharmacokinetic parameters suggest that MTX when administered at 160 mg/kg undergoes rapid biodistribution and accumulation.

Pharmacokinetic parameters obtained for MTX after a single bolus *iv* injection using compartmental and non-compartmental analyses in our study are in accordance with the results of similar investigations. Ren et al. [25] showed that AUC value calculated by compartmental analysis for MTX *iv* injected in a dose of 8 mg/kg to rats was 8.3 μg/mL*h (*i.e.* 1,095 μmol/L*min), which agrees with our results (Table 2). However, the results of the same study demonstrated that, when conjugated to poloxamer and further loaded in the obtained micelles, favourable drug bioavailability can be achieved by adjusting the molar ratio between the entrapped and conjugated MTX [25].

Calculated pharmacokinetic parameters in our study had similar values when using two-compartmental and non-compartmental analyses, although compartmental analysis could not be applied for all animals. Although compartmental modelling has a longer history and has been considered as the standard method, there are several limitations. There is no such thing as a compartment in reality; they are convenient mathematical constructs which facilitate model drug distribution. Unambiguous identification of the 'correct' model is often impossible because more than one model of comparable complexity is consistent with the available data. On the other hand, non-compartmental methods do not require the assumption of a specific compartmental model for either drug or metabolite, and involve the application of the trapezoidal rule for the measurements of the area under a plasma concentration-time curve [26, 27].

It was reported in the literature that high doses of MTX lead to the increased MTX efflux *via* multidrug-resistance transporters from the ABC superfamily [28]. MTX can be transported by multiple SLC and ABC transporters, such as SLC22A6, SLC22A8, SLCO1B3, ABCG2 and ABCC. It is evident that systemic effects often depend on these multiple SLC and ABC drug transporters, having different tissue expression patterns and being regulated in a complex fashion, such as through transcription, sorting and phosphorylation [29]. Membrane influx and/or efflux transporters are one of the major determinants of MTX pharmacokinetics, as well as of adverse drug reactions and clinical response profiles. With progress in pharmacogenomics, the improvement of the prediction of patients' therapeutic outcome treated with low doses of MTX offers a powerful tool for the translation of transporter single nucleotide polymorphisms (SNPs) into the personalized treatment strategies [30]. Besides, many research teams have attempted to hybridize MTX with nanocarriers to form advanced MTX drug delivery systems to overcome these transport protein-related limitations [15].

In a study investigating the pharmacokinetic behaviour of MTX after the administration of the high dose of 12 g/m^2 by infusion in children and young adults with osteosarcoma, it was determined that higher mean C_{max} concentrations, higher exposures, and lower mean clearance of MTX were associated with poorer outcome, which suggests the need of incorporating careful pharmacokinetic monitoring into future osteosarcoma treatment protocols. However, further studies are required to elucidate the causative mechanism by which very high MTX exposures are associated with poor clinical outcomes [31].

Dose-dependent changes in pharmacokinetics and metabolism were confirmed for another chemotherapeutic, alkylating anticancer agent cyclophosphamide, a prodrug that requires enzymatic bioactivation to manifest its anticancer cytotoxic activity. It was shown that following the dose escalation of cyclophosphamide, dividing the high dose over 2 days instead of one single infusion may favourably impact

the metabolism of cyclophosphamide in terms of bioactivation. Furthermore, in a split regimen, renal elimination of cyclophosphamide was decreased [32].

In patients with osteogenic sarcoma, using the pharmacokinetic analysis, MTX serum concentrations during time were explained by a two-compartment open model under the assumption that the elimination rate was proportional to both volume of parenteral solution and the amount of water intake. Besides, the amount of MTX in the peripheral compartment was found about 10-fold larger than that in the central compartment after about 40 h of administration, which may cause a delayed elimination of MTX and the occurrence of severe side effects [33]. MTX intracellular accumulation and folate depletion in cells were shown to represent the main mechanisms of chronic toxicity of MTX in patients [34].

Many scientists attempted to model pharmacokinetics of drugs that accumulate in tissues and return to the circulation after different periods of time. The pharmacokinetics of protease inhibitor amprenavir has been described using a two-compartment model with clearance to a recycling compartment and release back into the gut [35]. However, the existence of secondary peaks as a consequence of drug accumulation and delayed elimination is difficult to explain using classical pharmacokinetic models. In our study, in 3 out of 20 investigated animals, there were secondary peaks in a period between 6 and 8 hours after *iv* administration of MTX and the two-compartment model did not fit well the experimental concentration values.

Fractional order pharmacokinetic models have recently proved to be better suited to represent the time-course of anomalous concentration data. Based on real experimental data corresponding to low and high doses of MTX, the fractional calculus is a promising strategy to predict state dependent optimal chemotherapy treatments in adults and children. However, in doing so, experiments on animals need to be performed first [36].

Fractional calculus, dealing with derivatives of non-integer order, allows the formulation of fractional differential equations (FDEs), which have recently been applied to pharmacokinetics for one-compartment and multi-compartmental models. Multi-compartmental models were formulated by mixing different fractional orders in a consistent manner and the method for the numerical solution of these systems based on a numerical inverse Laplace transform algorithm was proposed. FDEs are particularly useful for modelling datasets that have power-law kinetics, accounting for anomalous diffusion and deep tissue trapping [37]. Amiodarone is an antiarrhythmic drug known for its non-exponential pharmacokinetics, which has important clinical implications due to its accumulation following the long-term administration. The fractional two-compartment model was used to analyse the amiodarone *iv* dataset that has already been analysed with power-law time dependent fractal kinetics [38], as well as a Mittag-Leffler function [39]. This model provided a good fit to the data for the 60 day period of this study, with evident non-exponential character of the curve [37].

Conclusion

MTX showed linear pharmacokinetics following *iv* administration up to the dose of 80 mg/kg. The administration of a high dose of MTX (160 mg/kg) resulted in the similar pharmacokinetic behaviour as when applied in the twice lower dose (80 mg/kg), which can be explained by dose-dependent changes in the expression of SLC and ABC transport proteins and intracellular metabolism. Furthermore, the classical two-compartment model could not explain the pharmacokinetics of MTX in a small percentage of experimental animals, which opens up new strategies for the use of fractional order pharmacokinetic models in MTX therapy optimisation.

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The risk of metabolic syndrome in patients with arterial hypertension in relation to psychological and biological risk factors

Rizik od nastanka metaboličkog sindroma kod bolesnika sa arterijskom hipertenzijom u odnosu na psihološke i biološke faktore rizika

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Abstract

Background/Aim. A type of personality and negative emotional reactions could be important for clustering biological risk factors for a cardiovascular disease in patients with arterial hypertension (AH). This study investigated if the patients with AH and psychological characteristics of the Distressed Type of personality with elevated anxiety/depression/aggression, have a higher risk of metabolic syndrome (MS) and explored value of the assessed parameters for MS occurrence. **Methods.** A total of 85 patients with AH were included in the cross-sectional observational study. Type D Scale-14 (DS-14) was used to detect Type D (Distressed) personality. The Hospital Anxiety and Depression Scale (HADS) assessed the levels of anxiety and depression and the Buss Perry Aggression Questionnaire (BPAQ) was used for the assessment of aggression. The explored biological parameters included: blood pressure, lipid status, body mass index (BMI), the occurrence of diabetes mellitus (DM) and MS. **Results.** Type D patients were frequently more anxious, aggressive and had more frequent MS compared to non-type D. Type D females were younger, more anxious and had a greater prevalence of DM than those with non-type D personality. A multivariate analysis revealed that in type D personality patients with AH, depression had predictive value for MS. **Conclusion.** The occurrence of both MS and AH was in correlation with the type D personality, anxiety and depression. Early detection/treatment of depression in patients with AH and Type D personality could decrease a risk of metabolic syndrome.

Key words:
metabolic syndrome; hypertension; cardiovascular diseases; risk factors; personality; questionnaires; depression; comorbidity.

Apstrakt

Uvod/Cilj. Tip ličnosti i negativne emocionalne reakcije mogu biti značajni za grupisanje bioloških i psiholoških faktora rizika od nastanka karidovaskularnih bolesti kod bolesnika sa arterijskom hipertenzijom (AH). Cilj rad bio je da se istraži da li bolesnici sa AH i psihološkim karakteristikama ličnosti sklonih distresu, sa povišenim nivoima anksioznosti/depresivnosti/agresivnosti, imaju veći rizik od prisustva metaboličkog sindroma (MS) i prediktivnu vrednost ovih parametara od pojave MS. **Metode**. Ukupno 85 bolesnika bilo je uključeno u opservacionu studiju preseka. *Type D Scale* (DS-14) je korišćena u cilju određivanja D (distres) tipa ličnosti, *the Hospital Anxiety and Depression Scale* (HADS) za određivanje nivoa anksioznosti i depresivnosti, a *the Buss Perry Aggression Questionnaire* (BPAQ) za utvrđivanje agresivnosti. Ispitivanje bioloških parametara uključivalo je određivanje nivoa krvnog pritiska, lipidnog statusa, indeksa telesne mase (ITM), prisustva dijabetesa melitusa (DM) i MS. **Rezultati**. Bolesnici D tipa ličnosti imali su veću zastupljenost povišene anksioznosti, agresivnosti i MS u odnosu na one bez D tipa ličnosti. Žene D tipa ličnosti su bile mlađe, imale povišenu anksioznost i veću prevalencu DM u odnosu na one koje nemaju tip D ličnosti. Mulitvarijantna analiza pokazala je da je depresija nezavisan faktor rizika za pojavu MS kod bolesnika sa AH i D tipom ličnosti. **Zaključak.** Udruženost MS i AH je u korelaciji sa tipom D ličnosti, anksioznošću i depresijom. Rano otkrivanje/lečenje depresije kod bolesnika sa AH i D tipom ličnosti može biti korisno za smanjivanje rizika od metaboličkog sindroma.

Ključne reči:
metabolički sindrom; hipertenzija; kardiovaskularne bolesti; faktori rizika; ličnost; upitnici; depresija; komorbiditet.

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Introduction

The National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) recognized that multiple metabolic elements were cardiovascular risk factors and renamed the constellation of these metabolic risk factors as "The Metabolic Syndrome" (MS) [1]. The criteria included any of the following factors: obesity, defined as waist circumference ≥ 102 cm in males and ≥ 88 cm in females [based on the 1998 National Institute of Health (NIH) obesity clinical guidelines], while arterial hypertension (AH) was defined as blood pressure ≥ 130/85 mmHg, fasting glucose > 110 mg/dL, triglycerides ≥ 150 mg/dL and high density lipoprotein cholesterol (HDL-c) < 40 mg/dL based on the Joint National Committee guidelines [1].

In psychosomatic cardiology, biological and psychological factors play a key role in the onset and prognosis for a heart disease. Arterial hypertension is a major risk factor and if AH is associated with MS, the development of atherosclerosis and a heart disease is more certain [2]. Psychosomatic approach indicates psychological factors: suppressed anger and hostility as toxic for psycho-physiological pathway leading to hypertension. Such psychological features increase autonomic arousal, which causes peripheral resistance and contributes to the development of hypertension [3]. Type D (distressed) personality construct has a tendency to experience negative affect in social relations and to inhibit its expression. This type of personality is similar to neuroticism, meaning that individuals express their tension mainly by somatization [4]. They also have a higher risk of cardiovascular morbidity and mortality in both genders, regardless of cultural background. Type D personality in patients with hypertension, together with depression and anxiety [5] could increase the risk of the coronary artery disease (CAD). There are different findings about the correlation between biological and psychological parameters in cardiac patients. In some studies, patients with serious hypertension had higher anxiety, depression and neuroticism than normotensives [6]. In other studies, there was no relation between type D personality and psychological distress in elderly hypertensive patients [7].

Gender differences were also spotted. Controlled anger, an unhealthy lifestyle, and a high level of hopelessness were more frequent predictive factors for hypertension in men [8] than in women who expressed anger more openly. Other studies found that women who express or suppress anger have twice the risk of cardiovascular morbidity than men [9].

In our clinical practice, we have noticed anger issues and negative emotions in patients with hypertension. Considering different findings about the influence of emotional characteristics on the onset of CAD risk factors, we wanted to determine psychological characteristics of patients diagnosed with arterial hypertension (AH) as a possible link between AH and the presence of MS.

The aim of our study was to investigate if the patients with AH and psychological characteristics of type D personality, experiencing anxiety or depression, have a higher risk of MS in comparison to those without distressed personality. Also, it was assessed if any of the psychological parameters was predictive of MS in the patients with AH in our study.

Methods

Subjects

Our sample was a part of a larger pool of patients with AH and CAD treated at the Institute for Prevention, Treatment and Rehabilitation of Rheumatic and Cardiovascular Diseases "Niška Banja", where the approval from the Institutional Ethics Committee for this cross-sectional observational study protocol was obtained. The research was done during the 6-month-period, from January to September 2015. The total sample of 148 patients were consecutively recruited on admission in an out-patient setting. After explaining the purpose of the study, an informed consent was obtained from each subject.

The inclusion criteria for the study were: patients diagnosed with AH without a history of CAD. Their regular AH treatment included diuretics, β-blockers, ACE inhibitors, angiotensin receptor blockers, calcium channel blockers and α1-blockers, alone or combined. The exclusion criteria were: CAD confirmed by percutaneous coronary angiography or myocardial infarction, severe medical conditions such as stroke, dementia, renal insufficiency, psychiatric disorder and malignant illness.

Patients filled the questionnaires to assess psychological variables in the waiting room, after their regular cardiologic check-up. Finally, 85 subjects completed questionnaires and were analyzed. The differences in the presence of psychological and biological factors between D type and non-D Type personality, as well as the gender differences among D subgroup personality were assessed.

Instruments

The demographic data such as: gender, age, marital status and smoking habits were explored by the semi-structured general questionnaire. Biological parameters of the patients: systolic/diastolic blood pressure, total cholesterol, HDL-c, low density lipoprotein cholesterol (LDL-c), triglycerides, blood glucose, body mass index (BMI), the occurrence of diabetes mellitus (DM) and MS, were collected from the medical database of the patients.

The self-assessment questionnaires for psychological variables were used. The Type D Scale-14 (DS-14) detected type D personality [10]. It consists of two seven-item subscales measuring emotional and behavioral dimensions: negative affectivity (NA) and social inhibition (SI). A 5-point Likert scale measured the intensity of these dimensions: zero (not true) to four (true). A total score of ≥ 20 denotes type D personality, while the cut off score ≥ 10 on both subscales indicates the presence of NA and SI as personality traits, and an overall tendency to experience and act in an inhibited way in social situations.

The Hospital Anxiety and Depression Scale (HADS) assessed the levels of anxiety and depression [11]. This is a suitable instrument for non-psychiatric subjects because it explores emotional and cognitive symptoms, but without somatic symptoms of anxiety and depression, making it applicable for medical and cardiac patients. A 7-item subscale defines the intensity of each emotion. The item scores are classified on a scale

range from zero (not present) to three (severe problem). Scores ≥ 10 on both subscales indicate the presence of the elevated anxiety and depression that could be clinically significant and the total HADS score ≥ 20 indicates the actual emotional distress experienced in the past week.

The Buss Perry Aggression Questionnaire (BPAQ), a self-measuring instrument of 29 items, was used for the assessment of aggression [12]. Every item ranges from one (extremely not characteristic of me) to five (extremely characteristic of me). The Scale includes four subscales that measure hostility (cognitive component), anger (emotional), verbal and physical aggression (behavioral component). Higher scores on each subscale and a higher total result indicate greater aggressiveness and certain features of hostile tendency.

Statistical analysis

Statistical Package for Social Science (SPSS 18.0) was used for all statistical analyses. For continuous variables, Student's *t*-test, Welch's test (two-tailed), and the Mann-Whitey test were used to assess the differences in mean scores of variables between the groups when appropriate, while the chi-squared (χ^2) test was used for the frequency comparison. The Pearson's correlation was performed to estimate the association between characteristics, which occur both in a positive or negative manner. The association between MS and psychological and biological factors was estimated through a univariate logistic regression analysis.

Multivariate regression models were used for all significant parameters from the univariate analysis to determine the predictive value of those factors for MS.

Results

General characteristics of the sample

In the total sample, the age range was from 42 to 75 years. The average age of the subjects was 64.52 ± 7.92. Out of 85 patients, there were 53 women. One third of the sample (36.47%) was without a partner and the majority of subjects were non-smokers. Only five patients in the type D subgroup and three in the non-type D subgroup were consuming cigarettes for longer than a year (9.41%). The type D personality group had 50 (58.82%) subjects vs. 35 (41.17%) subjects in the non-type D group. There were 34 females in the D type subgroup (68%).

The psychological parameters

NA was the most prominent characteristic of type D personality, while the SI, anxiety, depression and total distress scores were not out of range. In the same subgroup of patients, aggression subscales revealed hostility as the most prominent dimension, followed by anger, physical aggression and verbal aggression, respectively.

Comparison between type D and non-type D subgroups

A comparison between type D (n = 50) and non-type D (n = 35) patients revealed a significant difference in all evaluated psychological variables. Anxiety, depression and distress (total HADS) score, total Aggression score, and all four dimensions of aggressiveness were more prominent in the type D subgroup. All biological variables were without significant difference present, except for MS, which was significantly higher in the type D subgroup (Table 1).

Table 1

Data related to parameters studied in the type D *versus* non-type D subgroup

Variables	Type D (n = 50)	Non-type D (n = 35)	*p*
Age (years), mean ± SD	63.14 ± 8.33	66.59 ± 6.88	0.041‡
Gender (M/F), n	16/34	16/19	0.134§
Type D score, mean ± SD	28.45 ± 6.23	13.26 ± 4.28	< 0.001†
Anxiety score, mean ± SD	9.51 ± 3.58	4.50 ± 2.09	< 0.001†
Depression score, mean ± SD	8.27 ± 3.72	4.61 ± 2.25	< 0.001†
Distress score, mean ± SD	17.70 ± 6.55	9.11 ± 3.38	< 0.001†
Anger score, mean ± SD	19.62 ±6.52	15.44 ± 5.45	0.001†
Physical aggression score, mean ± SD	16.84 ± 5.85	12.85 ± 4.48	0.001†
Verbal aggression score, mean ± SD	14.33 ± 4.46	12.05 ± 3,49	0.026†
Hostility score, mean ± SD	23.70 ± 6.54	18.17 ± 5.50	< 0.001†
Aggression score, mean ± SD	74.84 ± 16.09	58.52 ± 14.77	< 0.001†
Blood glucose (mmol/L), mean ± SD	6.28 ± 1.68	5.71 ± 1.08	0.258†
Cholesterol (mmol/l), mean ± SD	5.75 ± 1.18	5.79 ± 0.88	0.500†
HDL-c (mmol/L), mean ± SD	1.21 ± 0.30	1.20 ± 0.30	0.285†
LDL-c (mmol/L), mean ± SD	3.57 ± 0.99	3.86 ± 0.69	0.429†
Triglyceride (mmol/L), mean ± SD	1.91 ± 0.99	1.73 ± 1.06	0.960†
Systolic blood pressure (mmHg), mean ± SD	155.60 ± 11.85	160.61 ± 15.55	0.098†
Diastolic blood pressure (mmHg), mean ± SD	91.58 ± 10.27	92.02 ± 11.08	0.865†
Body mass index (kg/m^2), mean ± SD	29.29 ± 3.43	29.74 ± 4.36	0.882†
Diabetes mellitus, n (%)	14 (28)	7 (20)	0.644§
Metabolic syndrome, n (%)	30 (60)	13 (37.1)	0.046§
Average number of risk factors, mean ± SD	2.76 ± 1.14	2.41 ± 1.05	0.124†

†Mann-Whitney test; ‡*t* test; §chi-squared test (χ^2).
SD – standard deviation; M – male; F – female; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol.

Table 2

Psychological and biological risk factors in the type D hypertensive patients –gender differences

Variables	Type D men (n = 16)	Type D women (n = 34)	*p*
Age (years), mean ± SD	67.13 ± 7.16	61.31 ± 8.27	0.030‡
Anxiety score, mean ± SD	7.87 ± 2.80	10.25 ± 3.66	0.035†
Depression score, mean ± SD	7.68 ± 2.86	8.54 ± 4.06	0.624†
Distress score, mean ± SD	15.12 ± 5.25	18.88 ± 6.85	0.084†
Anger score, mean ± SD	18.25 ±6.77	20.25 ± 6.41	0.393†
Physical aggression score, mean ± SD	18.58 ± 6.44	16.05 ± 5.48	0.127†
Verbal aggression score, mean ± SD	14.00 ± 3.52	14.48 ± 4.87	0.625†
Hostility score, mean ± SD	21.75 ± 5.19	24.60 ± 6.95	0.140†
Aggression score, mean ± SD	73.62 ± 15.61	75.40 ± 16.50	0.570†
Blood glucose (mmol/L), mean ± SD	5.67 ± 0.80	6.56 ± 1.90	0.210†
Cholesterol (mmol/L), mean ± SD	5.31 ± 1.45	5.97 ± 0.98	0.061†
HDL-c (mmol/L), mean ± SD	1.14 ± 0.28	1.23 ± 0.31	0.651†
LDL-c (mmol/L), mean ± SD	3.15 ± 1.09	3.79 ± 0.88	0.073†
Triglycerides (mmol/L), mean ± SD	1.73 ± 0.80	2.05 ± 1.13	0.482†
Systolic blood pressure (mmHg), mean ± SD	155.68 ± 15.12	155.57 ± 10.27	0.654†
Diastolic blood pressure (mmHg), mean ± SD	93.12 ± 10.62	90.85 ± 10.18	0.397†
Body mass index (kg/m^2), mean ± SD	28.31 ± 3.11	29.75 ± 3.52	0.188†
Diabetes mellitus, n (%)	1 (6.25)	10 (28.6)	0.039§
Metabolic syndrome, n (%)	7 (43.7)	23 (65.7)	0.337§
Average number of risk factors, mean ± SD	2.31 ± 1.07	2.97± 1.12	0.068†

†Mann-Whitney test; ‡*t* test; §chi-squared test (χ^2).
SD – standard deviation; M – male; F – female; HDL-c – high density lipoprotein cholesterol; LDL-c – low density lipoprotein cholesterol.

The group was taking a stable dose of anti-hypertensive and anti-lipid medications, but their biochemical parameters were in the upper normal range. Despite the therapy, the average systolic and diastolic blood pressure values were higher for the entire group. BMI was over the upper limit, indicating that the group was mildly overweight. There were no statistically significant gender differences in the levels of biological and psychological parameters throughout the sample.

After dividing the sample into subgroups, gender differences among D type patients showed that women had a higher prevalence of DM than men (Table 2).

In order to find which parameters were related to the occurrence of MS in our patients with AH, a univariate regression was performed. The obtained data indicated that in the type D subgroup, anxiety and depression were significant risk factors for MS (Table 3).

Table 3

Risk factors for metabolic syndrome

Parameter	OR	95% CI	*p*
Gender	1.340	0.558–3.214	0.512
Type D personality	2.721	1.112–6.657	0.028
Negative affectivity	1.068	0.995–1.146	0.067
Social inhibition	1.045	0.949–1.151	0.368
Anxiety	1.163	1.030–1.321	0.015
Depression	1.214	1.057–1.395	0.006
Total aggression	1.022	0.996–1.049	0.092
Hostility	1.065	0.994–1.142	0.075

OR – odds ratio; CI – confidence interval.

All statistically significant factors obtained from the univariate regression analysis were processed in a multivariate logistic regression, but none of the parameters was found to be significant. In the multivariate regression analysis, which included type D personality, age and depression, it was revealed that depression can be considered as a significant predictive factor for MS [odds ratio (OR) 1.172; $p = 0.043$ (Table 4)]. The depression score increased by one, increasing the chance of MS by 17% and adjusting the remaining parameters in the model (Table 4).

Table 4

Risk factors for metabolic syndrome – Multiple regression analysis

Models	Parameters	OR	95% CI	*p*
2	Age	0.981	0.925–1.040	0.516
	Type D	1.526	0.545–4.271	0.421
	Depression	1.172	1.005–1.366	0.043
1	Age	0.982	0.926–1.041	0.533
	Type D	1.336	0.416–4.291	0.627
	Depression	1.150	0.968–1.366	0.113
	Anxiety	1.043	0.877–1.240	0.638

OR – odds ratio; CI – confidence interval.

Discussion

According to the findings about CAD risk factors in the literature [5, 6], we noticed that a hostile attitude, easily experienced negative emotions in social interactions, and increased anxiety and depression, are important for clustering risk factors in patients with AH. In this out-patient sample, we assumed that those with type D personality had a greater MS risk, which could lead to cardiovascular consequences in the future. Type D personality was presented in more than half of the sample, corroborating our clinical assessment about psychological issues in patients with AH.

These findings are quite different from those reported by Ringoir's et al. study [7], where the subjects with AH

were less distressed and only 8% were found with type D personality, while 5% experienced anxiety and/or depression. On the other hand, our results were more similar to those from a cross-cultural analysis of a larger sample performed in South Eastern Europe. Type D personality was found in 35% of patients with cardiovascular diseases, which was significantly associated with the prevalence of high blood pressure, smoking and depression, but not with a severe heart disease [13]. The similarity between the sociocultural characteristics of patients in these regions may be the reason for such findings. Suppressed negative emotions in their social relations and unsatisfactory life conditions are often present. Living without a partner at an old age, a lack of social support, low monthly income or a high ambition and a failure to achieve life goals, may be the reason for the increased cardiovascular reactivity and distressed reactions. The high prevalence of type D personality in persons with AH in our sample ought to be explored in the light of these potential influencing factors.

The literature data suggest that a sub-syndrome depression is more frequently associated with AH in comparison to healthy subjects [14]. In a prospective study conducted in Finland [15], major depression was three times more likely to develop in men with AH, while a research in Taiwan indicated a higher incidence of hypertension in patients diagnosed with depression [16]. Our subjects were not clinically depressed (did not meet the criteria for this diagnosis), but the level of depressiveness was higher in 19% of the sample, while anxiety was present in a quarter of the sample. Although the patients were tenser and more dysphoric than depressed, their mood was unstable and they get annoyed by something that is beyond their control up to few times a day, and the total distress score was not out of the range. These findings were more consistent with the conclusions seen in different meta-analysis cohort studies, which dealt with the relation of these two major health problems [14–16], where depression was ruled out as a potential risk factor for AH.

The findings that consider emotional reaction were in agreement with those suggested for people with type D personality. A hostile attitude and cynicism create cognitive disposition to perceive other people in a negative context and to experience angry feelings and verbal or physical aggressive behavior in a destructive but non-constructive way. The suggestion that the psychological factor is potentially "toxic" for the cardiovascular system is confirmed in other studies [17]. In our patients' population, hostility was the most prominent dimension, followed by verbal aggression, and the inner tension was sometimes relieved through explosive reactions to the close ones. There is a belief that controlled negative emotions could stimulate catecholamine release and contribute to AH [14, 15]. Suppressed, longstanding emotional tension causes an increase in hypothalamic-pituitary-adrenal (HPA) axis activity [18]. Such elevation of the cortical and catecholamine release/synthesis enhances atherosclerosis [19]. Despite pharmacologic treatment, average blood pressure, BMI and all biochemical values (serum levels of total cholesterol, HDL-c, LDL-c fractions, triglycerides, blood glucose) were slightly elevated in the whole sample. The finding was similar to the observation of Strike et al. [20] who found that hostile attitudes and angry feelings were connected with risk behaviors such as smoking, unhealthy diets, and obesity, as well as higher total cholesterol and LDL-c, and DM as major issues for the onset and progression of CAD.

Our assumption that type D patients with AH were more vulnerable in clustering all biological parameters was surprisingly not confirmed, as only MS, but not other biological parameters, was more prevalent in the type D personality group. The results were in accordance with the findings of the study conducted in our country. This research involved 79 patients with CAD, where MS was more prevalent in type D subjects with AH [21]. Also, in some large cohort prospective studies [22–24], MS and AH were clustered in subjects with type D personality, suggesting an increasing risk of CAD and diabetes.

The association between two biological factors could be explained by the influence of the increased inner tension on the HPA axis (catecholamine and cortisol instability) in type D patients with consecutive dyslipidaemia, one of the basic factors of MS. In addition, psychological parameters in our type D hypertensive patients, such as depression, anxiety and hostility, are often associated with behavioral risks (unhealthy eating habits, smoking, a lack of physical activity), thus contributing to a higher prevalence of MS. Beside biological causes, some attention should be given to psychological adversities, due to their possible importance of risk factor clustering.

Metabolic syndrome was more prevalent in the type D subgroup, thus we assumed psychological characteristics could mediate this relation. Regression analysis (univariate) confirmed that in patients with type D personality, anxiety and depression might be independent risk factors for MS, while the multivariate regression analysis indicated that none of the parameters is predictive of MS in our patients with AH (Model 1).

Nevertheless, after we had included only type D personality, depression and age in the multivariate regression (Model 2), the result indicated depression to be a significant predictive factor for MS. It is in accordance with findings that MS is associated with the incidence of depression and with a low recovery rate from depression in older adults [25]. In our sample, a depression level was significantly higher in the type D group, but none of the patients had a depressive disorder. The result indicates a possible benefit of further psychiatric evaluation of the patients for early recognition of depressive symptoms and treatment, if necessary. According to our experience in treating psychological issues in patients with AH, supportive psychotherapy, counseling and relaxation techniques are helpful for coping with stressors and achieving better emotional balance. It would be useful to conduct a prospective study with the follow-up of our subjects, to explore their cardiovascular and psychiatric outcomes in relation to type D personality, emotional and biological parameters.

Study limitation

It is not possible to argue that the studied sample of 85 patients is relatively small, especially those with type D personality. In addition, the study lacks some regular follow-ups that might reveal changes in the evaluated risk factors. These two deficiencies restrict our findings making it difficult to derive accurate conclusions. However, preliminary data obtained from our country and from an interesting study group can contribute to this pool of knowledge related to this topic. Our future research will be focused on psychological and biological changes of parameters, the health outcome of these hypertensive patients and characteristics of other groups of hypertensive subjects with different clinical comorbidities.

Conclusion

This study found the high prevalence of type D personality among patients with arterial hypertension. The type D personality patients were more distressed, anxious, depressed and hostile, and had a higher prevalence of metabolic syndrome compared to the non-type D personality hypertensive patients. The presence of both MS and AH positively correlated to type D personality, anxiety and depression. However, the elevated depression had a predictive value for MS in patients with hypertension and type D personality. It could be considered as a possible link between hypertension and MS. The female type D personality hypertensive patients had more prominent anxiety and more frequent MS and DM than male patients. An early detection of depression, psychological support or treatment of patients with arterial hypertension could be beneficial for their emotional well-being and a decreased risk of MS, especially in those with type D personality.

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Patient-related outcome measures and clinical evaluation of dental implant therapy in the elderly population – a cross-sectional study

Subjektivne mere ishoda i klinička evaluacija terapije dentalnim implantatima kod starije populacije – studija preseka

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Abstract

Background/Aim. Oral health disorders are crucial regarding general health and quality of life of the elders. The aim of this cross-sectional study was to evaluate the long-term clinical and patient-centered outcomes of dental implants, placed in partially and fully edentulous people older than 65 years. **Methods.** A total of 38 participants with an overall number of 168 implants were selected and underwent clinical and radiological examination. The implant survival rate, implant failure rate and other complications were recorded and analyzed. All participants agreed to respond to the Oral Health Impact Profile-14 (OHIP-14) questionnaire and another questionnaires on the Visual Analog Scale (VAS) related to their experiences and satisfaction with the overall implant treatment. Univariate and multivariate regression models were used to verify the relation between the OHIP score and the VAS questionnaires' items. **Results.** The implant survival rate was 94.3%. The number of implants without any complication was 73.2% (123), while biological and technical ones occurred in 17.3% (29) and 9.5% (16) implants, respectively. Regarding quality of life, significant difference was found only between those who wear fixed and removable restauration (p = 0.001). The multivariate regression model showed that the degree of satisfaction with shape and size of dentures was significantly associated with lower OHIP scores, indicating a better quality of life. **Conclusion.** According to the results obtained, it can be concluded that dental implant therapy in elderly people can be considered as predictable long-term treatment option regarding high implant survival rate, minimal complications and significantly better quality of life found in the group with fixed dentures.

Key words:
dental implants; aged; surveys and questionnaires; quality of life; patient satisfaction.

Apstrakt

Uvod/Cilj. Poremećaj oralnog zdravlja je od značaja za opšte zdravlje i kvalitet života starijih osoba. Cilj studije preseka bio je da se procene klinički parametri i subjektivne mere ocene dugogodišnje terapije dentalnim implantatima, primenjene kod bezubih i krezubih osoba starijih od 65 godina. **Metode.** Ukupno, 38 ispitanika sa 168 implantata bilo je uključeno u studiju preseka i podvrgnuto kliničkom i radiološkom pregledu. Stopa preživljavanja implantata, stopa gubitka implantata i druge komplikacije praćene su i analizirane. Svi ispitanici su popunjavali upitnik *Oral Health Impact Profile*-14 (OHIP-14) i odgovarali na pitanja uz korišćenje Vizuelno analogne skale (VAS) koja su se odnosila na njihovo iskustvo i zadovoljstvo celokupnom implantološkom terapijom. Univarijantni i multivarijantni regresioni modeli korišćeni su za proveru korelacije između uticaja implantološke terapije na kvalitet života i zadovoljstvo pacijenata terapijom. **Rezultati.** Stopa preživljavanja implantata bila je 94,3%. Zastupljenost implantata bez komplikacija iznosila je 73,2% (123), dok su se biološke i tehničke komplikacije dogodile kod 17,3% (29) i 9,5% (16) implantata, redom. U odnosu na kvalitet života, statistički značajna razlika pronađena je samo kod poređenja grupa sa mobilim i fiksnim zubnim nadoknadama (p = 0,001). Multivarijantni regresioni model pokazao je da je stepen zadovoljstva oblikom i veličinom zubnih nadoknada značajno povezan sa nižim ukupnim skorom OHIP-14 upitnika, što ukazuje na bolji kvalitet života. **Zaključak.** Na osnovu dobijenih rezultata može se zaključiti da se terapija dentalnim implantatima kod osoba starije životne dobi može smatrati predvidivim dugoročnim terapijskim izborom s ob

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zirom na visoku stopu preživljavanja implantata, minimalne komplikacije, kao i statistički značajno bolji kvalitet života ustanovljen u grupi sa fiksnim protetskim nadoknadama.

Ključne reči:
zubi, implantati; stare osobe; ankete i upitnici; kvalitet života; bolesnik, zadovoljstvo.

Introduction

A demographic revolution is in progress throughout the world. The proportion of elderly people (aged 65 and over) is growing faster than any other age group [1]. Today, 8.5% of people worldwide belong to this group and the number is projected to double, i.e. there will be 1.6 billion people over the age of 65 by 2050 [2]. Concerning these facts, the World Health Organization (WHO) established the concept of „Active Aging – A Policy Framework", in which amongst other health issues, oral health is emphasized as an essential. The influence of oral health disorders is crucial regarding general health and quality of life in elders [2]. Recently published epidemiological study has revealed that patients wearing dentures, with larger number of missing or decayed teeth, as well as those with dry mouth are more prone to have poorer Oral Health Related Quality of Life (OHRQoL) [3]. Although incidence of edentulism has been reported to decline [4], gradual tooth loss continues and presents influential determinant of poor OHRQoL among elderly people [3]. It is also known from the literature that oral health issues have the great impact not only on the well-being and social activities of people [5], but also on chewing efficacy and nutritional intake [6]. Elderly people today demand both functionally and socially acceptable dental solution. Due to the advancement of dental implant therapy, elderly population could benefit from possibility of receiving sustainable implant supported restorations and quality dental care including replacement of single teeth, multiple teeth, or fully edentulous conditions. Numerous study have demonstrated that the age is not a risk factor for dental implant outcome [7–9]. For instance, Park et al. [10] have retrospectively evaluated clinical and radiographic outcomes of 902 dental implants placed in 346 people older than 65 years. Patients were monitored for a period of 2–17 years after implant surgery and results revealed that survival rates were 95.39%. Although the survival of implants is understandable, the current state of the literature indicates that patient-related outcome measures (PROMs) may represent major aspects of the implant success for the patients [11]. In fact, patients need to function with prosthesis, thus their final evaluation should be considered paramount. Yet, in the literature, scientific evidence regarding implants in the elderly group has mostly focused on the provision of implants and related prostheses. There is less evidence concerning complications, prosthodontic maintenance needs and patient satisfaction in those who have aged with dental implants. Also, a review article evaluating OHRQoL in subjects with implant-supported prostheses concluded that in the majority of prospective studies, OHRQoL was assessed prior to treatment and post-treatment within 12 months of implant placement [12]. As life expectancy is increased, maintenance is inevitably required and complications may develop, so the level of satisfaction will possibly decline over time.

Therefore, the aim of this cross-sectional study was to investigate the long-term clinical and PROMs of dental implants placed in partially and fully edentulous elderly people, as well as prevalence of biological and technical complications during maintaining period.

Methods

The study followed guidelines established by the Declaration of Helsinki for research involving humans [13] and was approved by the institutional Ethics Committee.

Study population

This cross-sectional study was performed involving elderly participants aged over 65 in time of treatment with dental implants. The patients were selected using a database search and all potential participants were recalled for check-ups. Fifty-six patients with 252 implants were identified. However, 10 patients have deceased, six refused to attend check-up and two patients could not be located. The definitive study group consisted of 38 participants with an overall number of 168 implants. The patients, who accepted to participate in the study, received detailed explanations through an information session and all recruited participants signed an informed consent form. The information regarding age, sex, general health, systemic diseases, smoking habits, time of implant surgery, applied type of implantation and loading protocol, position and number of implants were retrieved either retrospectively from the patients' dental records or directly through face-to-face interview. All participants underwent a clinical and radiological (digital OPT) examination and completed questionnaires related to their experiences and satisfaction with the overall implant treatment and its impact on quality of life.

Clinical evaluation

The survival rate was assessed according to the success criteria of Albrektsson et al. [14]. Implant failure was considered based on implant loss, presence of mobility, pain, discomfort, neuropathy or removal due to severe peri-implant infection or implant fracture. Also, the clinical examination included a basic periodontal examination with the use of manual periodontal probe. Outcomes measured were the presence or absence of peri-implant suppuration, the modified plaque and sulcus bleeding indexes [15] and the

probing depth. Furthermore, the prevalence of complications, regardless of its type (technical or biological), was followed for all participants.

Patient-related outcome measures (PROMs)

For the quality of life analysis the Oral Health Impact Profile-14 (OHIP-14) test, developed by Gary D. Slade [16] in 1997 and later adapted to native language [17], was used. All participants completed the OHIP-14 giving answers in relation to the period after the prosthetic rehabilitation on dental implants were completed. Every item has five possible answers: never, occasionally, often, very often and always. The categories are marked gradually on a five-point scale, where 0 means never and 5 mean always. The final score was obtained by summing all the points awarded, with the lower score indicating a better result (improved quality of life). Also, patients' satisfaction regarding comfort, esthetics, ability to maintain hygiene, chewing ability and implant therapy in general, was evaluated using Visual Analog Scale (VAS) [18]. Participants expressed a subjective impression on the given question, marking the response to 100-millimeter scale, with the most negative impression at the zero point and the most positive at the point 100.

Statistical analysis

All statistical analyses were done using Statistical Package for Social Science (SPSS software package, version 24.0; *SPSS Inc., Chicago, IL, USA*). Mean, median, standard deviation (SD) and range were used for description of numeric data. Descriptive data were expressed as a percentage for discrete measures. Categorical variables were compared using chi square test (χ^2). Numeric data were analyzed using Kruska-Wallis test and Mann-Whitney *U* test according to sample distribution detected with One-sample Kolmogorov-Smirnov test. Univariate and multivariate regression models were used to assess the relationship between parameters. Differences were considered significant when the *p* value was less than 0.05.

Results

The 38 participants who received 168 dental implants were examined in this study. There were 20 males and 18 females. The mean patient age at the time of implant placement was 68.4 years (range 65–84 years) and at the control appointments 72.34 years (range 67–87 years). The follow-up duration was 54.06 ± 48.072 months. Eight implants in 4 participants were removed due to the failure during the follow-up period. Four failures were caused by peri-implantitis, three because of technical complication and one implant was lost in the first six months, before loading. The overall survival rate of implants was 94.3% (Figure 1). During examination, it has been established that 73.2% (123) implants were without any complication while biological and technical ones occurred in 17.3% (29) and 9.5% (16) implants, respectively. Table 1 displays the overall OHIP score according to baseline characteristics of study participants, denture status and implant surgery. Statistical significant difference was observed only between those who wear fixed and removable dentures. In Table 2, results are depicted of non-

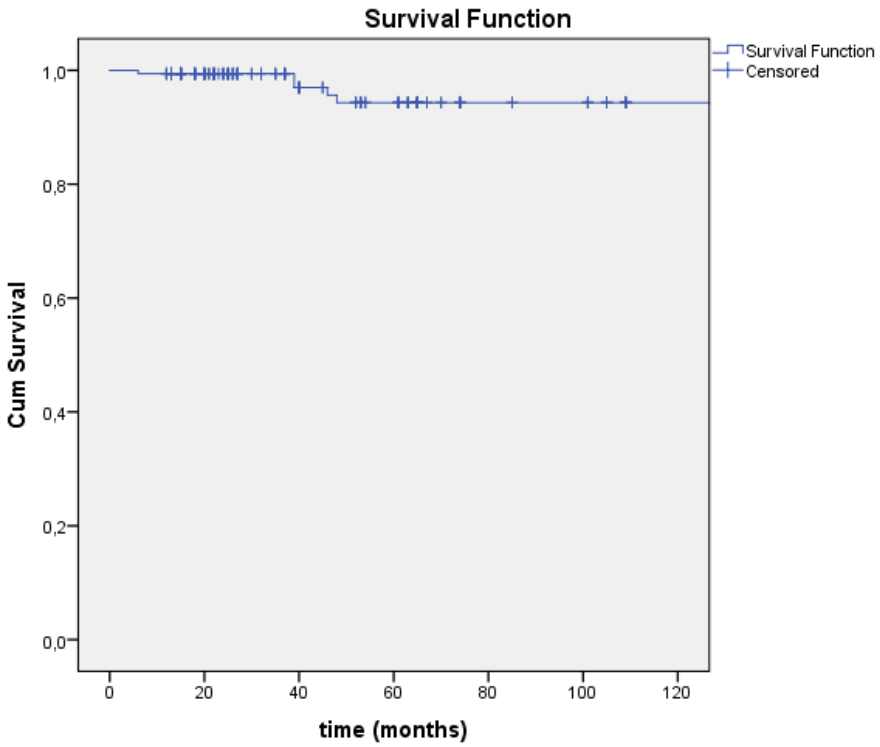

Fig. 1 – Kaplan-Meier implants survival estimate.

parametric correlations between the overall OHIP score and VAS questions from the query form. Further, the linear regression analysis was carried out to determine the contributions of the explanatory variables [age, gender, the American Society of Anasthesiologists (ASA) classification, type of denture and antagonist teeth, complication, failure, additional surgery and satisfaction evaluated with Visual Analog Scale regarding comfort, aesthetics, speaking ability, ability to maintain hygiene, chewing ability and implant therapy in general] on overall OHIP scores as a dependent variable. In the univariate linear regression model baseline participants' characteristics, denture status and implant surgery did not show significant association with the overall OHIP score. On the other hand, 7 out of 8 VAS questions were found to have a significant impact on the overall OHIP score. In the multivariate regression model, however, only the VAS 4 question proved to be an independent predictor of the overall OHIP score.

Table 1

Overall the OHIP-14 score according to participants' characteristics, denture status and implant surgery

Parameter	OHIP-14 score mean ± SD (min–max)	Significance
Gender		
male	2.30 ± 5.53 (0–25)	[a]$p = 0.150$
female	3.11± 3.86 (0–13)	
Age group (years)		
65–74	2.81 ± 5.37 (0–25)	
75–84	1.70 ± 2.00 (0–5)	[b]$p = 0.569$
≥ 85	6 ± 7.07 (1–11)	
ASA classification		
1	2.25 ± 3.62 (0–13)	
2	3.80 ± 6.73 (0–25)	[b]$p = 0.973$
3	1.80 ± 1.84 (0–5)	
4	1.40 ± 1.67 (0–4)	
Type of denture		
fixed denture	1.77 ± 4.87 (0–25)	[a]$p = 0.001$
removable denture	4.67 ± 4.05 (0–13)	
Type of antagonist teeth		
natural teeth	4.38 ± 8.47 (0–25)	
metal – ceramic teeth	1.31 ± 2.75 (0–11)	[b]$p = 0.068$
acrylic teeth	3.29 ± 3.58 (0–13)	
complications		
yes	3.93 ± 6.77 (0–25)	[a]$p = 0.433$
no	1.96 ± 3.03 (0–13)	
failure		
yes	4.00 ± 6.08 (0–11)	[a]$p = 0.822$
no	2.57± 4.73 (0–25)	
additional surgery		
yes	2.58 ± 2.43 (0–8)	[a]$p = 0.182$
no	2.73 ± 5.57 (0–25)	

OHIP – Oral Health Impact Profile; ASA – American Society of Anesthesiologists; SD – standard deviation.
[a]Mann-Whitney U test, [b]Kruskal – Wallis test.

Table 2

Nonparametric correlations between VAS questions and the overall OHIP-14 score for each participant

VAS question	(ρ)	p
1 (How do you rate your satisfaction with your denture?)	-0.372	0.022*
2 (How long did it take you to get used to your denture?)	-0.249	0.131
3 (Do you like the esthetical appearance of your denture?)	-0.474	0.003*
4 (Do you like shape and size of your denture?)	-0.339	0.037*
5 (Do you like the color of your teeth?)	-0.260	0.115
6 (How do you rate your chewing ability?)	-0.497	0.002*
7 (How do you rate cleanability of your denture?)	-0.159	0.340
8 (How do you rate the overall treatment experience?)	-0.542	0.000*

VAS – Visual Analog Scale; OHIP – Oral Health Impact Profile; ρ – Spearman's rank correlation coefficient.

Discussion

An obvious trend of accelerated aging in the world's population has gained considerable interest in dental implant rehabilitation of elderly group by the scientific community. As osseointegration is strongly governed by the patients' wound healing response, successful outcomes for dental implants could be expected to be less favourable in elderly patients due to age-related circumstances with slowed bone metabolism, weaken immune defence and chronic diseases. On the other hand, nowadays, elderly patients do not only expect optimal function and comfort, but are also interested in esthetics and other psychosocial parameters related to their perception of implant treatment. The present study investigated the long-term clinical and patient-related outcome measures of dental implants placed in elderly people using a cross-sectional study model.

Consequently, there are usual limitations of this study design. A prospective, long-term observational study would have provided results with a higher level of scientific evidence but one of the leading problems when investigating an elderly population with dental implants is accounting for all patients initially included in the study.

Results of this study revealed that the survival rate of implants placed in people older than 65 years was 94.3% which is in accordance with values found in previous studies [10, 19]. The great deal of failed implants (5 out of 8) were lost due to biological complications and the most common reason for implant failure was peri-implantitis (4 out of 8). Peri-implantitis is generally considered to be strongly connected with plaque retention and poor oral hygiene. In our study dental plaque was found in 100% of implants with registered peri-implantitis ($p = 0.000$). These findings are in accordance with the study of Serino and Ström [20], who found peri-implantitis in a greater percentage at implant sites with poor oral hygiene compared to sites with proper oral hygiene. Oral hygiene maintenance amongst the elders is compromised as a result of both limited motoric skills and required complex technique, therefore more simplified solutions should be considered for elderly people. Another interesting finding was that both implant failure rate and the incidence of other types of complications, were not found to be the parameters that significantly influenced patient's quality of everyday life. As it has been well demonstrated that subjects who requested implants had the poorest oral health related quality of life prior to treatment [21], it seems that those two important parameters did not contribute to the decreasing of satisfaction with overall treatment. Besides, the majority of complications were solved and patients usually accepted it as normal service during maintenance period. Equally important was the finding that necessity for additional surgery did not have the significant impact on participants' quality of life. It is in contrast with findings of De Bruyn et al. [22] who found that patients prefer straightforward over complex implant surgery that includes bone grafting procedures. The authors of this article share opinion, that in case of proper approach and detailed explanation of additional surgery inevitability, it can be expected that patient consent will be obtained without disturbing their judgement of the overall treatment and altering the quality of life.

Further analysis of the OHIP data distributed by gender showed no significant difference between men and women, although it was found slightly higher overall score for OHIP in the female group. It coincides with generally accepted way of thinking that women are more motivated, detail orientated and more inclined to express their dissatisfaction. This was confirmed in the study of other authors who found that women rated satisfaction with their dentures much lower than men [18, 23]. Regarding age groups, our study revealed no significant difference concerning OHRQoL. Nevertheless, it is obvious that middle-old participants reported the best implant treatment related quality of life. It can be assumed that those participants accepted implant-supported restorations as an option to improve their quality of life after becoming fully aware of their ages, medical issues and general health conditions. On the other hand, people in the early-old ages, compared their previous quality of life when they were younger with better general and oral health status, so they had higher expectations. On the third level, as people getting old, the ability for objective evaluation of their own life status constantly decreases, which makes it difficult for them to evoke emotional and sociological memories of previous life period. High demanding criteria are probably the most influential factor, why in the group of very old participants (over 85) the highest score for the overall OHIP was registered. It is important to highlight that there was no significant difference between overall scores for the OHIP of various medical patient groups, according to the ASA classification. This information is very important because the majority of people older than 65 have one or more chronic health disorders and still those people have the oral health issues, that have to be addressed adequately. The most contributing parameter for OHRQoL was the type of denture. Participants with fixed implant restorations showed significantly greater satisfaction and improvement of quality of life, compared to those with removable prostheses. It can be concluded that people regardless of their age, prefer more fixed prosthodontic solutions than mobile ones. Possible reasons are superior functional, esthetic and phonetic features of fixed over mobile dentures. To the best of our knowledge, there are no clinical studies which compared elders' quality of life with reference to the type of denture.

Non-parametric correlation was done in order to determine whether there is a mutual relation between participants' perception of the psychosocial impact of delivered restorations on their well-being and psychometric evaluation of their satisfaction with implant therapy. It can be stated that the elders are mostly concerned about aesthetical appearance as well as shape and size of their implant restorations. Furthermore, moderate correlation was found between the overall OHIP score and elders' rates of chewing ability with their new prostheses (the VAS 6 question). Therefore, the functional component of implant supported dentures can also be regarded as the factor of the immense importance which significantly contributed to the enhancement of the elders' quality of life. In addition to those questions specifically related to denture, strong

negative correlation was found between the elders' rate of overall treatment experience (the VAS 8 question) and overall score for OHIP. In other words, the greater the elders' satisfaction with treatment approach and protocol, the better perception about quality of life improvement was found. The overall score for the OHIP regarding the type of antagonist teeth, did not differ significantly amongst natural dentition, metal-ceramic and acrylic teeth. Yet, those elders with natural teeth with the highest score in this group were the least satisfied. These findings follow the logical pathway as they compared mobile or fixed dentures to their own teeth. On the contrary, other factors such as teeth colour or cleanability of dentures were not found to correlate significantly with the overall score for the OHIP. Although professionals may think that these two factors are dominant ones, from the participants' point of view, they are irrelevant with regard to their experience of quality of life improvement. Presumably, the elders are not as objective as doctors in relation to teeth colour, thus being unable to differentiate minor distinctions between numerous shades. Similarly, the elders are less interested about dentures cleanability, while from the doctor's standpoint it is tremendously important prerequisite for the long-term success of implant therapy.

According to the results of univariate and multivariate regression model analysis, it can be suggested that the degree of satisfaction with shape and size of dentures could describe almost 50% of variabilities amongst population in terms of general attitude about quality of life improvement after implant-prosthodontic therapy in the elderly group. Due to this fact, in conjunction with significantly better quality of life found in the group with fixed dentures, it can be postulated with great level of certainty, that the elderly people, above all, appreciate the comfort obtained by well-shaped and size-limited fixed restorations in comparison with bulky removable dentures.

Conclusion

Within the limitations of the study, it can be concluded that dental implant therapy in the elderly people can be considered as predictable long-term treatment option regarding high implant survival rate, minimal complications and improved quality of life. Also, main approach to implant-prosthodontic rehabilitation of the elderly people and making decision about type of implant restorations should be based on thorough examination and treatment planning, concerning general and intraoral health status, minimal surgical invasiveness, with understanding the participant's preferences regarding function, esthetics and oral hygiene maintenance.

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Influence of physical activity on prevention and occurrence of spinal deformities in children during development

Uticaj fizičke aktivnosti na prevenciju i pojavu deformiteta kičmenog stuba kod dece u razvoju

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Abstract

Background/Aim. The published data indicate that the appearance of spinal deformities in children is significantly influenced by physical activity. The aim of our study was to examine the influence of physical activity on prevention and occurrence of spinal deformities in children. **Methods.** The study was conducted as observational, clinical study in the period from 2016 to 2018. Participants were children with spinal deformities, which were examined, for the first time, by physiatrists and pediatric surgeons. The sample included 100 children with spinal deformities, aged 7–17 years. The control group consisted of 100 children without spinal deformity, of similar age. The study instrument was a questionnaire based on a survey filled by children or parents/legal guardians. The questions were related to different parameters of the possible significance for the existence of spinal deformity and especially to the influence of physical activity. The collected data were processed using methods of descriptive and analytical statistics. **Results.** Scoliosis the most common deformity of the spinal column, represented in about 67% of children ($p = 0.0006$). Respondents from both groups did not differ significantly in terms of gender. Children in the group with spinal deformities were older (11.5 ± 3.1 years vs. 10.4 ± 3.1 years, $p = 0.016$), with increased body weight (43.9 ± 16.0 kg vs. 39.3 ± 16.6 kg, $p = 0.046$) and height (151.7 ± 17.2 cm vs. 145.8 ± 18.2 cm, $p = 0.019$), as well as with less physical activity (81.0% vs. 92.02%, $p = 0.001$). Over 80% of children were regularly engaged in physical activity, more often recreationally and on average 2.5–3 hours per week. **Conclusion.** Children in the spinal deformity group were significantly less involved in physical activity than the control group, but there was no significant difference in the frequency and duration of time spent in physical activities during the week. It is important for children to be involved in physical activities of a recreational nature, and according to our research, 3 hours during the week.

Key words:
adolescent; child; exercise; kyphosis; scoliosis; spine; spinal curvatures; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Publikovani podaci ukazuju na to da na pojavu deformiteta kičmenog stuba kod dece značajan uticaj ima fizička aktivnost. Cilj istaživanja je bio da se ispita uticaj fizičke aktivnosti na prevenciju i pojavu deformiteta kičmenog stuba kod dece. **Metode.** Istraživanje je sprovedeno po tipu opservacione, kliničke studije u periodu od 2016. do 2018. godine. Učesnici studije su bila deca sa deformitetima kičme, koja su prvi put pregledana od strane fizijatra i dečjeg hirurga. Uzorak je obuhvatio 100 dece sa deformitetima kičme, uzrasta 7–17 godina. Kontrolnu grupu je činilo 100 dece bez deformiteta kičme, sličnog uzrasta. Instrument studije bio je upitnik na bazi ankete koji su popunjavala deca ili roditelji/staratelji. Pitanja su se odnosila na različite parametre od mogućeg značaja za postojanje deformiteta kičme, a posebno na upražnjavanje fizičkih aktivnosti. Prikupljeni podaci su procesuirani korišćenjem metoda deskriptivne i analitičke statistike. **Rezultati.** Skolioza je bila najčešći deformitet kičmenog stuba, zastupljena kod 67% dece ($p = 0.0006$). Ispitanici iz obe grupe nisu se bitno razlikovali prema polu. Deca u grupi sa deformitetima kičmenog stuba bila su statistički značajno starijeg uzrasta (11,5 ± 3,1 god. vs.10,4 ± 3, 1 god, $p = 0.016$), povećane telesne mase (43,9 ± 16,0 kg vs. 39,3 ± 16,6 kg, $p = 0.046$) i visine (151,7 ± 17,2 cm vs.145,8 ± 18,2 cm, $p = 0.019$) i bila su manje fizički aktivna (81,0% vs. 92,0%, $p = 0,001$). Preko 80% dece se redovno bavilo fizičkim aktivnostima, češće rekreativno

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i u proseku 2,5–3 sata nedeljno. **Zaključak.** Deca iz grupe sa deformitetima kičme su bila značajno manje uključena u fizičke aktivnosti u odnosu na kontrolnu grupu, ali nije bilo značajne razlike u učestalosti i trajanju vremena provedenog u fizičkim aktivnostima tokom nedelje. Za decu je važno da se bave fizičkim aktivnostima, rekreativno, a prema našem istraživanju 3 sata nedeljno.

Ključne reči:
adolescenti; deca; vežbanje; kifoza; skolioza; kičma, krivine; ankete i upitinici.

Introduction

Spine deformities are pathological deviations of the curvature of the spinal column from normal physiological curves. Nonstructural deformities of the spine are due to postural dysfunction, lower limb inability, inflammatory, posttraumatic and other conditions [1]. Structural deformities of the spine include deformities resulting from pathological changes in the structure and morphology of spinal vertebrae that are of etiologically different causes [1–3]. The results of systematic examinations of children in primary and secondary schools show that the deformities of the spinal column are increasing from year to year. The development of spinal deformity in children in the developmental period is associated with the gender, body weight, body height and age of the child, family burden of the spinal column deformities, hereditary diseases and other conditions, as well as with insufficient physical activity [3, 4]. The diversity of psychophysical abilities of children by age groups indicates that aerobic muscle endurance starts from early childhood, and anaerobic endurance and their strength from puberty and later [5]. In children aged 8–10 years, the elasticity and flexibility of the locomotor system is particularly expressed, while in children from 10–17 years, the strength of muscles dominates, along with the development of movements, speed and coordination. Reduced physical activity, rapid growth and poor life habits lead to the weakness of musculature of the trunk in the stage of rapid growth of children and the appearance of dysfunctional deformities of the spinal column [4, 6]. The changed statics have a tendency to progress with the onset of deformities, such as kyphosis and scoliosis, which can have permanent physical, psychological and social consequences on the growth and development of children. Physical activity is the basis for the preservation of health, it has a favorable effect on growth and it is equally important in all life cycles of the child's development. Regular physical activity, through exercises of the appropriate type, intensity and duration, is a prerequisite for preventing the development of spinal deformity in children [6]. Early detection of spinal deformities is of a great importance for the application of preventive measures to prevent the progression of deformities and possible unwanted complications [7]. Bearing in mind the different results in the available literature on the impact of physical activity on deformities of the spinal column, the aim of this study was to examine and determine the effectiveness of regular and individually oriented physical activity on the prevention and appearance of spinal column deformities in children in the developmental period.

Methods

The study was conducted as prospective, observational, clinical study at the University Children's Hospital (UCH) in Belgrade in the period from July 1, 2016 to August 1, 2018. Participants were children aged between 7-17 years, who, due to deformity of the spine, were examined by pediatric orthopedist and physiatrist. The group of respondents were children with deformities of the spinal column (D) (n = 100). The control group (C) was composed of the children without spinal deformity (n = 100), who came for physical examination of the pediatric orthopedist or physiatrist at the UCH, because of another reason and whose normal spinal column was confirmed, at least by one of mentioned physicians. The participants of both groups were otherwise healthy, because the children with congenital anomalies, associated diseases of the spine, syndromes, neuromuscular and metabolic diseases were excluded from the study. Parents or legal guardians of children completed questionnaires in cases where children were unable to fill them. The purpose of the research had been explained to respondents, before they confirmed in writing their voluntary consent to fill in the questionnaire.

The questionnaire, on the basis of the survey, was conducted on a sample of respondents from both examined groups. It contains 10 questions, which are defined quantitatively through 4 groups of questions.

The first group of questions was referred to demographic data on children: gender, age, weight and height.

The second group of questions was related to the type of spine deformity that has been detected in a child, based on a clinical finding: kyphosis, scoliosis and lordosis, as well as family burden on hereditary diseases, in particular hereditary deformities of the spine and associated morbidity.

The third group of questions has been related to the physical activity: the first question defined whether the child was engaged in physical activity, with the answers provided with yes and no; the second question referred to the weekly physical activity rhythm, and the response was offered in 2 scales: 1–3 times and more than 3 times. The third question has been related to the time that a child spends on physical activity during the week, and the response is also offered in 2 scales: 1–3 hours and over 3 hours.

The data collected from the questionnaire were analyzed by comparing the results with respect to the control group.

The questionnaire wass the original work of the author within the doctoral dissertation based on the available literature and many years of clinical experience as a specialist in social medicine.

During the study, all legal regulations, defined by the provisions of the UCH Ethics Committee were respected and harmonized with the European Guidelines in this field.

Statistical analysis

Statistical data analysis was done using the SPSS 20.0 for Windows (SPSS Inc.Chicago, IL, USA). The collected

data were processed using descriptive and analytical statistics and displayed both in tables and graphically. The significance of the difference was obtained by Student's *t*-test (in the form of contingency tables), the Fisher's, Mann-Whitney's and χ^2 test, as well as, by two-way ANOVA test for independent variables on multiple levels. Statistical significance was set as $p < 0.05$.

Results

According to subjects' gender among the subjects of the experimental group (D), 53 (53%) of children with spinal deformities were males, while in the control group, 45 (45%) were males without statistically significant difference between groups ($\chi^2 = 1.281$; $p = 0.258$) (Table 1).

The average age, body weight, and body height, as well as body mass index (BMI) of children with deformities of the spinal column and children in the control group are shown in Table 1.

Among 100 children with spinal deformities, 27% had kyphosis, 6% had lordosis and 67% had scoliosis. The most common spine deformity was scoliosis with very high statistical significance ($p = 0.0006$).

Considering the significant difference in the incidence of various types of spinal deformities (scoliosis, kyphosis and lordosis), demographic data observed within each of them are presented in Table 2.

Deformity of the spine as a family burden was found in 29 (29%) of children in the group with deformities and in 14 (14.0%) of children without deformity of the spine (Figure 1), with statistically significant difference ($\chi^2 = 6.666$; $p = 0.010$). Different inherited diseases of the family members were presented in 11 (11%) children with spinal deformities and in 15 (15%) those without the deformity of the spine, without statistically significant difference ($\chi^2 = 0.707$; $p = 0.400$). Other diseases as family burden, such as hypertension, diabetes mellitus type 2, disorders of thyroid gland etc, were found in 31 (31%) children with deformities and in 28 (28%) children without deformity of the spine, also without statistically significant difference ($\chi^2 = 0.216$; $p = 0.642$).

Physical activity was performed in 81% of children with spinal deformities and in 92.0% of children without spinal deformity, with statistically significant difference between examined groups ($\chi^2 = 5.181$; $p = 0.001$) (Table 3).

The children of both examined groups, with and without spinal deformities were equally engaged in physical activity, 3 times a week, on average 2.5–3 hours per week (range 1–7 h), without any statistically significant difference between them ($U = 4{,}940.5$; $p = 0.879$) (Table 3).

Median time spent in physical activity per week, in children with spinal deformities was 3 hours (range 1–8), while in children without deformity it was 2.5 hours (range

Table 1

Demographic data of participants

Parameter	Values	*p*
Total number (%)		
D	100 (100)	
C	100 (100)	
Gender (M/F), n (%)		
D	53/47 (53/47)	0.258
C	45/55 (45/55)	
Age (year), mean ± SD; median (range)		
D	11.5 ± 3.1; 11.0 (7.0 –17.0)	
C	10.4 ± 3.1; 9.5 (7.0–17.0)	0.016
Body weight (kg), mean ± SD; median (range)		
D	43.9 ± 16.0; 43.0 (18.0–91.0)	
C	39.3 ± 16.6; 34.0 (18.0–90.0)	0.046
Body height (cm), mean ± SD; median (range)		
D	151.7 ± 17.2; 152.0 (116.0–186.0)	
C	145.8 ± 18.2;143.5 (116.0–200.0)	0.019
BMI (kg/m^2), mean ± SD; median (range)		
D	18.5 ± 4.2; 17.9 (11.4–31.9)	
C	17.7 ± 3.6; 17.0 (11.5–32.0)	0.134

D – group of children with deformities of the spinal column; C – control group; M – males; F – females; BMI – body mass index; SD – standard deviation.

Table 2

Demographic data of participants with deformities of the spinal column in regard to the type of spine deformity

Parameter	Values			*p*
Number of participants, n (%)				
scoliosis		67 (67)		
hyphosis		27 (27)		
lordosis		6 (6)		
Gender (M/F), n (%)				
scoliosis	36/31	(54/46)		
kyphosis	13/14	(48/52)		0.705
lordosis	4/2	(67/33)		
Age (years), mean ± SD; median (range)				
scoliosis	11.45 ± 2.996;	11.00	(7–17)	
kyphosis	12.07 ± 3.245;	12.00	(7–17)	0.213
lordosis	9.67 ± 2.658;	9.00	(8–15)	
Body weight (kg), mean ± SD; median (range)				
scoliosis	44.14 ± 16.94;	40.0	(18.0–91.0)	
kyphosis	45.07 ± 13.58;	49.0	(20.0–69.0)	0.485
lordosis	43.93 ± 16.05;	43.00	(18.0–91.0)	
Body height (cm), mean ± SD; median (range)				
scoliosis	152.07 ± 16.28;	152.0	(116–186)	
kyphosis	153.67 ± 18.03;	158.0	(118–185)	0.162
lordosis	151.72 ± 17.21;	152.0	(116–186)	
BMI (kg/m^2), mean ± SD; median (range)				
scoliosis	18.474 ± 4.586;	17.72	(11.4–31.9)	
kyphosis	18.710 ± 3.611;	18.40	(11.9–27.6)	0.946
lordosis	18.135 ± 2.466;	17.36	(15.1–21.4)	

M – males; F – females; BMI – body mass index; SD – standard deviation.

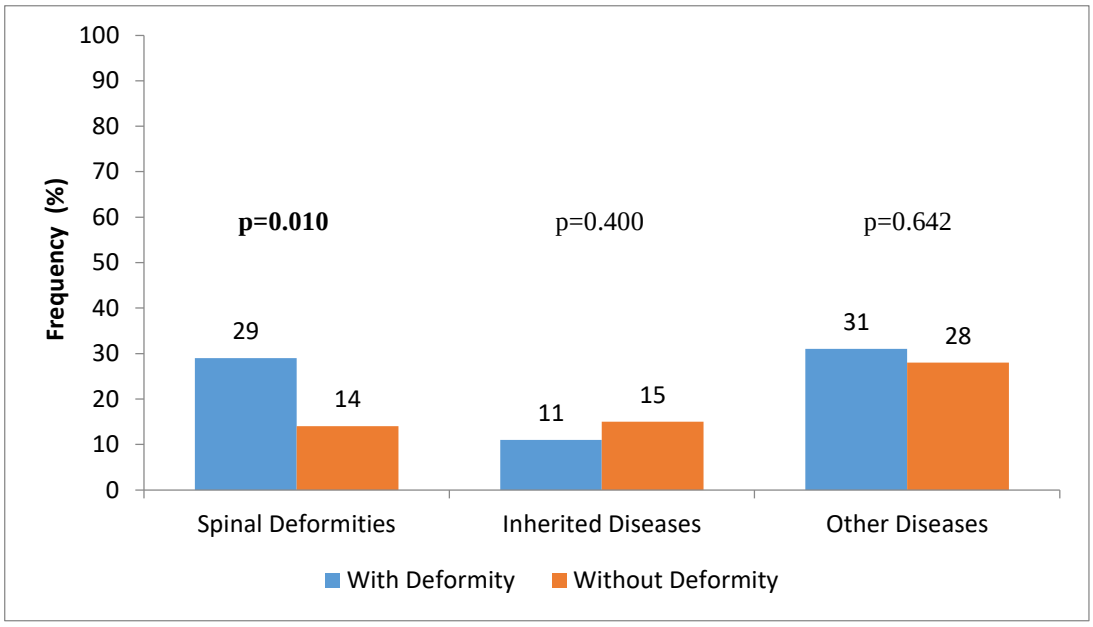

Fig. 1 – Distribution of children according to the association with family burden.

1–9), which was without statistically significant difference (U = 4535.0; p = 0.244) (Table 3).

Physical activity in the D group included recreational activity in 62% of the respondents, while 38.0% of the children had continuous training. In the control group, 61% of the children were engaged in recreational activity and 39% had continuous training, with no statistically significant difference between these two groups (χ^2 = 0.021; p = 0.884).

Also, significantly, more frequent spinal deformities existed in children who had deformity of the spine as a family burden.

In the countries of the European Union, in 10% of children, deformities of the spinal column occur due to insufficient physical activity during adolescence. The exercising physical activity is equally important in all ages, especially in school and at prepubertal age of the child. Reduced physical activity, sudden growth and development of children with bad habits of life, along with family burden, lead to deformity of the spinal column [10].

Table 3

Distribution of participants in regard to physical activity

Parameter	Values			*p*
Number of participants (%)				
D	81 (81)			0.001
C	92 (92)			
Frequency (times/week), mean ± SD; median (range)				
D	3.7 ± 1.7;	3.0	(1.0–7.0)	0.879
C	3.9 ± 1.9;	3.0	(1.0–7.0)	
Duration (h/week), mean ± SD; median (range)				
D	3.2 ± 1.5;	3.0	(1.0–8.0)	0.244
C	3.0 ± 1.7;	2.5	(1.0–9.0)	

D – group of children with deformities of spinal column; C – control group; SD – standard deviation.

Discussion

The most common spine deformities are scoliosis, kyphosis and lordosis [8]. The scoliosis in our study is more frequent, in almost two-thirds of patients, because the most difficult and more complex patients are treated at the UCH as a tertiary healthcare center. The results of the published studies suggest that the incidence of deformity of the spinal column is significantly influenced by the factors of the environment where the children come from, by the age, gender, body mass index, and especially the type and frequency of sport activities [9].

Gunawardena et al. [6], from Japan, in their randomized controlled trial suggest that there is a greater interest for sports in boys vs. girls, which is reflected in the increased incidence of spinal deformity in girls compared to boys in the same developmental period.

Comparing both groups of children with and without the deformity of the spinal column, our research showed that they did not differ much more in relation to the gender, but the older age and increased body weight and height were significantly more frequent in the group with spinal deformities. Although, this is not quite relevant, since these children were certainly older and therefore much heavier and taller than the children in the group without spinal deformities and, in particular, there was no significant difference in BMI among the study groups. Comparing demographic data and BMI of participants in regard to the type of spine deformity, we found that there were no statistically significant difference in the gender distribution, age, body weight and height and BMI among three observed groups of participants with scoliosis, kyphosis and lordosis.

The studies of Plaszewski et al. [9] in 2015 and Tsirikos and Jain [11] in 2011 were aimed to form official protocols on long-term health and treatment of children and adolescents with kyphosis and scoliosis, with the particular importance of prevention and correction of deformities. It was found that physical activities improve and enhance muscle strength, flexibility, bone vitality, BMI, and cognitive function, which emphasize the importance of preventing these deformities in relation to their corrective treatment [9, 11–13].

The World Health Organization has made recommendations on the importance of physical activity by age groups. At the age of 5–17 years, physical activity is recommended through everyday play, sports and recreation and planned exercises within physical education [14]. When choosing sports for children, the age, gender, height, weight of a child, health status and family burden should be taken into account. Moderate to more intense physical activity is recommended in children at least 60 min a day, up to three times a week [15, 16].

The American College of Sports Medicine was the first to formulate guidelines on the amount of physical activity that should be pursued in order to achieve the optimal functional capacity of vital parameters, physical endurance and quality of life [17].

Our research showed that children in the spinal deformity group were significantly less involved in physical activity than those in the control group. Although all of them were regularly engaged in physical activity, on average twice as many of them did it recreationally and not as a continouos

training, which was, on average, 2.5–3 hours per week, or 3 times a week for about 60 min.

Conclusion

Scoliosis is the most common deformity of the spinal column in children.

Children, in the spinal deformity group, are significantly less involved in physical activity than children in the control group, but there is no significant difference in the frequency and duration of the time spent in physical activities during the week. It is important for children to be involved in physical activities of a recreational nature, and according to our research, 3 hours during the week.

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Hyperhomocysteinemia and inflammatory biomarkers are associated with higher clinical SYNTAX score in patients with stable coronary artery disease

Hiperhomocisteinemija i biomarkeri inflamacije povezani su sa višim kliničkim SINTAKS skorom kod bolesnika sa stabilnom koronarnom arterijskom bolešću

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Abstract

Background/Aim. Previous studies have confirmed a positive correlation between homocysteine levels and a greater risk for acute coronary syndrome and stroke, but there are no available data to support an association between homocysteine and inflammatory markers and the severity of coronary artery disease according to the clinical SYNTAX score in patients with stable angina. The aim was to determine the association between homocysteine and inflammatory biomarker levels: interleukin (IL)-6, high sensitive C-reactive protein (hs-CRP), fibrinogen, erythrocyte sedimentation rate (ESR) and the severity of coronary artery disease according to clinical SYNTAX score. **Methods**. Eighty-two patients with stable angina pectoris (average age 65 ± 8 years, 28.9% females) underwent coronary angiography and were divided into three groups according to the clinical SYNTAX score: the group I < 22 (39 patients), the group II 23–32 (16 patients), the group III > 33 (27 patients). The severity and complexity of coronary artery disease were calculated by clinical SYNTAX score, multiplying the SYNTAX score with the modified ACEF score, based on the patients' left ventricular ejection fraction, age and creatinine clearance (derived with Cockcroft–Gault equation). **Results**. Homocysteine levels were significantly higher in patients with high clinical SYNTAX score [the group I: median (interquartile range – IQR): 10.20 (3.97), the group II: 10.45 (5.77), the group III: 14.70 (7.50), $p = 0.005$]. Patients in the group III had significantly higher homocysteine levels compared to the group I ($p = 0.001$). We also found a positive association between inflammatory biomarkers (IL-6, hsCRP, fibrinogen, ESR) and the severity of coronary artery disease according to the clinical SYNTAX score ($p = 0.017$, 0.001, 0.032, 0.049 respectively). We detected significantly lower plasma levels of vitamin B12 in the group III and group II in comparison with the group I (the group I: median (IQR): 238 (160), the group II: 171 (160), the group III: 172 (102), $p = 0.022$), which indicates its important role in homocysteine metabolism. **Conclusion.** The elevated plasma levels of homocysteine, IL-6, hsCRP, fibrinogen, ESR were detected in patients with high clinical SYNTAX score (> 33). Our results showed that hyperhomocysteinemia and some inflammatory biomarkers can predict more severe and extensive coronary artery disease in stable angina patients.

Key words:
coronary disease; inflammation mediators; homocysteine; angina, stable.

Apstrakt

Uvod/Cilj. Prethodne studije potvrdile su pozitivnu korelaciju između nivoa homocisteina i većeg rizika od nastanka akutnog koronarnog sindroma i moždanog udara, ali nije bilo istraživanja koja su ispitivala povezanost između vrednosti homocisteina i inflamacijskih markera i težine koronarne arterijske bolesti prema kliničkom SINTAKS skoru kod bolesnika sa stabilnom anginom pektoris. Cilj ovog istraživanja bio je da se utvrdi povezanost između koncentracije homocisteina i inflamacijskih biomarkera: interleukina (IL)-6, visoko senzitivnog C-reaktivnog proteina (hs-RCP), fibrinogena i sedimentacije eritrocita (SE) i stepena težine koronarne arterijske bolesti prema kliničkom SINTAKS skoru. **Metod.**

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Kod 82 bolesnika sa stabilnom anginom pektoris (prosečne starosti 65 ± 8 godina, 28,9% žena) urađena je koronarografija, nakon čega su podeljeni u tri grupe prema kliničkom SINTAKS skoru: I grupa < 22 (39 bolesnika), II grupa 23–32 (16 bolesnika), III grupa > 33 (27 bolesnika). Stepen težine koronarne arterijske bolesti određen je prema kliničkom SINTAKS skoru, množenjem SINTAKS I skora i modifikovanog ACEF skora, koji uzima u obzir ejekcionu frakciju leve komore, starost bolesnika i klirens kreatinina (izvedenog pomoću Cockcroft-Gault-ove jednačine). **Rezultati**. Vrednosti homocisteina bile su značajno više kod bolesnika sa visokim kliničkim SINTAKS skorom [I grupa: medijana (interkvartilini raspon – IQR): 10,20 (3,97), II grupa: 10,45 (5,77), III grupa: 14,70 (7,50), $p = 0{,}005$]. Bolesnici III grupe imali su značajno više vrednosti homocisteina u poređenju sa I grupom ($p = 0{,}001$). Takođe smo detektovali pozitivnu korelaciju između inflamacijskih markera (IL-6, hs-CRP, fibrinogena i SE) i težine koronarne arterijske bolesti prema kliničkom SINTAKS skoru (p = 0.017, 0.001, 0.032, 0.049 redom). Detektovali smo značajno niže vrednosti vitamina B12 u grupama III i II u odnosu na grupu I (I grupa: medijana (IQR): 238 (160), II grupa: 171 (160), III grupa: 172 (102), $p = 0{,}022$) što ukazuje na njegovu važnu ulogu u metabolizmu homocisteina. **Zaključak**. Povišene koncentracije homocisteina, IL-6, hsCRP, fibrinogena i SE u plazmi detektovane su kod pacijenata sa visokim kliničkim SINTAKS skorom (> 33). Naši rezultati pokazali su da hiperhomocisteinemija i pojedini inflamacijski biomarkeri mogu ukazati na prisustvo ozbiljnije i ekstenzivnije koronarne arterijske bolesti kod bolesnika sa stabilnom anginom pektoris.

Ključne reči:
koronarna bolest; zapaljenje, medijatori; homocistein; angina, stabilna.

Introduction

Amino acid homocysteine (HCy), participates in the initiation of endothelial dysfunction, and increases oxidative stress [1, 2], leading to accelerated atherosclerosis. HCy has been associated with hypercoagulability state and increased thrombus burden [3, 4] and it has been recognized as a risk factor for acute coronary syndrome and ischemic stroke [5, 6]. Recent studies [7, 8] concluded that hyperhomocysteinemia may develop as a consequence of chronic immune activation, which implies the importance of simultaneous measurement of both inflammatory markers and homocysteine levels, as well as vitamin B12 and folic acid.

Coronary artery disease (CAD) is mostly caused by atherosclerosis, which is considered to be an inflammatory disease [9, 10]. Inflammatory factors have a substantial role in the initiation and progression of CAD [11], and circulating markers reflect the inflammatory process within the coronary artery wall. During clinical practice, we have found that a certain number of patients without traditional risk factors for CAD had significant changes in coronary arteries. Thus, it is necessary to determine if some other risk factors may contribute to the formation and progression of CAD. Several studies have shown that fibrinogen is related to the increased cardiovascular (CV) risk [12] and plaque progression in patients with acute coronary syndrome and stable angina [13–15]. The AtheroGene study [16], which investigated 1,806 patients with documented CAD and stable angina pectoris, concluded that high fibrinogen and C-reactive protein (CRP) values were predictive for future CV risk, but did not provide additional information on top of traditional risk factors. CRP has been studied in patients with unstable and stable angina pectoris [17]. Very recent study [18] found a positive association between CRP levels and intrahospital mortality in patients with ST-elevation myocardial infarction. Another important inflammatory marker, interleukin (IL)-6, is engaged in pathogenesis of CAD, participating in plaque formation and its destabilization [19]. High levels of IL-6 have been detected in patients with unstable CAD, in comparison with stable angina patients [20].

The assessment of CAD severity can be done by using different scores, and according to the number of the diseased coronary arteries. The clinical SYNTAX (Synergy Between Percutaneous Coronary Intervention With Taxus and Cardiac Surgery) score (CSS), which combines SYNTAX I and modified the ACEF (age, creatinine, ejection fraction) score provides additional clinical characteristics based on the patients' left ventricular ejection fraction (LVEF), age and creatinine clearance (CrCl) derived using the Cockcroft-Gault [21] equation. It has been shown that CSS has predictive ability for adverse clinical outcome after percutaneous coronary intervention (PCI) [22–24] by incorporating clinical variables, but it also can be used in the assessment of the severity of CAD.

The aim of this clinical study was to investigate and determine the correlation between inflammation markers and metabolism of homocysteine and CAD and its severity in patients with stable angina pectoris.

Methods

The study included 82 patients with stable angina pectoris, and all had positive myocardial ischemia noninvasive tests, either on a treadmill exercise test or pharmacological echocardiography dobutamine stress test. Patients with acute coronary syndrome, active inflammatory diseases, infections and malignant diseases, as well as with previous myocardial infarction, history of coronary revascularization and severe valvular disorders were excluded. Several standard laboratory parameters were measured: fasting glucose, total and low density lipoprotein (LDL) cholesterol, triglycerides, creatinine, erythrocyte sedimentation rate (ESR), leukocyte count (Le), high sensitive C-reactive protein (hs-CRP) and fibrinogen.

The homocysteine level, expressed in μmol/L, was determined using a commercially available test on System Siemens nephelometric analyzer by immunonephelometry method in EDTA plasma samples. Sample coefficient of variation (CV) was 4.2%, and reference range 4.995–15.000 μmol/L. For serum IL-6 measurement we used DPC Immu-

lite 2000, Siemens analyzer by chemiluminescence immunometric assay. Sample coefficient of variation (CV) was 4.0%, and reference range 0.0–5.9 pg/mL.

Subjects assessed as positive for ischemic heart disease underwent coronary angiography in order to determine the severity of CAD according to the CSS [22, 23]. According to the severity of CAD, we divided all the patients into the three groups regarding the CSS: the group I (< 22 points), the group II (23–32 points), and the group III (> 33). We, also, estimated the severity of CAD according to the number of the affected coronary vessels (1-vessel, 2-vessel and 3-vessel disease). For the assessment of CAD severity, we used CSS, calculated multiplying the value of SYNTAX I score and modified ACEF score [24,25] based on the patients' left ventricular ejection fraction (LVEF), age and CrCl derived using the Cockcroft-Gault equation.

Statistical analysis was done using SPSS statistical software 25.0. Average values and standard deviation were used for data with a normal distribution. The median and interquartile range (IQR) were used for the data without normal distribution. A significant difference between the groups was measured using the Mann Whitney test for two independent groups and K independent samples (Kruskal Wallis) and categorical variables were compared by the chi-square test (χ^2).

The association between HCy serum levels and the severity of angina, clinical and anatomic SYNTAX score was estimated with logistic regression analysis. The results between groups were described as odds ratios (OR) (Mantel-Haenszel current OR) with a 95% confidence interval (CI). Cluster analysis with Ward's method was used for finding the cut-off points. For statistically significant differences we used $p < 0.05$.

Results

A total of 82 patients with the symptoms of stable angina (average age 65 ± 8 years, 28.9% females) underwent coronary angiography and were divided into three groups according to CSS: the group I (< 22; n = 39), the group II (23–32; n = 16), the group III (> 33; n = 27).

Patients' clinical characteristics and laboratory parameters from all the three groups are summarized in Table 1. There were significant differences between all three groups regarding age, physical activity, triglycerides, creatinine clearance and diastolic blood pressure on the admission to the hospital ($p < 0.05$). On the other hand, there was no statistically significant correlation between gender, active smoking, hypertension, family history, diabetes mellitus, fasting glucose, total and LDL cholesterol, atherosclerosis index, body mass index (BMI), acidum uricum, LVEF, end-diastolic, end-systolic diameter of left ventricular and the severity of CAD according to CSS. Homocysteine, inflammatory biomarkers (IL-6, hs-CRP, fibrinogen, ESR, leukocytes), folic acid, vitamin B12, prothrombin time (PT), activated partial thromboplastin time (APTT), as well as the number of affected and treated coronary arteries in all 3 groups are presented in Table 2.

There was a statistically significant positive correlation between homocysteine levels and the severity of CAD according to clinical SYNTAX score. Homocysteine levels were significantly higher in patients with high Clinical SYNTAX (> 33). Patients in group III had significantly higher HCy levels compared to group I (the group I: median (IQR): 10.20 (3.97), the group II: 10.45 (5.77), the group III: 14.70 (7.50), Kruskal Wallis test, $p = 0.005$); (Figure 1).

Then, we evaluated the odds ratio (OR) for CCS according to HCy values (I group HCy < 15 μmol/L, II group > 15 μmol/L) using multivariable logistic regression analysis (Mantel-Haenszel common OR with 95% confidence intervals). The patients with HCy > 15 μmol/L had more severe CAD according to CSS. We found that the OR between group III and group I was 8.125 with 95% CI (2.258–29.241, $p = 0.001$), and the relative risk was 4.695 (1.715–12.821). The high-risk patients for CAD were in the group with HCy values > 15 μmol/L (Figure 2). In multiple logistic regression analysis, where the Clinical SYNTAX score was a dependent variable, and homocysteine levels were independent variables we found statistically significant differences in HCy levels between group III (> 33) and group I (< 22); (Odds ratio = 1.230, 95% CI = 1.079–1.403, $p = 0.002$ and Odds ratio = 1.153, 95% CI = 1.015–1.309, $p = 0.028$, respectively). In multiple logistic regression analysis, where the multivessel disease was a dependent variable, and homocysteine levels were independent variables we found significant differences in HCy levels between 3-vessel and 2-vessel disease (Odds ratio = 1.217, 95% CI = 1.041–1.422, $p = 0.014$).

We detected significantly lower plasma levels of Vitamin B12 in group III compared to group I, which indicates its important role in HCy metabolism (the group I: median (IQR): 238 (160), the group II: 171 (160), the group III: 172 (102), Kruskal Wallis test, $p = 0.022$). Our results showed that HCy values were significantly higher in groups II and III, where vitamin B12 values were significantly lower. On the other hand, we did not find differences in folic acid values between all three groups (Table 2). We found that the inflammatory biomarkers (IL-6, hs-CRP, fibrinogen, ESR) were all in positive correlation with the severity of coronary artery disease according to CSS (Table 2). The presence of CAD was associated with higher values of IL-6 [the group I: median (IQR): 2.49 (2.67), the group II: 3.10 (3.91), the group III: 4.80 (4.52), Kruskal Wallis test, $p = 0.017$]; (Figure 3).

We detected significant differences in hs-CRP values between 3 groups, and additional statistical analysis showed differences between group III and group I, and group III and group II [the group I: median (IQR): 2.75 (5.77), the group II: 1.01 (2.78), the group III: 5.17 (8.84), Kruskal Wallis test, $p = 0.001$]. Comparison of the groups demonstrated significant differences in fibrinogen (Figure 4) and ESR values between group III and group I.

Fibrinogen in the group III was higher than in the groups I and II, which was statistically significant [the group I: median (IQR): 3.30 (0.90), the group II: 3.55 (0.85), the group III: 3.70 (0.60), Kruskal Wallis test; $p = 0.032$]. Patients with higher CSS had higher values of ESR [the group I: median (IQR): 18 (25), the group II: 22.5 (30), the group III: 26 (29), Kruskal Wallis test; $p = 0.049$].

Table 1

Patients' clinical characteristics and laboratory parameters from all the three groups according to the clinical SYNTAX score

Parameters	Clinical SYNTAX score			*p* (test)
	I (n = 39) < 22	II (n = 16) 23–32	III (n = 27) > 33	
Gender, n (%)				
female	8 (20.5)	1 (6.3)	10 (37.0)	> 0.05 (x^2)
male	31 (79.5)	15 (93.7)	17 (63.0)	
Age (years), median (IQR)	62.0 (13.0)	68.5 (11.5)	71.0 (9.0)	0.001 (KW)
Active smoking, n (%)	31 (79.49)	11 (68.75)	14 (51.85)	> 0.05 (x^2)
Hypertension, n (%)	38 (97.4)	13 (81.25)	26 (96.30)	> 0.05 (x^2)
Family history, n (%)	27 (69.23)	12 (75.00)	21 (77.78)	0.731 (x^2)
Diabetes mellitus, n (%)	13 (33.33)	3 (18.75)	11 (40.74)	0.332 (x^2)
Physical activity, n (%)	17 (43.59)	7 (43.75)	2 (7.41)	0.008 (x^2)
Glucose (fasting) (mmol/L), median (IQR)	6.00 (2.40)	5.60 (1.05)	6.40 (2.50)	> 0.05 (KW)
Triglycerides (mmol/L), median (IQR)	1.84 (1.21)	1.44 (0.86)	1.46 (0.75)	0.036 (KW)
Cholesterol (mmol/L), median (IQR)	5.24 (1.79)	5.29 (1.99)	4.74 (1.58)	> 0.05 (KW)
HDL cholesterol (mmol/L), median (IQR)	1.14 (0.31)	1.18 (0.23)	1.10 (0.36)	> 0.05 (KW)
LDL cholesterol (mmol/L), median (IQR)	3.00 (1.70)	3.00 (1.36)	2.92 (1.26)	> 0.05 (KW)
Atherosclerosis index, median (IQR)	2.98 (1.34)	2.59 (1.19)	2.32 (1.19)	> 0.05 (KW)
Atherogenic index of plasma, median (IQR)	0.22 (0.34)	0.17 (0.24)	0.11 (0.35)	> 0.05 (KW)
Body mass index (kg/m^2) median (IQR)	29.41 (5.07)	26.49 (4.74)	27.76 (4.80)	> 0.05 (KW)
Creatinine clearance (mL/min), median (IQR)	89.2 (15.4)	85.8 (21.3)	75.3 (29.8)	0.029 (KW)
Creatinine (μmol/L), median (IQR)	89.2 (15.4)	80.5 (18.0)	84.0 (29.0)	> 0.05 (KW)
Acidum uricum (μmol/L), median (IQR)	342 (136)	333 (120)	331 (147)	> 0.05 (KW)
Systolic blood pressure (mmHg), median (IQR)	140 (30)	130 (28)	140 (30)	0.004 (KW)
Diastolic blood pressure (mmHg), median (IQR)	80 (10)	80 (10)	80 (10)	> 0.05 (KW)
LVEF (%), median (IQR)	60.00 (2.00)	57.00 (5.00)	58.00 (5.00)	> 0.05 (KW)
End diastolic diameter (mm), median (IQR)	53.00 (5.00)	56.00 (4.25)	54.00 (9.00)	> 0.05 (KW)
End systolic diameter (mm), median (IQR)	34.00 (7.00)	35.50 (4.50)	35.00 (7.00)	> 0.05 (KW)

x^2 – chi-square test; KW – Kruskal Wallis test; IQR – interquartile range; HDL – high density lipoprotein; LDL – low density lipoprotein; LVEF – left ventricular ejection fraction.
***p* – values < 0.05 indicate significant differences regarding parameters among all 3 groups.**

Table 2

Laboratory parameters across the three groups of the clinical SYNTAX score

Parameter	Clinical SYNTAX score group			*p* (Kruskal Wallis test)
	I (< 22)	II (23–32)	III (> 33)	
Leukocytes (× 10^9), median (IQR)	7.03 (1.41)	6.71 (2.53	7.32 (2.74)	>0.05
ESR (mm/h), median (IQR)	18.0 (25.00)	22.5 (33.00)	26.0 (29.00)	0.049
C-reactive protein (mg/L), median (IQR)	2.75 (5.77)	1.01 (2.78)	5.17 (8.84)	0.001
Fibrinogen (g/L), median (IQR)	3.30 (0.90)	3.55 (0.85)	3.70 (0.60)	0.032
Interleukin-6 (pg/mL), median (IQR)	2.49 (2.67)	3.10 (3.91)	4.80 (4.52)	0.017
Homocysteine (μmol/L), median (IQR)	10.20 (3.97)	10.45 (5.77)	14.70 (7.50)	0.005
Folic acid (nmol/L), median (IQR)	14.6 (14.23)	13.1 (13.57)	14.02 (14.72)	>0.05
Vitamin B12 (pmol/L), median (IQR)	238 (160)	171 (160)	172 (102)	0.022
Prothrombin time (second), median (IQR)	1.05 (0.08)	1.01 (0.07)	1.02 (0.12)	> 0.05
Activated prothrombin time (second), median (IQR)	31.62 (5.77)	30.77 (5.28)	33.82 (9.70)	>0.05

IQR – interquartile range; ESR – erythrocytes sedimentation rate;
***p* – values < 0.05 indicate significant differences regarding parameters among all 3 groups.**

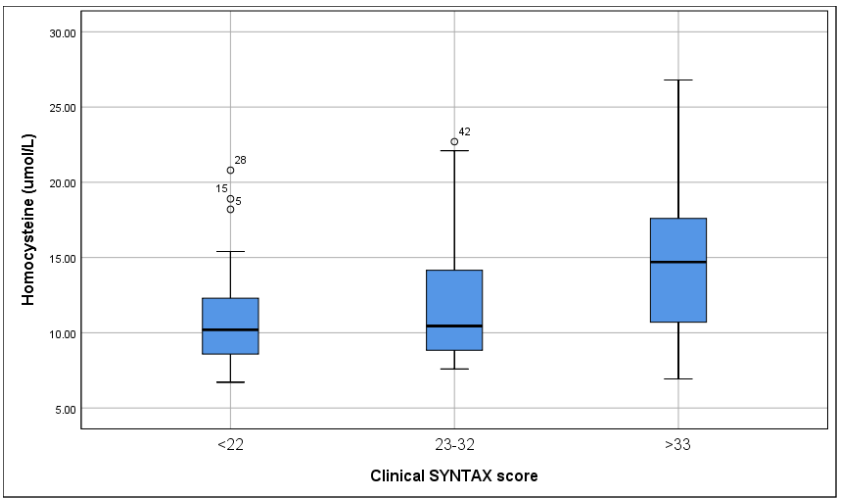

Fig. 1 – The correlation between plasma levels of homocysteine and the severity of coronary artery disease according to the clinical SYNTAX score.

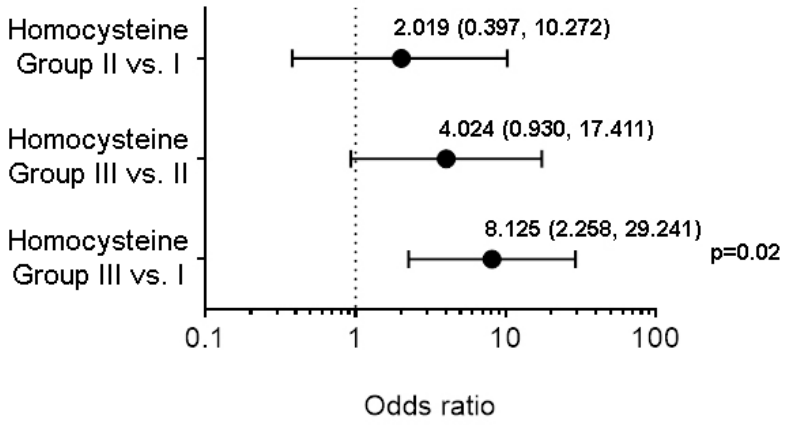

Fig. 2 – Odds ratio with 95% confidence intervals for the clinical SYNTAX score (CSS) according to the homocysteine (HCy) levels (HCy < 15 μmol/L, HCy > 15 μmol/L) in the study groups. Group I CSS < 22; Group II CSS 23–32; Group III CSS > 33.

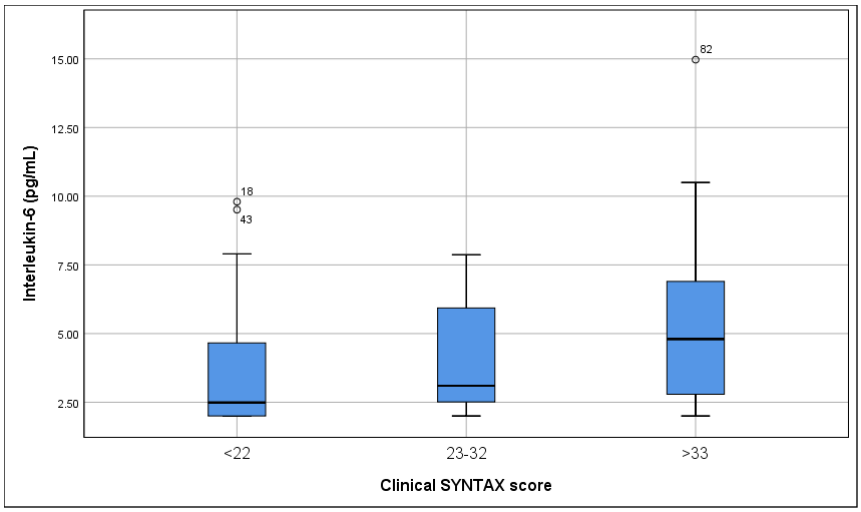

Fig. 3 – The correlation between plasma levels of interleukin-6 and the severity of coronary artery disease according to the clinical SYNTAX score.

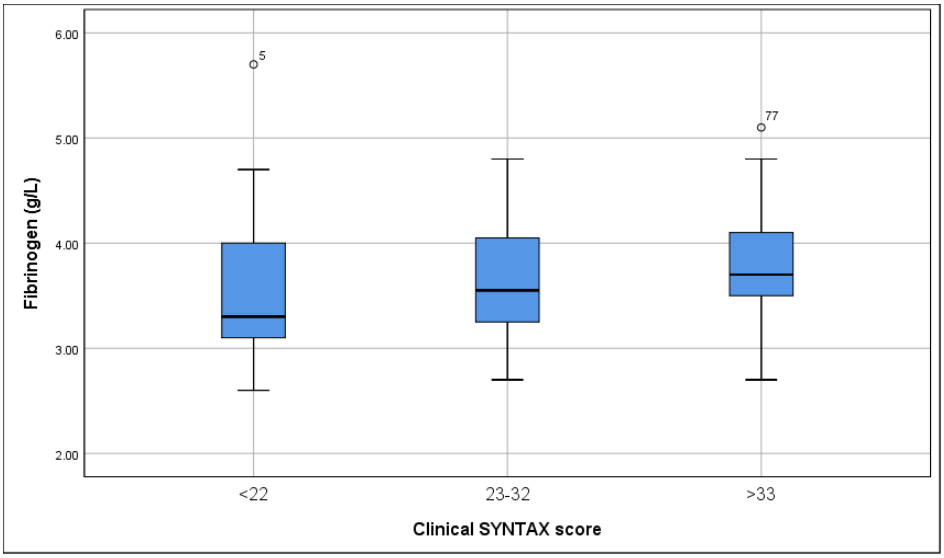

Fig. 4 – The correlation between plasma levels of fibrinogen and the severity of coronary artery disease according to the clinical SYNTAX score.

Discussion

The results of our study showed a significant correlation between the severity of CAD represented by the CSS and the levels of HCy and inflammatory markers (hs-CRP, ESR, IL-6, fibrinogen).

HCy reduces the production of nitric oxide (NO) and increases the proliferation of smooth muscle cells [26, 27]. HCy levels are influenced by vitamin B12 and folic acid, but also by a chronic immune response and renal function [28]. Unlike a recent study [29] in which a positive correlation between hyperhomocysteinemia and SYNTAX I score was found in patients with the acute coronary syndrome (ACS), we conducted the study where we found an association between the values of HCy and the CSS, but in stable angina patients. Two separate studies [30, 31] have shown that HCy levels were higher in patients with the three-vessel disease compared to those with single-vessel CAD. A positive correlation between hyperhomocysteinemia and acute coronary syndrome can be explained by its role in oxidative stress, endothelial dysfunction, and his prothrombotic activity, inducing the progression of stable to unstable atherosclerotic plaque [13, 14]. McCully [32] has shown that hyperhomocysteinemia can lead to accelerated atherosclerosis in the general population. The study we have done showed a positive correlation between HCy levels and the severity of CAD according to the CSS in patients with stable angina pectoris.

The results of our research are consistent with the results of the previous one, but a positive correlation was found, not only with HCy levels but also with the concentrations of the investigated inflammatory markers. Additional statistical analysis of the groups according to the CSS showed that the levels of the inflammatory markers (hs-CRP, ESR, IL-6, fibrinogen) were in correlation with the serum HCy levels and that a significant difference was detected between the group III and the group I. One explanation could be the synergistic action of HCy and inflammatory markers on the inflammation process in the blood vessel wall, which was the conclusion of the recent study [19] that detected the association of moderate hyperhomocysteinemia and cellular immune-mediated activity. Another assumption of the study was that the accumulation of HCy in the vessel wall might be due to a deficiency of vitamin B 12 which is related to chronic activation of the immune system. The results of a recent study [33] have shown that hyperhomocysteinemia in older patients with ACS is a significant predictor of total mortality and major adverse cardiovascular events (MACE). Our study included patients with stable angina pectoris, and it has shown the average age of 70 years, in the group III that had significantly higher levels of HCy than the group I, with an average age of 62 years, which is consistent with the fact that HCy levels raise with aging. We detected significantly lower plasma levels of vitamin B12 in the group III and the group II in comparison with the group I, which indicates its important role in HCy metabolism.

It is well known that inflammation is the initial step in atherosclerotic plaque formation, progression, and development of arterial thrombus burden [34]. Inflammatory mediators have an essential role in plaque destabilization and consequence rupture [35].

Some cohort studies [36] revealed that patients with multiple traditional risk factors did not have CAD, and that is one of the reasons why we conducted a study where we investigated traditional risk factors on one side, and HCy levels and the inflammatory markers on the other. Patients with more severe CAD (CSS > 33) were older, which can be explained by the cumulative effects of different CV risk factors in an extended period. Elderly patients have a high incidence of CAD and worse cardiovascular prognosis [37].

The results of our study showed significant correlation between inflammatory markers (hsCRP, ESR, IL-6, fibrinogen) and the severity of CAD according to the CSS ($p < 0.05$).

CRP is a biomarker of systemic inflammation, and elevated levels are detected in different conditions, such as in-

fection, injury and other inflammatory stimuli [38]. Recent study [18] involved patients with ST-elevation acute myocardial infarction (STEMI), and detected higher intrahospital mortality in those with higher CRP levels on admission to hospital. Other study [39] also showed a positive correlation between CRP and recurrent coronary events in ACS patients, but our study, to the best of our knowledge, was the first conducted to establish an association between CRP levels and the severity of CAD according to the CSS in patients with stable angina pectoris. In the early stage of inflammation, CRP provokes endothelial dysfunction, and therefore accelerates atherosclerosis by reducing NO release. Some studies [40, 41] have shown that high CRP levels are associated with future cardiovascular events in patients with unstable and stable coronary disease, but this is the first study in which the CSS was used for the severity assessment of CAD.

ESR has a positive association with traditional risk factors: gender, age, total cholesterol, body mass index (BMI), diabetes, and active smoking [42]. Reykjavik Study [43] has shown that ESR was an independent long-term predictor of CAD in both men and women. The results of our study are consistent with the study [44] in which ESR was related to the extent of atherosclerosis of coronary artery, but, unlike this study, we found an association with the extent of CAD according to the more accurate CSS.

IL-6 plays an important role in the pathogenesis of CAD [45], directly, leading to endothelial dysfunction, macrophage/ monocyte initiation, extracellular matrix degradation, and indirectly, stimulating the synthesis of coagulation factors. IL-6 also initiates the synthesis of other inflammatory markers in the liver [46]. The MESA study (Multi-Ethnic Study of Atherosclerosis) [47] with 6,617 participants without CV disease after 13.2 years of follow-up revealed a strong association and predictive value of IL-6 in the development of CV disease, heart failure, and total mortality. A large meta-analysis [48], which included 17 studies with 5,730 patients with CAD and 19,038 subjects in the control group, detected a strong association between IL-6 concentrations and CAD. Our results indicated that the elevated IL-6 values in the highest tercile were in positive correlation with CAD severity, and the CSS values were consistent with these findings. The elevated concentrations of IL-6 are detected at the very beginning of the inflammation in response to tissue damage and are a "warning signal" for the entire organism [49]. Concentrations of IL-6 correlate with obesity, which can explain the increased risk of CAD in obese patients. Our results are consistent with the previous study [50] because we found that 57.32% of patients were overweight (BMI 25–29.9 kg/m^2), and 26.83% were obese (BMI > 30 kg/m^2). Also, IL-6 stimulates the synthesis of the CRP [51], which can explain the results of our study where patients with a more severe CAD (CSS > 33), with high values of IL-6, also had elevated CRP values. The results of our study are entirely consistent with a recent study [52], which detected the elevated values in 100 patients with coronary angiography proven CAD, but we found a positive association with CAD severity according to CSS ($p < 0.05$). An explanation for the above may be the fact that the CSS takes into account the patients' age, renal function, and left ventricular ejection fraction.

A meta-analysis [53] comprising 31 studies with 154,211 subjects detected the correlation between fibrinogen concentration and the risk of CAD, stroke and other vascular mortality. A recent study [54] on 3,545 patients with stable angina pectoris during 7.3–10.2 years of follow-up showed that fibrinogen is a long-lasting independent marker of acute myocardial infarction and total mortality, and fibrinogen concentrations were the highest in patients with coronary angiographically most complex CAD, but not according to more sensitive CSS. Tabakci et al. [55] detected the severity and complexity of CAD in 134 patients, but patients were divided into three groups according to the values of SYNTAX score (SS control group = 0, SS intermediate group < 22, SS high-risk group > 22). In our study, we detected significantly higher fibrinogen values in the group of patients with the CSS > 33. De Luca et al. [56] detected a correlation between the severity of CAD by the number of affected blood vessels and the elevated fibrinogen levels. Very recent study [57] with 440 patients with acute myocardial infarction in whom 36 (8.2%) were identified as myocardial infarction with nonobstructive coronary arteries (MINOCA) and compared with myocardial infarction patients with obstructive CAD (MICAD), showed a significant increased fibrinogen concentration in both groups, which may be due to a myocardial infarction. Fibrinogen, as a precursor of fibrin, increases plasma viscosity, erythrocyte aggregation and has a thrombogenic potential because it connects thrombocytes in the formation of thrombus [58]. By comparing fibrinogen values among three groups, we found the highest values in patients with the CSS > 33, compared to small and intermediate-scale groups (< 32). A study of Cappelletti et al. [59] on 574 subjects, who performed coronary angiography, found that the elevated fibrinogen levels were associated with a critical narrowing of the main tree of the left coronary artery and the proximal segment of the left anterior descending artery (LAD). The results of our study was shown that there was a correlation between the concentration of fibrinogen and other investigated markers (ESR, CRP, IL-6) with significant stenosis (> 50%) of coronary arteries when we divided the subjects according to the CSS. The results of our study are in agreement with the results of other studies [60, 61], which have shown that baseline fibrinogen values may indicate the existence of a significant CAD and have the prognostic significance of future CV diseases.

To our knowledge, this is the first study where a significant difference in HCy and inflammatory marker levels were found between three groups according to the CSS. The results obtained in this study are consistent with previous studies, but we used the CSS for the assessment of the severity of CAD which included patients' clinical features like left ventricular ejection fraction, age, and creatinine clearance, besides anatomical variables.

Conclusion

The elevated plasma levels of homocysteine, IL-6, CRP, fibrinogen, ESR were detected in patients with high CSS (> 33), which confirmed previous findings that long-term, chronic inflammation of the coronary wall arteries preceded the formation of atherosclerotic plaques. We detected significantly lower plasma levels of vitamin B12 in the group III and the group II in comparison to the group I. Our results showed that both hyperhomocysteinemia and some inflammatory biomarkers could predict more severe and extensive CAD in stable angina patients. Higher values of tested parameters can be a useful prognostic indicator of the development of more severe clinical picture in patients with CAD.

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Polyphenol rich horseradish root extracts and juice: *in vitro* antitumor activity and mechanism of action

Antitumorska aktivnost i mehanizam delovanja polifenolima bogatih ekstrakata i soka korena rena *in vitro*

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Abstract

Background/Aim. Plant polyphenols are well known to show antimutagenic, anticarcinogenic, antiviral and antioxidative activity. The aim of this study was to investigate bioactive potential of *Armoracia rusticana* root juice and extracts: their polyphenol content, as well as *in vitro* antitumor activity and cell-death mechanism. **Methods.** Liquid-liquid extraction of polar and non-polar compounds was used and polyphenolic compounds were identified and quantified by high performance liquid chomatography (HPLC) analysis. Antiproliferative activity was examined *in vitro* on human cervix carcinoma (HeLa), breast adenocarcinoma (MCF7, MDA-MB-231), colon adenocarcinoma (HT-29), lung adenocarcinoma (A549), prostate adenocarcinoma (PC-3), melanocyte carcinoma (Hs 294T), hepatocyte carcinoma (Hep G2), as well as rat hepatocyte carcinoma (H-4-II-E), and normal human fetal lung (MRC-5) cell line using sulforhodamine B assay. The mechanism of cell-death in cell line was determined using Cell Death Detection ELISA^{PLUS} kit. **Results.** Dichloromethane extracts had the highest content of catechin, p-hydroxybenzoic, syringic and gallic acid (pulp, E1), and epicatechin (juice, E3). The results showed strong and non-selective antiproliferative activity of chloroform and dichloromethane extracts and root juice – highest being towards liver, breast and lung tissue cells. IC_{50} values of extracts and juice had low range of concentrations (IC_{50} = 3.49–26.5 μg/mL) and high range of dilutions (IC_{50} = 418–1,590). High and unfavorable potential of horseradish juice and chloroform juice extract (E4) to induce necrotic cell death was detected. **Conclusion.** Strong and non-selective *in vitro* antiproliferative activity of chloroform and dichloromethane extracts and root juice of horseradish was detected, with necrosis as a main mechanism of induced cell death. In order to utilize horseradish root bioactive potential further investigations that will pinpoint active components with more favourable apoptosis/necrosis inducing properties are needed.

Key words:
horseradish; phenols, antineoplastic agents; in vitro techniques; cell, death; apoptosis.

Apstrakt

Uvod/Cilj. Poznato je da polifenoli biljaka poseduju antimutagenu, antikancerogenu, antivirusnu i antioksidativnu aktivnost. Cilj ovog rada bio je ispitivanje bioaktivnog potencijala soka i ekstrakata korena *Armoracia rusticana*: određivanje polifenolnog sastava, *in vitro* antitumorske aktivnosti i mehanizma ćelijske smrti **Metode.** Primenom tečno-tečne ekstrakcije izolovane su i razdvojene polarne od nepolarnih komponenti, a HPLC metodom identifikovana su i kvantifikovana polifenolna jedinjenja. Antiproliferativna aktivnost ekstrakata i soka korena rena ispitana je *in vitro* na tumorskim ćelijskim linijama: karcinoma grlića materice (HeLa), adenokarcinoma dojke (MCF7 i MDA-MB-231), adenokarcinoma debelog creva (HT-29), adenokarcinoma pluća (A549), adenokarcinoma prostate (PC-3), karcinoma kože (Hs 294T), karcinoma jetre (Hep G2), kao i na ćelijskim linijama karcinoma jetre pacova (H-4-II-E) i normalnim fetalnim ćelijskim linijama pluća (MRC-5) upotrebom sulforodamin B testa. Mehanizam ćelijske smrti određen je detekcijom apoptoze i nekroze upotrebom Cell Death Detection ELISA^{PLUS} kompleta. **Rezultati.** Dihlormetanski ekstrakti korena rena imali su najveći sadržaj katehina, p-hidroksibenzoeve, siringinske i galne kiseline (pulpa, E1), i epikatehina (sok, E3). Utvrđena je

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snažna i neselektivna antiproliferativna aktivnost hlonajsnažnijim delovanjem na ćelijske linije jetre, dojke i pluća. Dobijene IC_{50} vrednosti bile su u niskom rasponu koncentracija (IC_{50} = 3,49–26,5 μg/mL) i u visokim razblaženjima (IC_{50} = 418–1590). Sok i hloroformski ekstrakt soka rena (E4) pokazali su snažnu, nepoželjnu sposobnost indukcije nekroze. **Zaključak.** Hloroformski i dihlormetanski ekstrakti, kao i sok korena rena ispoljili su su snažnu i neselektivnu antiproliferativnu aktivnost *in vitro*, sa nekrozom kao dominantnim roformskih i dihlormetanskih ekstrakata i soka korena rena, sa mehanizmom ćelijske smrti. U cilju iskorišćenja bioaktivnog potencijala korena rena, neophodna su dalja ispitivanja i izolacija aktivnih komponenti sa povoljnijim odnosom indukcije apoptoze i nekroze.

Ključne reči:
ren; fenoli; antineoplastici; in vitro; ćelija, smrt; apoptoza.

Introduction

Horseradish (*Armoracia rusticana*, G. Gaertn, B. Mey. and Scherb.) is perennial plant from Brassicaceae family that is cultivated for its aromatic, fleshy root. Horseradish root possesses intense and opulent taste that produces the feeling of cooling during consumption due to the presence of sulfur compounds called glucosinolates (GSLs) [1–4]. Epidemiological and pharmacological studies have shown that GSLs and their degradation products – isothiocyanates (ITC), may reduce the risk of developing cancer in humans [5]. The most commonly found natural ITC is allyl isothiocyanate (AITC) i.e. "burning oil" [6] that is derived from sinigrin – GSL abundant in Brassicaceae family especially in mustard, horseradish and wasabi [7]. Agneta et al. [8] detected 16 different GSLs in horseradish juice.

Plant polyphenols are well known to show biological and pharmacological activity, such as antimutagenic, anticarcinogenic, antiviral and antioxidative [9]. Phenolic acids (chlorogenic, caffeic, and ferulic), flavonoids (quercetin, genistein, catechins, isoflavones), quinones, coumarins, stilbenes, curcuminoids and lignans possess potent antioxidant and also anticarcinogenic and antimutagenic activities [10–14]. Over 20 phenolic compounds have been identified in some varieties of the Brassicaceae family, which include kale, curly kale, white and black cabbage, cauliflower and tronchuda cabbage [15, 16]. The most important phenolic compounds in *Brassica* species are flavonoid glycosides, such as glycosides of kaempferol and quercetin, and their derivatives hydroxycinnamic and sinapic acid [17, 18]. Phenolic acids, among them gallic, protocatechuic, p-hydroxybenzoic, vanillic, syringic, salicylic, p-coumaric, caffeic, ferulic and sinapic acid were identified in the kale (*Brassica oleracea* var. *Acephala)* [19]. On the other hand, little is known about the polyphenolic composition of horseradish. Based on earlier research, *Armoracia rustucana* contains a small amount of flavonoids, mainly kaempferol and quercetin [20]. Cirimbei et al. [21] demonstrated great potential of the aqueous plant extract from *A. rusticana* and its main flavonoids, kaempferol and quercetin, to protect DNA from damage induced on human lymphocytes by the oxidative agent hydrogen peroxide.

In order to gain insight into its bioactive potential, present study focused on the evaluation of *Armoracia rusticana* root juice and extracts: (1) their polyphenol content, (2) *in vitro* antitumor activity and (3) cell-death mechanism using mammalian cell lines. To the best of our knowledge this is the first study evaluating sequential horseradish root extracts as well as horseradish root juice.

Methods

Chemicals and standards

Reference standards of ≥ 98% purity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All used chemicals and solvents were of p.a. purity grade and were supplied by: Sigma-Aldrich (St. Louis, MO, USA), Acros Organics (New Jersey, USA), Lach-Ner s.r.o. (Neratovice, Czech Republic), Zorka Pharma, Šabac (Serbia), J.T. Baker (Deventer, Netherlands), AppliChem Panreac, (Darmstadt, Germany), Carlo Erba Reagenti (Milan, Italy), and Promochem (Wessel, Germany).

Preparation of horseradish root juice and extracts

Horseradish roots were obtained from Bački Petrovac, Serbia (45°21′5.32″N 19°37′21.65″E) in January 2016. Roots (2.64 kg) were peeled, chopped and grinded using Philips juicer (HR1858/55/BD, 650W). From 1.475 Kg of peeled horseradish 200 mL of juice and 1.25 kg of pulp was obtained. Juice and pulp were aliquoted in smaller portions and frozen (-20 °C) prior to extraction. Modified method of Kupchan [22] and Sarker et al. [23], based on the liquid-liquid extraction, was applied for the preparation of horseradish pulp and juice extracts. This method enables isolation and separation of polar and non-polar compounds. Mixtures of methanol and water (70:30, *v/v*), chloroform, dichloromethane or n-butanol were used as solvents for: 1) "crude extract" extraction (methanol), 2) extractions from methanol extract (chloroform, dichloromethane) and 3) further consecutive extractions (n-butanol and water) of horseradish root pulp and juice. The obtained extracts were gently evaporated to dryness using rota-evaporator. Dried extracts were sealed tight and kept at 4 °C until use.

Eight horseradish extracts from pulp, i.e. E1 (dichloromethane), E2 (chloroform), E7 (water) and E8 (n-butanol) and from juice E3 (dichloromethane), E4 (chloroform), E5 (water) and E6 (butanol) as well as horseradish juice J9 were used to examine chemical composition, *in vitro* antiproliferative activity and cell death activity.

Identification and quantification of phenolic acids and flavonoids by HPLC method

Horseradish root extracts and juice were analyzed using Shimadzu Prominence (Shimadzu, Kioto, Japan) high per-

formance liquid chromatography (HPLC) equipped with binary pump LC-20AT, thermostat CTO-20A and automatic dispenser SIL-20A connected to SPD-20AV UV/V detector. Chromatograms were recorded using different wavelengths for individual compounds: 280 nm for hydroxybenzoic acids, catechin and epicatechin, 320 nm for hydroxycinnamic acids, and 360 nm for flavonoids. Separation was performed on a Luna C-18 RP column, 5 mm, 250 x 4.6 mm with a C18 guard column, 4 × 30 mm (both from Phenomenex, Torrance, CA, USA). Two mobile phases, A (acetonitrile) and B (1% formic acid) were used at flow rates of 1 mL min^{-1} with the following gradient profile: 0–10 min from 10 to 25% B; 10–20 min linear rise up to 60% B, and from 20 min to 30 min linear rise up to 70% B, followed by 10 minutes reverse to initial 10% B with additional 5 min of equilibration time. Reference substances (flavonoids and phenolic acids) and samples were dissolved in 50% methanol.

In vitro antiproliferative activity

Cell lines

Human cervix epithelioid carcinoma (HeLa, ECACC 93021013), breast adenocarcinoma (MCF7, ECAACC 86012803; MDA-MB-231, ECAACC 92020424), colon adenocarcinoma (HT-29, ECAACC 91072201), lung adenocarcinoma (A549, ECACC 86012804), prostate adenocarcinoma (PC-3, ECACC 90112714), melanocyte carcinoma (Hs 294T, ATCC HTB-140), hepatocyte carcinoma (Hep G2, ECACC 85011430), as well as rat hepatocyte carcinoma (H-4-II-E, ATCC CRL-1548) and normal human fetal lung (MRC-5, ECACC 05090501) were used for the estimation of antiproliferative effects of horseradish root juice and extracts.

Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH, Pashing, Austria) with 4.5% glucose, supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA Laboratories GmbH, Pashing, Austria), 100 IU mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin (Galenika, Belgrade, Serbia). They were cultured in 25 cm^3 flasks (Corning, New York, USA) at 37 ºC in atmosphere of 5% CO_2, with high humidity, and subcultured twice a week. Single cell suspension was obtained using 0.1% trypsin (Serva, UK) with 0.04% EDTA.

Preparation of samples

Extracts were dissolved and further diluted in DMSO (to obtain five working concentration) and culture medium (1 μg/L of working concentration + 199 μg/L of culture medium; a = 200) to achieve required final concentrations. Ranges of final concentrations of extracts depended on extract yields and were in the range from 0.0625–1 mg/mL. Final concentration of DMSO in the samples was ≤ 0.05% (v/v).

Horseradish root juice was diluted in 0.9% NaCl to obtain four additional working dilutions and further in culture medium (1 μg/L of working concentration +199 μg/L of culture medium; a = 200) to achieve final dilutions in the range from 200–3200. The final concentrations of juice dilutions were in the range from 0.12–19.33 mg/mL, calculated on dry weight of juice (concentration of horseradish juice was 385.80 mg/mL).

Sulforhodamine B (SRB) assay

Cell lines were harvested and plated into 96-well microtiter plates (Sarstedt, Newton, NC, USA) at seeding density of 4–8 × 10^3 cells per well, in a volume of 199 μL, and pre-incubated in complete medium supplemented with 5% FCS, at 37 ºC for 24 h. Working concentrations of juice, extracts or solvents (1 μL) were added to the test and control wells. Microplates were then incubated at 37 ºC for an additional 48 h. Cell growth was evaluated by the colorimetric SRB assay, according to previously described procedure [24].

Absorbance was measured on a microplate reader (Multiscan Ascent, Labsystems) at 540/620 nm. Cell growth activity was expressed as a percent of the control and calculated as At/Ac × 100 (%), where At is the absorbance of the test sample and Ac is the absorbance of the control. The concentration-cell growth (dose effect) curves were drawn for each treatment and IC_{50} values (concentration that inhibit cell growth by 50%) were determined, using OriginPro 8 SRO (Origin-Lab Corporation, Northampton, USA).

In order to identify selectivity towards tumor cells compared to healthy tissue, non-tumor/tumor IC_{50} ratios were calculated as NT/T = $IC_{50}^{MRC-5}/IC_{50}^{repective\ tumor\ cell\ line}$ for extracts and as 1/NT/T for juice [25].

Cell death detection

Apoptosis and necrosis were detected using the Cell Death Detection ELISAPLUS kit (Roche, Version 11.0). Cell death detection was performed in HeLa, MCF7 and HT-29 cell lines using the most active extracts from cell growth experiments (E4 and J9), according to previously described procedure 25. Depending on the cell line used concentrations of extracts were 10 μg/mL (E4 in HeLa and MCF7) and 20 μg/mL (E4 in HT-29), while juice dilutions were a = 20 (J9 in HeLa and HT-29) for the 2 h exposition time.

Respective enrichment factors (ef) for apoptosis and necrosis (efA and efN) were calculated as ef = test/control using average absorbance-blank values for each sample and control [26, 27]. From these values, apoptosis/necrosis ratios were calculated indicating that apoptosis is a dominant mode of cell death if efA/efN > 1 or that necrosis is a dominant mode of cell death if efA/efN < 1.

Statistical analysis

Cell growth experiments were carried out in at least four repetitions (n = 4). Enrichment factors in cell death experiments were calculated using average absorbance-blank values (n = 2) from pooled quadruplicates (n = 4) for each sample and control. Results were expressed as mean ± standard deviation (SD). A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p < 0.05$.

Results

Polyphenolic compounds in Armoracia rusticana root juice and extracts

Major polyphenolic compounds present in investigated horseradish root juice and extracts were identified and quantified by HPLC analysis (Figure 1). Phenolic acids (gallic, protocatechuic, caffeic, ferulic, isoferulic, chlorogenic, p-hydroxybenzoic, p-coumaric, syringic, and synapic acid) and flavonoids (catechin, epicatechin, quercetin, kaempferol, luteolin, apigenin and isorhamnetin) were identified by matching their retention times (RT) and on-line ultraviolet (UV) spectra with those of standards. The content of total and individual phenolic compounds, quantified at 280, 330 or 360 nm, depending on their maximal response, is listed in Table 1. Catechin (flavan-3-ol) was detected at relatively high levels in all samples (0.75–33.17 mg/g), where E1 had the highest catechin content. Besides that, E1 had the highest content of p-hydroxybenzoic (6.68 mg/g), syringic (3.65 mg/g) and gallic acid (3.34 mg/g), while E3 had the highest content of epicatechin (2.80 mg/g). The horseradish juice J9 contained mostly catechin and gallic acid.

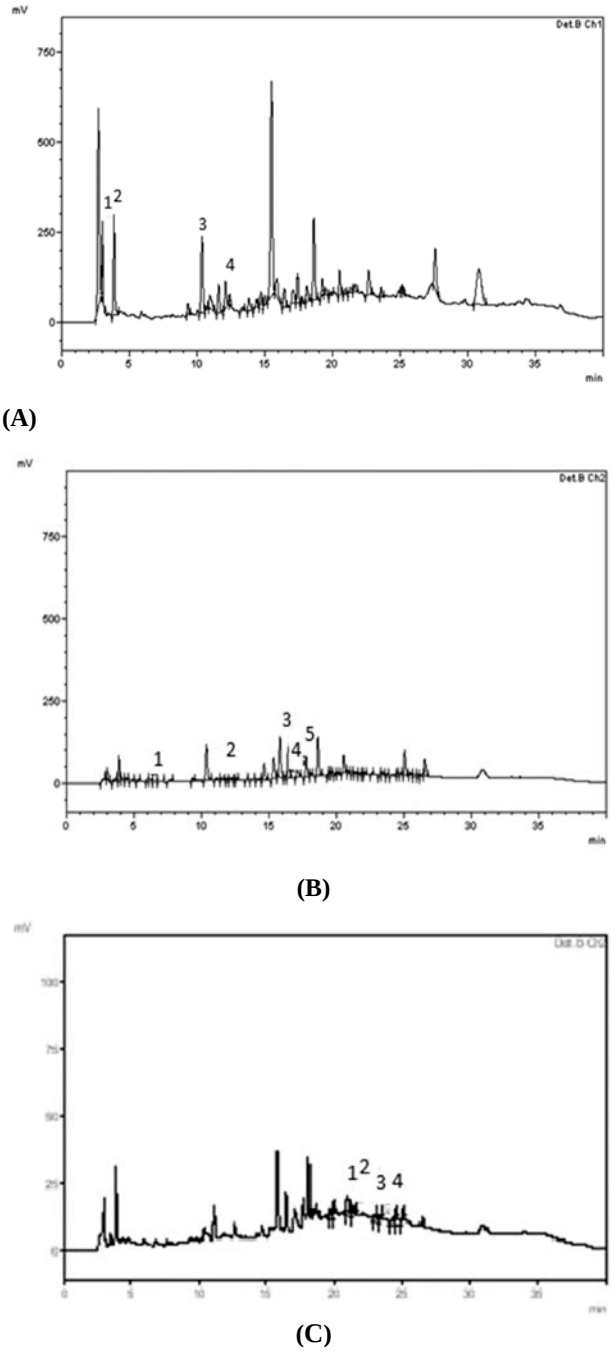

Fig. 1 – Phenolics in E1 extract. HPLC chromatograms on: A) 280 nm: 1-gallic acid; 2-catechin; 3-p-hydroxybenzoic acid; 4-syringic acid; B) 320 nm: 1-chlorogenic acid; 2-caffeic acid; 3-p-coumaric acid; 4-ferulic acid; 5-sinapic acid; C) 360 nm: 1-quercetin; 2-luteolin; 3-apigenin; 4-isoramnetin.

Horseradish extracts obtained from juice contained higher concentrations of epicatechin, ferulic and chlorogenic acid (dichloromethane); ferulic, isoferulic, p-coumaric acid and quercetin (chloroform); protocatechuic, syringic, chlorogenic, isoferulic acid and quercetin (n-butanol); and catechin, epicatechin, gallic and p-hydroxybenzoic acid (water) compared to extracts obtained from pulp.

In vitro antiproliferative activity

Obtained results revealed strong and non-selective antiproliferative activity of horseradish root extracts E4, E2, E3 and E1 and root juice J9 – highest being towards liver, breast and lung tissue cells (Table 2).

IC50 values of extracts and juice were obtained in low range of concentrations (IC50 = 3.49–26.5 μg/mL) and high range of dilutions (IC50 = 418–1590). Extract E4 and juice J9 possessed the highest cytotoxic activity towards rat hepatoma (IC50H-4-II-E = 3.49 μg/mL and IC50H-4-II-E = 1401.57) and human cervix carcinoma (IC50HeLa = 4.66 μg/mL and IC50HeLa = 1596.04), respectively (Table 2).

Higher antiproliferative activity of chloroform (E4), and n-butanol juice (E6) extracts was achieved compared to same extracts obtained from pulp (Table 2).

In order to identify selectivity towards tumor cells compared to healthy tissue, non-tumor/tumor ratios were calculated as NT/T for extracts and 1/NT/T for juice 25 (Table 3). Majority of extracts and juice demonstrated non-favourable (≤ 1) NT/T and 1/NT/T ratios indicating higher antiproliferative affinity towards healthy tissue. Most favourable (> 1) ratios were obtained in rat hepatoma cells using extracts E4 (NT/TH-4-II-E = 2.03) and E1 (NT/TH-4-II-E = 1.63), breast adenocarcinoma cells using E6 (NT/TMCF7 = 1.64) and cervix carcinoma cells using juice J9 (1/NT/THeLa = 1.18) indicating higher cell growth inhibition affinity towards tumor compared to healthy cells (Table 3).

In vitro cell death detection

In cervix carcinoma (HeLa) and colon adenocarcinoma cells (HT-29), horseradish juice J9 induced significant levels of apoptosis (efA = 2.59–4.73) compared to control, but also significant levels of necrosis (efN = 2.75–4.43) resulting in

Table 1

Polyphenolic compound contents (mg/g) in *Armoracia rusticana* root juice and extracts

Polyphenolic compounds	Pulp extracts (mg/g) mean ± SD				Juice extracts (mg/g) mean ± SD				Juice (mg/L) mean ± SD
	E1	E2	E7	E8	E3	E4	E5	E6	J9
Gallic acid	3.34 ± 0.17	1.87 ± 0.09	0.18 ± 0.01	0.60 ± 0.03	1.99 ± 0.09	0.21 ± 0.01	0.32 ± 0.01	0.53 ± 0.02	1.38 ± 0.06
Protocatechuic acid	0	0	0.36 ± 0.017	0	0	0	0.03 ± 0.001	0.03 ± 0.001	0.07 ± 0.002
Epicatechin	0	0	0.05 ± 0.002	0	2.80 ± 0.14	0	0.06 ± 0.003	0	0
Catechin	33.17 ± 1.66	8.36 ± 0.42	1.23 ± 0.06	1.07 ± 0.05	12.44 ± 0.62	1.24 ± 0.06	1.63 ± 0.08	0.75 ± 0.03	7.10 ± 0.35
Ferulic acid	0.01 ± 0.001	0	0	0.004 ± 0.001	0.45 ± 0.02	0.034 ± 0.001	0	0	0.01 ± 0.003
Caffeic acid	0.14 ± 0.006	0	0	0.003 ± 0.001	0.01 ± 0.001	0	0	0	0.01 ± 0.002
Syringic acid	3.65 ± 0.18	0.44 ± 0.02	0.02 ± 0.001	0.08 ± 0.003	0	0.10 ± 0.004	0.12 ± 0.004	0.06 ± 0.002	0.16 ± 0.01
Sinapic acid	0.65 ± 0.03	0	0	0.005 ± 0.001	0.11 ± 0.003	0	0	0	0.002 ± 0.001
p-coumaric acid	1.66 ± 0.08	0	0	0.01 ± 0.002	0.09 ± 0.004	0.01 ± 0.001	0	0	0.01 ± 0.002
Chlorogenic acid	0.07 ± 0.001	0	0.003 ± 0.001	0	0.20 ± 0.01	0	0	0.02 ± 0.001	0.02 ± 0.001
p-hydroxybenzoic acid	6.68 ± 0.33	0.26 ± 0.01	0.14 ± 0.01	0	0	0.11 ± 0.004	0.15 ± 0.01	0	0.64 ± 0.03
Isoferulic acid	0.02 ± 0.001	0	0	0.01 ± 0.001	0	0.04 ± 0.002	0	0.02 ± 0.002	0
Quercetin	0.23 ± 0.01	0.02 ± 0.001	0	0	0	0.04 ± 0.001	0	0.002 ± 0.001	0
Kaempferol	0	0.09 ± 0.003	0	0	0	0.02 ± 0.001	0	0	0
Luteolin	0.01 ± 0.001	0	0	0	0	0	0	0	0
Apigenin	0.02 ± 0.001	0	0	0	0	0	0	0	0
Isorhamnetin	0.02 ± 0.001	0	0	0	0	0.003 ± 0.001	0	0	0
Total	49.64	11.034	1.98	1.77	18.084	1.81	2.31	1.40	9.38

Pulp extracts: E1 – dichloromethane, E2 – chloroform, E7 – water, E8 – n-butanol; Juice extracts: E3 – dichloromethane, E4 – chloroform, E5 – water, E6 – n-butanol; Juice: J9. SD – standard deviation.

Table 2

Cell growth effects after 48 h exposure to horseradish extracts and juice (SRB test)

Extract, Juice/Cell line		HeLa	MCF7	HT-29	MRC-5	A549	H-4-II-E	MDA-MB-231	Hs294T	HepG2	PC-3
		IC_{50} (μg/mL), mean + standard deviation									
Pulp extracts	E1*	>31.25^{c}	>31.25^{c}	>31.25^{b}	>31.25^{c}	>31.25^{b}	19.19 ± 3.51^{b}	26.01 ± 2.45^{c}	>31.25^{c}	>31.25^{c}	>31.25^{b}
	E2*	21.31 ± 4.30^{b}	16.54 ± 1.20^{b}	28.46 ± 0.54^{b}	18.80 ± 3.88^{b}	28.11 ± 3.57^{b}	17.63 ± 0.88^{b}	19.44 ± 3.82^{b}	20.66 ± 3.00^{b}	15.42 ± 0.46^{b}	>31.25^{b}
	E7‡	>500^{f}	>500^{f}	>500^{e}	>500^{g}	>500^{e}	>500^{e}	>500^{f}	>500^{f}	>500^{f}	>500^{d}
	E8†	>125^{d}	>125^{d}	>125^{c}	>125^{d}	>125^{c}	>125^{c}	114.52 ± 0.28^{d}	>125^{d}	109.76 ± 9.67^{d}	>125^{c}
Juice extracts	E3*	>31.25^{c}	18.52 ± 2.19^{b}	>31.25^{b}	16.74 ± 0.33^{b}	>31.25^{b}	19.41 ± 3.45^{b}	26.50 ± 4.15^{c}	>31.25^{c}	>31.25^{c}	>31.25^{b}
	E4*	4.66 ⊥ 1.09^{a}	6.54 ± 0.66^{a}	12.17 ± 2.84^{a}	7.10 ± 1.87^{a}	11.43 ± 3.77^{a}	3.49 ± 1.24^{a}	5.52 ± 1.47^{a}	6.70 ± 1.81^{a}	5.68 ± 1.54^{a}	9.01 ± 1.89^{a}
	E5‡	>500^{f}	>500^{f}	>500^{e}	>500^{g}	>500^{e}	>500^{e}	>500^{f}	>500^{f}	>500^{f}	>500^{d}
	E6‡	239.86 ± 81.98^{e}	146.97 ± 4.29^{e}	460.97 ± 11.28^{e}	240.34 ± 40.20^{e}	354.66 ± 11.03^{d}	>500^{e}	>500^{f}	>500^{f}	>500^{f}	>500^{d}
Juice	J9§**	241.72^{e}	494.76^{f}	338.34^{d}	286.23^{f}	923.28^{f}	275.26^{d}	317.93^{e}	437.52^{e}	411.80^{e}	799.52^{e}
		IC_{50} (dilution), mean ± standard deviation									
	J9‖	1596.04 ± 386.77	779.77 ± 67.48	1140.26 ± 135.54	1347.86 ± 63.72	417.86 ± 3.61	1401.57 ± 240.37	1213.49 ± 97.68	881.79 ± 161.88	936.86 ± 79.22	482.54 ± 33.47

Investigated in 1.95–31.25*, 7.81–125† and 31.25–500‡ μg/mL range of concentrations. Investigated in 200–3200‖ range of dilutions or 0.12–19.33§ mg/mL range of concentrations (calculated based on dry weight of juice; concentration of horseradish juice was 385.80 mg/mL). Means within each column with different letter (a-g) differ significantly ($p < 0.05$). Calculated for mass concentrations of extracts and juice.**

Pulp extracts: E1 – dichloromethane, E2 – chloroform, E7 – water, E8 – n-butanol; Juice extracts: E3 – dichloromethane, E4 – chloroform, E5 – water, E6 – n-butanol; J9 – juice. SRB – sulforhodamine B; HeLa – human cervix epithelioid carcinoma; MCF7 – breast adenocarcinoma; HT-29 – colon adenocarcinoma; MRC-5 – normal human fetal lung; A549 – lung adenocarcinoma ; H-4-II-E – rat hepatocyte carcinoma ; MDA-MB-23 – breast adenocarcinoma; Hs294T – melanocyte carcinoma; HepG2 – hepatocyte carcinoma; PC-3 – prostate adenocarcinoma.

low efA/efN ratios (efA/efN = 0.94–1.07; Table 4). In cervix carcinoma (HeLa), breast (MCF7) and colon (HT-29) adenocarcinoma cells, extract E4 induced low levels of apoptosis (efA = 0.74–1) compared to control, but significant levels of necrosis (efN = 1–4.5) resulting in low efA/efN ratios (efA/efN = 0.22–0.74; Table 4).

Higher induction of necrosis was observed using juice extract E4 compared to horseradish juice J9 (Table 4).

Table 3

Non-tumor/tumor (NT/T) ratio of extracts and juice in different cell lines

Extract, Juice/ Cell line		HeLa	MCF7	HT-29	A549	H-4-II-E	MDA-MB-231	Hs294T	HepG2	PC-3
		NT/T								
Pulp extracts	E1	1	1	1	1	1.63	1.2	1	1	1
	E2	0.88	1.14	0.66	0.67	1.07	0.97	0.91	1.22	< 0.6
	E7	1	1	1	1	1	1	1	1	1
	E8	1	1	1	1	1	> 1.09	1	> 1.14	1
Juice extracts	E3	< 0.54	0.9	< 0.54	< 0.54	0.86	0.63	< 0.54	< 0.54	< 0.54
	E4	0.21	1.08	0.58	0.62	2.03	1.29	1.06	1.25	0.79
	E5	1	1	1	1	1	1	1	1	1
	E6	1	1.64	0.52	0.68	< 0.48	< 0.48	< 0.48	< 0.48	< 0.48
		1/NT/T*								
Juice	J9	1.18	0.58	0.85	0.31	1.04	0.90	0.65	0.69	0.36

***calculated for juice dilutions.**
Favourable (>1) ratios indicate higher cell growth inhibition of the extract/juice towards tumor compared to healthy cells
Pulp extracts: E1 – dichloromethane, E2 – chloroform, E7 – water, E8 – n-butanol; Juice extracts: E3 – dichloromethane, E4 – chloroform, E5 – water, E6 – n-butanol; J9 – juice.
HeLa – human cervix epithelioid carcinoma; MCF7 – breast adenocarcinoma; HT-29 – colon adenocarcinoma;
MRC-5 – normal human fetal lung; A549 – lung adenocarcinoma; H-4-II-E – rat hepatocyte carcinoma;
MDA-MB-23 – breast adenocarcinoma; Hs294T – melanocyte carcinoma; HepG2 – hepatocyte carcinoma;
PC-3 – prostate adenocarcinoma.

Table 4

Apoptosis and necrosis induced by horseradish root juice J9 and extract E4 in HeLa, HT-29 and MCF7 cell line

Cell line	Sample	Apoptosis		Necrosis		efA/efN
		absorbance	efA	absorbance	efN	
HeLa	J9	1.446	4.72	0.031	4.43	1.07
	Control*	0.306		0.007		
	E4	0.304	0.74	0.001	1.00	0.74
	Control**	0.411		0.001		
HT-29	J9	1.750	2.58	0.022	2.75	0.94
	Control*	0.677		0.008		
	E4	0.750	0.99	0.009	4.50	0.22
	Control**	0.761		0.002		
MCF7	E4	0.294	1.00	0.005	5.00	0.20
	Control**	0.295		0.001		

E4 – chloroform juice extract; J9 – juice; HeLa – human cervix epithelioid carcinoma; HT-29 – colon adenocarcinoma; MCF7 – breast adenocarcinoma; *0.9% NaCl; **dimethyl sulfoxide (DMSO); efA – enhancement factor for apoptosis; efN – enhancement factor for necrosis.

Discussion

Earlier reports demonstrated that the methanol-aqueous extract of horseradish root was rich in polyphenols: malic acid, gallic acid, ferulic acid and epigallocatechin-3-gallate. The most abundant compound of the extract was allyl isothiocyanate [26]. Herz et al. [27] showed that prominent compounds in the aqueous extract from horseradish root were the amino acids arginine and proline, citric acid, phenolic compounds (caffeic acid and kaempferol derivatives), the main glucosinolates, 2-propenyl-GLS and 3-methylsulfinyl-propyl-GLS, and fatty acid derivatives. The study of Marzocco et al. [28] reported the highest content of sinigrin in the methanol extract from horseradish root.

The obtained HPLC results pointed out rich content of total polyphenolics in dichloromethane extracts from pulp E1 (49.64 mg/g) and juice E3 (18.084 mg/g).

In the study of Calabrone et al. [29] total polyphenolic contents of methanol, methanol/water (70/30, v/v) and methanol/water (50/50, v/v) horseradish root extracts that were

determined spectrophotometrically were: 1.80 ± 0.17, 2.36 ± 0.14 and 2.61 ± 0.20 mg/g DW, respectively. Similar results were presented in Tomsone et al. [30] study, where total phenolics in horseradish roots extracts obtained by convectional and Soxhlet extraction ranged from 334.29 mg GAE/100 g DW to 985.87 mg GAE/100 g DW depending on solvents used. The differences in total polyphenolics comparing HPLC and Folin–Ciocalteau method could be explained by the fact that spectrophotometric method is not an absolute measurement of the amount of phenolics, because some other substances such as organic acids, residual sugars, amino acids, proteins and other hydrophilic compounds interfere with this assay. Also, different classes of phenolic contribute to different extents to the absorbance produced by reaction with the Folin–Ciocalteau reagent when compared to gallic acid [31].

Antioxidative activity of phenolic acids is based on scavenging of reactive electrophiles and oxygen radicals [32]. The proposed mechanism by which phenolic acids induce apoptosis in tumor cells is by activation of proapoptotic factors [33]. Anticarcinogenic activity of flavonols is partially due to their antioxidative activity, since it has been shown that quercetin and kaempferol increase intracellular levels of natural antioxidant glutathione[34].

In this study, antiproliferative activity of extracts and juice horseradish root in cancer cell lines were evaluated. All extracts and juice contained catechin and gallic acid.

As confirmed by previous research (based on IC_{50} values) among twenty-four evaluated flavonoids and phenolic acids kaempferol > gallic acid > quercetin > caffeic acid (in this order of activity) had most prominent antiproliferative activity [35]. Numerous studies have shown that gallic acid exhibit antitumor and antiproliferative activity toward many types of human cancer cells [36], such as human cervix carcinoma [37], prostate cancer, and lung cancer [38]. On the other hand, catechin, epicatechin, p-coumaric acid, p-hydroxybenzoic, protocatechuic, syringic, sinapic, chlorogenic and ferulic acids had no significant cell growth activity [35].

Extracts E4 (chloroform from juice) and E2 (chloroform from pulp) that showed highest antiproliferative activity contained similar classes of polyphenols but in lower concentrations compared to E1 (dichloromethane from pulp) that had widest range and highest concentrations of polyphenols. Higher activity of extracts E4 and E2 compared to E1 can be attributed to kaempferol, one of the flavonoids with most potent antiproliferative activity [35], while higher activity of extract E4 compared to E2 can be explained by additional presence of isorhamnetin with proven powerful antiproliferative activity – even higher than activity of kaempferol, quercetin or gallic acid (based on IC_{50} values) [39]. Kaempferol exerted a dose-dependent reduction in cell viability and DNA synthesis on human lung cancer cell line A549 at different doses from 0–70 μM [40]. Hung [41] has shown that kaempferol at concentration of 35 μM significantly reduced the number of viable estrogen receptor-positive MCF7 breast cancer cells. Isorhamnetin suppressed cell proliferation of the human colon adenocarcinoma cell line HT-29, and induced arrest of cell growth in G2/M phase [42].

Higher concentrations of phenolics in extracts obtained from juice compared to same extracts obtained from pulp resulted in higher antiproliferative activity of chloroform (E4), and n-butanol juice (E6) extracts due to presence of quercetin. Higher concentration of cell growth active gallic acid in water extract from juice (E5) did not result in its higher antiproliferative activity compared to same extract from pulp, most likely due to its low concentration.

Numerous studies have shown that ITC exhibit antitumor activity, inhibiting *in vitro* growth of many types of human cancer cells, such as leukemia [43], prostate cancer [44], breast cancer [45], lung cancer [46], carcinoma of the cervix [47] and colorectal carcinoma [48]. A ITC inhibits *in vitro* proliferation of various types of human cancer cells in low micromolar range of concentrations [49–52, 43] while being less toxic to normal cells [51]. In recent study, antiproliferative activity of three isothiocyanates: sulforaphane, benzyl isothiocyanate (BITC) and phenylethyl isothiocyanate, on human cervix carcinoma cell line (HeLa), melanoma cell line (Fem-x), colon cancer cell line (LS 174), and peripheral blood mononuclear cells (PBMC) was evaluated and selectivity of BITC towards malignant cells was confirmed [53]. Due to their nonpolar nature (chloroform) it is highly unlikely that extracts E4 and E2 contained or that their activity can be attributed to isothiocyanates or sinigrin. Due to their more polar nature (dichloromethane), activity of extracts E3 and E1 can be attributed to activities of detected polyphenols as well as isothiocyanates and sinigrin.

Both water extracts, E5 (from juice) and E7 (from pulp) showed lowest antiproliferative activity, confirming that extraction of active components occurred in earlier steps of extraction (in chloroform, dichloromethane and n-butanol extracts).

High and non-selective cell growth activity of horseradish root juice towards all evaluated cell lines was achieved due to high mass concentration of juice (385.80 mg/mL; based on dry weight of the juice). Evaluated ranges of concentrations of extracts depended on extract yields and were from 0.0625–1 mg/mL. On the other hand, final concentration of juice was in the range from 0.12–19.33 mg/mL. This was almost 20 fold higher compared to extracts. When activity of extracts and juice was compared using IC_{50} values, it was clear that highest activity of juice (IC_{50}^{HeLa} = 241.72 μg/mL), that could only be attributed to gallic acid was from 8–70 fold lower compared to the most potent extracts (E1–E4).

Results in this study indicate unfavorable, high horseradish juice J9 and extract E4 potential to induce necrotic cell death of tumor cells with a possible consequence of simultaneous damage to adjacent healthy tissue (that follows necrosis). Further investigation of active components should pinpoint ones with more favourable apoptosis/necrosis inducing activities.

Conclusion

The highest and non-selective *in vitro* antiproliferative activity of chloroform and dichloromethane extracts and juice of horseradish root was detected, with necrosis as a

main mechanism of induced cell death. Chloroform extracts showed high antiproliferative activity most likely due to the presence of kaempferol, while the highest activity of chloroform extract from juice can be explained by additional presence of isorhamnetin. Due to more polar nature of dichloromethane, the activity of these extracts that was lower compared to the activity of non-polar extracts could be attributed to detected polyphenols as well as to isothiocyanates and/or sinigrin. In order to utilize horseradish root bioactive potential further investigations that will pinpoint active components with more favourable apoptosis/necrosis inducing properties are needed.

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Relationship between the frequency of falls, fear of falling and functional abilities in women aged 65 and over

Povezanost učestalosti pada, straha od pada i funkcionalne sposobnosti kod žena starosti 65 godina i više

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Abstract

Background/Aim. An increased tendency to fall and subsequent occurrence of injuries due to decline in the functional abilities are some of the many problems in older persons. The aim of the study was to determine the differences in functional abilities and the expressiveness of fear of falling between the women aged 65 and over who experienced a fall in the past 12 months (G1) and those that did not (G2). **Methods.** In this cross-sectional study, 236 women aged 65 and over were included. Fall history was based on self–reporting, the fear of a fall was estimated based on The Falls Efficacy Scale International – FESI, while the functional ability was measured by The Lawton Instrumental Activities of Daily Living – IADL. **Results.** Just under half of the women in the sample reported that one or more falls occurred in the last 12 months. Through the connectivity analysis, in both groups (G1 and G2), coefficients of correlation achieved statistical significance between the functional ability and fear of falling. A strong and negative correlation between IADL and FESI score in both groups, G1 (ϱ = -0.695; $p < 0.001$) and G2 (ϱ = -0.657; $p < 0.001$), was confirmed. **Conclusion.** The improvement of functional ability in women aged 65 and over could lower the risk of falling in this population.

Key words:
nesrećni padovi; stare osobe; žene; strah; motorna aktivnost.

Apstrakt

Uvod/Cilj. Povećana tendencija ka padu s posledičnom pojavom povreda zbog smanjenja funkcionalne sposobnosti je jedan od mnogih problema koji se javljaju kod starijih osoba. Cilj rada je bio utvrditi razlike u funkcionalnim sposobnostima i izraženosti straha od pada između žena starosti 65 godina i više koje su doživele pad u poslednjih 12 meseci i onih koje to nisu. **Metode.** U ovu studiju preseka je bilo uključeno 236 žena starosti 65 godina i više. Istorija pada je dobijena na osnovu ankete, strah od pada procenjen je na osnovu Internacionalne skale za procenu zabrinutosti zbog pada (*Falls Efficacy Scale International* – FESI), dok je funkcionalna sposobnost merena preko Lotonove skale za procenu instrumentalnih aktivnosti svakodnevnog života (*The Lawton Instrumental Activities of Daily Living* – IADL). **Rezultati.** Nešto manje od polovine žena u uzorku prijavile su jedan ili više padova u poslednjih 12 meseci. Koeficijenti korelacije između funkcionalne sposobnosti i straha od pada, kod grupa G1 i G2, dostigli su statističku značajnost. Potvrđena je jaka i negativna korelacija između IADL i FESI skora za obe grupe, G1 (ϱ = -0.695; $p < 0.001$) i G2 (ϱ = -0.657; $p < 0.001$). **Zaključak.** Poboljšanje funkcionalnih sposobnosti kod žena starosti 65 godina i više mogao bi smanjiti rizik od pada u ovoj populaciji.

Ključne reči:
accidental falls; aged; women; fear; motor activity.

Introduction

In a person's developmental cycle, aging is a necessary and inevitable stage that brings a number of challenges. An increased tendency to fall and subsequent occurrence of injuries, all due to changes in the functional behavior and decline in the functional abilities are some of the many problems for older persons. During the last decade, there has been a significant increase in life expectancy in the Republic of Serbia. Therefore, the percentage of people over 65 years of age has increased from 16.6% to 18.7%, thus representing a group of the population that has grown most in the country [1]. In this

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statistic data, women are ahead of men, so the average life expectancy for men is 73 years and for women 78 years. Because of this demographic aging, it is predicted that the proportion of people older than 65 years of age will increase from 17% to 24% in the next 30 years. That means that almost one-fourth of the population would be older than 65 and that the demographic dependency rate of the older population would have increased from 25% to 39% during the projection period. "The oldest" is the Region of South and East Serbia, where as many as 27% of the population is older than 60 years [1]. Old age is one of the key risk factors for falls. People of older age are at the highest risk of falling and getting severe injuries during a fall, some of which may end up with a fatal outcome [2]. With the progress of medicine, modern society has succeeded in prolonging life expectancy but consequently accompanied by chronic diseases, weaknesses, and comorbidities. Therefore, the measurement of functional abilities gained significance [3]. The three components included in the assessment of functional abilities are the following: self-care, self-sufficiency, and the ability of older people to lead an active life [4, 5].

A fall can cause concern about further falls, which could further lead to activity limitation or increased dependence. In addition, in the research, some authors point out that the presence of concerns over falling represents a link between falls and functional capacity [6]. The fear of falling is the main limiting factor of functional independence [7].

In the Republic of Serbia, pioneering research on the identification of older people at risk of falling [8], risk factors for falling and fear of falling [9] were conducted. In all of these studies, female gender has proved to be a predictive risk factor for falling. In order to confirm the aforementioned facts we have conducted a research whose aim was to determine the differences in functional capabilities and expressiveness of fear of falling between women aged 65 and over by whether they have experienced a fall in the last 12 months or not.

Methods

Cross-sectional study was conducted through home visits of the patronage services of the Health Care Center in Niš, Serbia, by interviewing persons aged 65 and over from the general population. Participants were randomly selected from the register of patients in the Primary Health Center of Niš. Criteria for inclusion in the study were as follows: individuals aged 65 and over, female gender, living in a house or apartment, ambulatory, and a signed informed consent. The criteria for exclusion were as follows: immobile persons and persons who were unable to understand and follow instructions.

The sample consisted of 236 individuals aged 65 and over, female, living in the territory of the municipality of Niš. The sample size was calculated on the basis of the Cochrane formula by Fisher, Laing and Stoeckel [10]. The total sample was divided into two groups. The first group (G1) consisted of participants with a positive history of falls thus they had an increased risk of falling, while participants who did not report any previous falls were in the second group (G2). The study was performed in accordance with the formal demands contained in the national and international regulations standards for researches that include human beings.

Basic demographic data of participants was obtained (age, marital status, residence, level of education, pre-retirement occupation).

Fall history was determined based on self-reporting for the period of the last 12 months. The functional ability was measured by The Lawton Instrumental Activities of Daily Living – IADL [11]. This scale is designed to measure the level of dependence in performing everyday activities. It consists of eight basic daily activities (ability to use the phone, going shopping, food preparation, housework, taking care of laundry, use of transportation, responsibility for own medicines, ability to take care of finances). Each answer ranges from 0 to 1, depending on the degree to which the problem has been identified. The highest level of functionality in this category is evaluated. The score ranges from 0 to 8 and shows the functional status of the person, where 0 points indicates a low level of functionality (the person is dependent in performing the instrumental activities of daily life), while the score of 8 indicates high functionality (the person is independent in performing these activities) [11].

Fear of falling was evaluated according to the Falls Efficacy Scale International – FESI [12]. This scale consists of 16 items on daily activities performing: house cleaning, dressing and undressing, preparing simple meals, bathing or showering, going to a store, climbing or descending up/ down stairs, walk in the neighborhood, reaching things at or above head or on the floor, answering your phone in a timely manner (before it stops ringing), walking on a slippery surface (*eg* wet and icy), visiting a friend or relative, crowding, walking on uneven surfaces (*eg* rocky ground or damaged sidewalk), climbing or descending a slope, going to a social event (*eg* going to church for a family gathering or meeting). Each item is rated on a scale from 1 (not concerned at all) to 4 (very concerned), while the total score ranges from 16 (not concerned about falling) to 64 (very pronounced fear of falling) [12].

Descriptive data and inferential statistics are presented. Normal distribution was checked using the Kolmogorov-Smirnov test and data independence across variables by using χ^2 test (for categorical responses). As a measure of association, φ coefficient and Cramer's *V* were reported and interpreted as small (0.10), medium (0.30) or large (0.50) effect sizes [13]. The frequencies, percentages, mean and standard deviations are listed for comparison purposes only because none of the data was normally distributed. Differences in continuous variables between two groups were tested using the non-parametric Mann-Whitney *U*-test for two independent groups. The effect size was calculated using the formula $r = Z/\sqrt{N}$, where N is the total number of the sample and interpreted as small = 0.10–0.29, medium = 0.30–0.49, or large effect size = 0.50–1.00 [13]. Statistically significant differences were found on two demographic variables (age and education level) with the effect sizes that were assessed as small. However, preliminary checks for ANCOVA (anal-

ysis of covariance) were conducted and several violations of the basic assumptions were noted, as well. Therefore, it was decided not to statistically remove the influence of age and education level as potentially confounding variables. Next, to test relationships between selected variables, Spearman's rank correlation test was used. All analyses were performed in SPSS, version 23 (IBM, Armonk, NY, USA), and a significance level of 0.05 was established to consider the results significant.

Results

The research included 236 women, with the average age of 75.19 years. The basic demographic characteristics of the patients are given in Table 1. Most of the respondents consisted of women that had not reported a fall in the past 12 months, that lived in the city, housewives with middle school education.

Of all the respondents included in the research, 105 (44.5%) was with a positive history of falls, that is, they have reported two or more falls in the last 12 months (Table 1).

Demographic characteristics of the patients included in the study

Comparison of groups in relation to functional abilities and fear of falling was carried out using the Mann-Whitney *U*-test for the planned group comparison with the subsequent calculation of the effect size *r*. The effect size was classified as small (0.10–0.29), moderate (0.30–0.49) or large (0.50–1.00) (Table 2).

Comparison of groups in relation to IADL and FESI scores

The instruments applied in this study, the IADL and the FESI, showed excellent internal consistency. The value of a Cronbach α coefficient for the IADL was 0.902 and 0.989 for the FESI.

Mann-Whitney *U*-test revealed a statistically significant difference in the IADL score with a small effect size between the two groups ($p = 0.001$, $r = 0.22$). More specifically, the median (Mdn) of the IADL score was lower in the group G1

Table 1

Demographic characteristics of female participants included in the study

Characteristics	G1	G2	*p*
Number (%)	105 (44.5)	131 (55.5)	
Age (years), mean	76.31	74.30	0.009
Place of living, n (%)			
urban	52 (49.5	57 (43.5)	0.430
rural	53 (50.5)	74 (56.5)	
Level of education			
no formal education	57 (54.3)	57 (43.5)	
elementary school	35 (33.3)	34 (26)	
secondary school	10 (9.5)	36 (27.5)	0.0010
high school	1 (1)	3 (2.3)	
faculty	2 (1.9)	1 (0.8)	
Employment position			
farmer	13 (12.4)	14 (10.7)	
administrative worker	3 (2.9)	14 (10.7)	
housewife	61 (58.1)	63 (48.1)	
production worker	19 (18.1)	19 (14.5)	0.173
trader	0 (0)	1 (0.8)	
hairdresser	0 (0)	1 (0.8)	
waiter	1 (1)	1 (0.8)	
other	8 (7.6)	18 (13.7)	

G1 – participants with a positive history of falls; G2 – participants who did not report the history of falls.

Table 2

Comparison of groups in relation to the IADL and FESI scores

Score	Group	Descriptive value of score			Comparison			
		mean (SD)	median (IQR)	mean rank	*U*	*z*	*p*	*r*
IADL	G1	5.27 (2.97)	6.00 (5.00)	102.86	5235.00	-3.33	0.001	0.22
	G2	6.58 (2.08)	8.00 (2.00)	131.04				
FESI	G1	40.24 (14.70)	42.00 (18.00)	150.78	3488.00	-6.33	0.000	0.43
	G2	26.79 (13.10)	19.00 (16.00)	92.63				

IADL – Lawton Instrumental Activities of Daily Living; FESI – Falls Efficacy Scale International; G1 – participants with a positive history of falls (*n* = 105); G2 – participants who did not report the history of falls (*n* = 131); SD – standard deviation; IQR – interquartile range; *r* – effect size.

(Mdn = 6.00) compared to the group G2 (Mdn = 8.00). By comparing the basic descriptive values, it could also be noted that the mean (M) IADL score was lower in the G1 than in the G2 group (M = 5.27 *vs* M = 6.58).

Mann-Whitney *U*-test revealed a statistically significant difference in the FESI score with a moderate effect size between the two groups ($p < 0.001$; $r = 0.43$). Specifically, the median of FESI score was higher in the group G1 (Mdn = 42.00) compared to the group G2 (Mdn = 19.00). By comparing the basic descriptive values, it could also be noted that the mean FESI score was higher in the G1 than in the G2 group (M = 40.24 *vs* M = 26.79).

The statistical significance of the relationship between functional ability and fear of falling in the groups G1 and G2 was investigated using Spearman's Rank Order Correlation (Table 3).

Table 3

Correlation of functional ability (IADL score) and fear of falling (FASI score)

	Group	IADL score	
		ρ	*p*
FESI score	G1	-0.695	0.000
	G2	-0.657	0.000

IADL – Lawton Instrumental Activities of Daily Living; FESI - Falls Efficacy Scale International; G1 – participants with a positive history of falls (*n* = 105); G2 – participants who did not report the history of falls (*n* = 131).
ρ – coefficient of correlation.

Correlation of functional ability and fear of falling

According to the results of statistical analysis of the correlation between functional ability and fear of falling, in the group G1, as well as in the group G2, correlation coefficients reached statistical significance.

First, a strong and negative correlation between the IADL score and the FESI score was confirmed in the group G1 ($\rho = -0.695$; $p < 0.001$). The higher values of the IADL score were associated with lower levels of the FESI score in participants of the group G1, and *vice versa*.

Second, a strong and negative correlation between the IADL score and the FESI score was confirmed in the group ($\rho = -0.657$; $p < 0.001$). The higher values of the IADL score were associated with lower levels of the FESI score in participants of the group G2, and *vice versa*.

Discussion

The prevalence of falls in our study (44.5%) was higher than in other studies that have assessed the falls in older females (32.4%) [11], (29.1%) [12], (33.2%) [14]. The mean age of participants in our study (75.19 years) was higher than in other studies, which could explain the higher prevalence of falls that was found, as age is one of the main risk factors for falls, culminating in persons older than 80 years [15–17]. The results of our study also support the fact that it was confirmed that there was a statistically significant difference in the age of the group of participants with a positive history of falls compared to the group of participants who did not report the history of falls ($p = 0.009$), with small effect size ($r = 0.17$). The participants from the group G1 were older than participants from the group G2 (Mdn = 77.00 *vs* Mdn = 74.00).

The authors of this study are not familiar with research conducted in the Republic of Serbia that evaluated the prevalence of falls along with the impact on the functional abilities of older persons, making it difficult to compare the data. The main reason for selecting the IADL is the decrease in functional performance of older persons in all IADL domains, which justifies interventions as well as further assessment, in order to prevent a continuous decline, promote safe living conditions for older persons and prolong their independence [18].

This study showed that the functional abilities were significantly different between women who experienced a fall in the previous year and those who had no history of falls. Older persons who did not report a fall had a higher score than participants in the group with a positive history of falls (M = 5.27 *vs* M = 6.58). Our results are equivalent to studies that have shown a relationship between reduced functional abilities and increased risk of falling in persons over the age of 65 [19–21].

Responsibility for the occurrence of fear of falling in older persons is attributed to the positive history of falls [22]. After the fall, a person reduces its daily activities due to fear of falling or reduction of activity, as a protective measure, is even recommended by family and health care workers [6, 23]. Some authors point out that the presence of fear of falling is the link between a fall and functional abilities [6], and that the main limiting factor of functional independence in older people is a fear of falling [7]. Our research fully confirms the results of these studies. The results of our study showed that participants from the group with a positive history of falls experienced greater fear of falling ($\rho = -0.695$; $p < 0.001$) compared to the group of participants who did not experience a fall ($\rho = -0.657$; $p < 0.001$).

Pioneering research conducted in the Republic of Serbia on the identification of the elderly at risk of falling and the risk factor for falling [8], and risk factors for the onset of fear of falling [9] showed that age was a predictive risk factor for decline.

A limitation of our study is that the assessment of functional ability (execution of functional tasks) was performed by the respondents themselves, which other authors also cite as a limitation [24]. It would be best to ask the participants to perform these tasks in a time interval in order to get the most accurate information on what a person can do, and in which situations a person really has a problem. Thus, the answers can overestimate or underestimate some of the given functional activities.

Conclusion

The results of this study indicate that older women with a positive history of falls showed less functional ability and had a more pronounced fear of falling.

In this regard, it is recommended that the enhancement of functional abilities in older women is included in fall prevention programs for reduction of risk factors for falls, which will also reduce the fear of falling.

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Moderate radioprotective role of zeolite in rats

Umerena radioprotektivna uloga zeolita kod pacova

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Abstract

Background/Aim. Exposure of living organisms to γ-radiation results in the overproduction of free radicals. The aim of the study was to test if the subacute administration of micronized zeolite (MZC) accomplishes radioprotective role based on the evaluation of the status of oxidative stress (OS) in the brain and 8-hydroxyguanosine (8-OH-dG) in the plasma of the rats exposed to the single γ-ray irradiation of 2 and/or 10 Gray (Gy). **Methods.** Wistar rats were on a four-week normal or 5% MZC supplemented diet and afterward exposed to the single γ-ray irradiation of 2 and 10 Gy. Groups of rats were: a) on a normal diet (the control group, and 2Gy and 10Gy groups); b) on 5% MZC supplemented diet (the control group – MZC, MZC + 2Gy, and MZC + 10Gy groups). We measured malondialdehyde (MDA), glutathione (GSH) total, and activity of total superoxide dismutase (tSOD) and manganese superoxide dismutase (MnSOD) in vulnerable brain regions (cerebellum, hippocampus, and cerebral cortex) and 8-OH-dG in plasma. **Results.** Lower MDA was found in the MZC+2Gy and MZC+10Gy groups compared to the 2Gy and 10Gy groups. Activity od total SOD was higher in the MZC+10Gy group than in the 10Gy one. GSH was the highest in the 10Gy group. Compared to the control group, 8-OH-dG was extremely higher in groups radiated with 10 Gy regardless of a diet, but slightly lower in the MZC+2Gy and 2Gy groups. **Conclusion.** Subacute MZC pretreatment accomplished partial radioprotective effect in irradiated rats compared to non-irradiated rats, based on suppressed SOD activity at 2 Gy, and reduced brain MDA when exposed to 2 Gy and 10 Gy.

Key words:
brain; oxidative stress; plasma; radiation, ionizing; rats; zeolites.

Apstrakt

Uvod/Cilj. Izlaganje živih organizama gama zračenju rezultira hiperprodukcijom slobodnih radikala. Cilj istraživanja je bio da se ispita da li subakutna ishrana dopunjena sa 5% mikronizovanog zeolita (MZC) ispoljava radiozaštitnu ulogu na osnovu statusa oksidativnog stresa (OS) u mozgu i 8-hidroksiguanozina (8-OH-dG) u plazmi pacova izloženih pojedinačnim dozama jonizujućeg zračenja od 2 i 10 Gray (Gy). **Metode.** Wistar pacovi su bili na četvoronedeljnoj normalnoj ishrani ili ishrani obogaćenoj sa 5% MZC, posle čega su bili izloženi pojedinačnom jonizujućem zračenju od 2 Gy, odnosno 10 Gy. Grupe pacova bile su: a) grupa pacova na normalnoj ishrani (kontrolna grupa i grupe 2Gy i 10Gy); b) grupa pacova na ishrani obogaćenoj sa 5% MZC (kontrolna grupa – MZC i grupe MZC+2Gy i MZC+10Gy). Meren je malondialdehid (MDA), glutation (GSH) i aktivnost ukupne (tSOD) i mangan superoksid dizmutaze (MnSOD) u osetljivim strukturama mozga (cerebelum, hipokampus i cerebralni korteks), a 8-OH-dG u plazmi. **Rezultati.** Biomarker MDA je bio niži u MZC+2Gy i MZC+10Gy grupama, u odnosu na grupe 2Gy i 10Gy. Aktivnost ukupne SOD je bila viša u grupi MZC+10Gy, u odnosu na grupu 10Gy. Najviši nivo GSH bio je u grupi 10Gy. U pređenju sa kontrolnom grupom, 8-OH-dG je bio izuzetno viši u grupama ozračenim sa 10 Gy, bez obzira na dijetetski režim i niži u grupama MZC+2Gy i 2Gy. **Zaključak.** Pacovi koji su bili na režimu ishrane obogaćene sa 5% MZC bili su delimično zaštićeni od zračenja, shodno redukovanoj moždanoj aktivnosti SOD pri 2 Gy i sniženom nivou MDA pri izlaganju zračenju od 2 i 10 Gy.

Ključne reči:
mozak; stress, oksidativni; plazma; zračenje, jonizujuće; pacovi; zeoliti.

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Introduction

Exposure of living organisms to γ-radiation results in the overproduction of free radicals through water radiolysis [1]. Generated reactive oxygen and nitrogen species develop oxidative stress (OS) and/or nitrosative stress, reflected in deteriorated cell morphology and physiology, including oxidation/nitration of proteins, lipids, and deoxyribonucleic acid (DNA) [2–4]. Insufficient cell antioxidative defense and adaptive mechanisms against free radicals (FRs) mostly ended with energy devastation and apoptosis [3].

Zeolite, a natural clinoptilolite, is a strong, nonselective adsorbent, ion/exchanger, catalyst, detergent or antidiarrheic with a wide range of uses for the treatment of stomach poisoning, poisoning by some harmful agents, alkaloids, mycotoxins, some strains of bacteria, dyspepsia or flatulence (endogenous gasses generated either by products of digesting particular food or of incomplete digestion in the stomach or small intestine, such as oxygen, ammonium, nitrogen oxide), in humans and animals [5, 6]. No selective adsorption and ion/exchange by zeolite limits its prolonged use, because it may decrease the overall bioavailability of nutrients, including essential metals, etc. and so it can endanger health. Zeolite does not pass into the systemic circulation after oral intake and remains within the gastrointestinal tract [7].

The extent of zeolite ion/exchange and adsorption (binding) capacity depends on the size of its surface area; thus, we used micronized zeolite (MZC) in our study [7].

The brain is particularly vulnerable to oxidative injury compared to other organs because it spends 15–20% of the entire body energy [8]. If oxidative stress is going to happen in the body affected by some stimulus (xenobiotics, inflammation, etc.) it may be expected that lipid peroxidation would be developed more in the brain than in other organs, since the brain has a higher lipid content compared to other body organs and that omega-three polyunsaturated fatty acids are susceptible to oxidation [9]. Also, the content of transition metals (especially iron and copper) is pretty high in the brain tissue, which additionally contributes to OS development and generation of reactive oxygen species (ROS), concretely hydroxyl radical ($HO^{\bullet}$), through Fenton-like reactions [8, 10]. Vulnerable brain regions such as the pyramidal neurons of CA1 and CA3 sectors of the hippocampus, the third layer of the cerebral cortex, and striatum are particularly vulnerable to free radicals toxicity [11].

Gamma rays induce metabolic oxidative stress and prolonged cell injury by oxidative damage of biomolecules, including DNA, chromatin materials, lipids, and proteins. 8-hydroxyguanosine (8-OH-dG) is a well-known biomarker of DNA oxidation [2, 3]. Looking for the systemic effect of the applied γ-radiation of rats in terms of OS, we measured the content of 8-OH-dG in plasma.

In this study, we used rats as animal models to define if the pretreatment of a four-week diet supplemented with 5% MZC could change radiation responses to applied single γ-ray irradiation of 2 and/or 10 Gray (Gy), based on the evaluation of the tested end-points referring to OS status in the cerebellum, hippocampus and cortex, and plasma 8-OH-dG, after 5 postirradiation days.

Methods

Experimental animals

Adult male Wistar rats (weights of 220–250 g) were kept under standardized housing conditions (temperature 23 ± 2 °C, lighting 12:12 light: dark, light on from 8:00 to 20:00 h) with free access to tap water and a custom pellet rat diet. Suspension of MZC was administered daily by gavage. The Ethical Committee for Experimental Animals of the Institute of Nuclear Science "Vinča" approved this experimental protocol (No. 6/12), which follows the "Guide for the Care and Use of Laboratory Animals".

Experimental design

Wistar rats on normal diet were randomly subdivided into three groups (n = 6): control (not treated) group, and 2Gy and 10Gy groups (rats subjected to the single doses radiation of 2 Gy or 10 Gy, respectively); and accordingly, rats on 5% MZC supplemented diet covered three groups (n = 6): MZC, MZC + 2Gy, and MZC + 10Gy groups. The MZC amount was calculated concerning the quantity of ingested food and rat body mass. The suspension of 0.85–1 g of MZC/day (corresponds to 5% of 17–20 g of custom pellet/day) was administered orally, by gavage, during four-weeks[12, 13]. Rats from the control group gained 133.85 ± 24.7 g body weight, and from the MZC group, 126.28 ± 31.42 g during the four-week diet. No statistically significant differences between them were observed.

^{60}Co gamma source was used and designed for radiobiological and radiation chemistry experiments in the Laboratory of Radiation Chemistry and Physics, Institute of Nuclear Sciences "Vinča". The animals were confined in custom-made individual cages, made of wire, sideways positioned, and subjected to the γ-ray irradiation.

The results of our previous pilot study, designed in line with the study of Kassayová et al. [14], ascertained maximal lethal dose (LD_{100}) of $\geq$ 12.5 Gy within five postirradiation days in rats. Also, Alya et al. [15] reported LD_{100} of 9 Gy for male and female Wistar rats within 16 postirradiation days, whereas the observed peak (mediana) was between 6th to 10th days. However, Mason et al. [16] established total-body irradiation sublethal dose of 4 Gy for rats and 5 Gy for mice (ranking radiosensitivity of the organs as follows: lung > hematopoietic system > gastrointestinal tract) within a reasonable time.

According to the abovementioned, we applied a total body irradiation of 0.167 Gy/min for 12 min, that corresponds to a total non lethal dose of 2 Gy, i.e. 200 rad, and for 60 min, that corresponds to a sublethal dose of 10Gy, i.e. 1,000 rad, in order to study chosen end-points within the appropriate period of postirradiation time of 5 days [16, 17].

After the applied treatments, animals continued with the same diet (normal or 5% MZC supplemented diet) for

the next 5 five postirradiation days, when they were sacrificed by decapitation (previously anesthetized with an injection of 50 mg of sodium pentobarbital/kg). The brains were removed immediately and stored at -80 °C until analyses were performed.

We measured OS parameters, including malondialdehyde (MDA), total superoxide dismutase – SOD (tSOD) activities and manganese superoxide dismutase (MnSOD) activities, and glutathione (GSH) in the cerebellum, hippocampus, and cerebral cortex and 8-OH-dG in the plasma of the rats.

Measurements of oxidative status

The cerebellum, hippocampus, and cerebral cortex were dissected from each frozen brain, and a crude mitochondrial fraction was prepared from each region [18]. Slices of brain structures were transferred separately into a saline solution (0.9% w/v). Aliquots (1 mL) were placed into a glass tube homogenizer (Tehnica Zelezniki Manufacturing, Slovenia). Homogenization was performed twice with a Teflon pestle at 800 rpm (1,000 g) for 15 min at 4 °C. The supernatant was centrifuged at 2,500 g for 30 min at 4 °C. The resulting precipitate was suspended in 1.5 mL of deionized water. The subcellular membranes were constantly mixed in the hypotonic solution for one hour, using a Pasteur pipette. Then, homogenates were centrifuged at 2,000 g for 15 min at 4 °C, and the resulting supernatant was used for the analysis.

The Lowry method was used to measure protein concentrations in the homogenates of the tested brain regions of the rats [19].

The activity of SOD (EC 1.15.1.1.; SOD) was measured spectrophotometrically. The principle of the method is related to the sequestration of superoxide anion radical ($O_2^{\bullet-}$) by SOD, which disables spontaneous epinephrine auto-oxidation (recorded at 480 nm). The kinetic of sample enzyme activity was followed in a carbonate buffer (50 mM, pH = 10.2, containing 0.1 mM EDTA), after the addition of 10 mM epinephrine [20]. The results were expressed as U tSOD per mg of protein.

The principle of MnSOD activity measurement (which applies to tSOD, as well) assumes the addition of cyanide anions to block CuZnSOD activity. Samples were prepared in a carbonate buffer (50 mM, pH = 10.2) with the addition of 8 mM KCN, containing 0.1 mM EDTA, and 10 mM of epinephrine [20]. The results were expressed as U MnSOD per mg of proteins.

Lipid peroxidation was measured as the quantity of MDA produced. Upon reaction with thiobarbituric acid, MDA forms a fluorescent red-complex at a ratio of 2:1, whose absorbance is measured at 532 nm [21].

The amount of GSH present within the tissues was determined by using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 mL of 100% methanol) in Tris-HCl buffer (0.4 M, pH = 8.9). The intensity of produced yellow-colored p-nitrophenol anion (corresponds to GSH concentration) was spectrophotometrically measured at 412 nm. Brain tissue was prepared in 10% sulfosalicylic acid for GSH determination [22].

8-OH-dG was measured in the plasma of the rats by using commercial HT 8-OH-dG ELISA Kit II (R&D Systems, Inc. 614 McKinley Place NE Minneapolis, USA).

Kruskal-Wallis, *post hoc* Dunn's tests and Spearman's nonparametric correlations were used for the statistical data analysis using GraphPad Prism, version 5.01. Differences were considered statistically significant at $p < 0.05$. Values were presented graphically as average ± standard deviation using GraphPad Prism.

Results

A decrease of MDA was obtained in the hippocampus ($p < 0.05$) and the cortex ($p < 0.0001$) of the rats in the MZC group and in the cortex ($p < 0.0001$) of the rats in the MZC + 2Gy and MZC + 10Gy groups compared to the C group; and in the cerebellum and the hippocampus ($p < 0.05$) and the cortex ($p < 0.0001$) of the rats in the MZC + 2Gy group compared to the 2Gy group; and in the cerebellum and the hippocampus ($p < 0.001$) and the cortex ($p < 0.0001$) of the rats in the MZC + 10Gy group compared to the 10Gy group (Figure 1A).

The increase of tSOD was documented in the cortex of the rats in the 2Gy ($p < 0.0001$), MZC + 2Gy ($p < 0.05$) and MZC +10Gy ($p < 0.0001$) groups compared to the C group. In the radiated groups (2Gy, 10Gy), lower tSOD activity was documented in the cortex of the rats in the MZC + 2Gy group ($p < 0.05$) and higher in the MZC + 10Gy group ($p < 0.0001$), respectively. Also, tSOD activity was lower in the cortex of the rats in the 10Gy group ($p < 0.0001$) compared to the 2Gy group (Figure 1B).

A reduced MnSOD activity ($p < 0.05$) was observed in all the examined tested brain regions of rats in the 10Gy group and the cortex of the rats in the MZC + 2Gy group ($p < 0.05$) compared to the C group. Compared to the 2Gy group, a decrease of MnSOD activity was observed in the cortex of the rats in the MZC + 2Gy group ($p < 0.0001$) and 10Gy [in the cerebellum ($p < 0.05$) and the cortex ($p < 0.0001$)] groups. In contrast, MnSOD activity was higher in the cortex of the rats ($p < 0.05$) in the 2Gy group compared to the C group and in the MZC + 10Gy group [in the hippocampus ($p < 0.05$) and the cortex ($p < 0.0001$)] compared to the 10Gy group (Figure 1C).

Higher GSH contents were documented in the MZC group [in the cerebellum ($p < 0.05$)]; the MZC + 2Gy and MZC +10Gy groups [in the cortex ($p < 0.0001$)]; the 2Gy group [in the hippocampus ($p < 0.05$) and the cortex ($p < 0.0001$)] and the 10Gy group in all examined brain structures ($p < 0.0001$) compared to the C group. GSH decrease was observed in the MZC + 10Gy group [in the cerebellum ($p < 0.05$) and the cortex ($p < 0.0001$)] compared to the 10Gy group. Also, the GSH level was profoundly higher in the cortex ($p < 0.0001$) of the rats in the 10Gy group than in the 2Gy group (Figure 1D).

Significantly higher values of 8-OH-dG were obtained in the plasma of the rats radiated with 10 Gy, regardless of

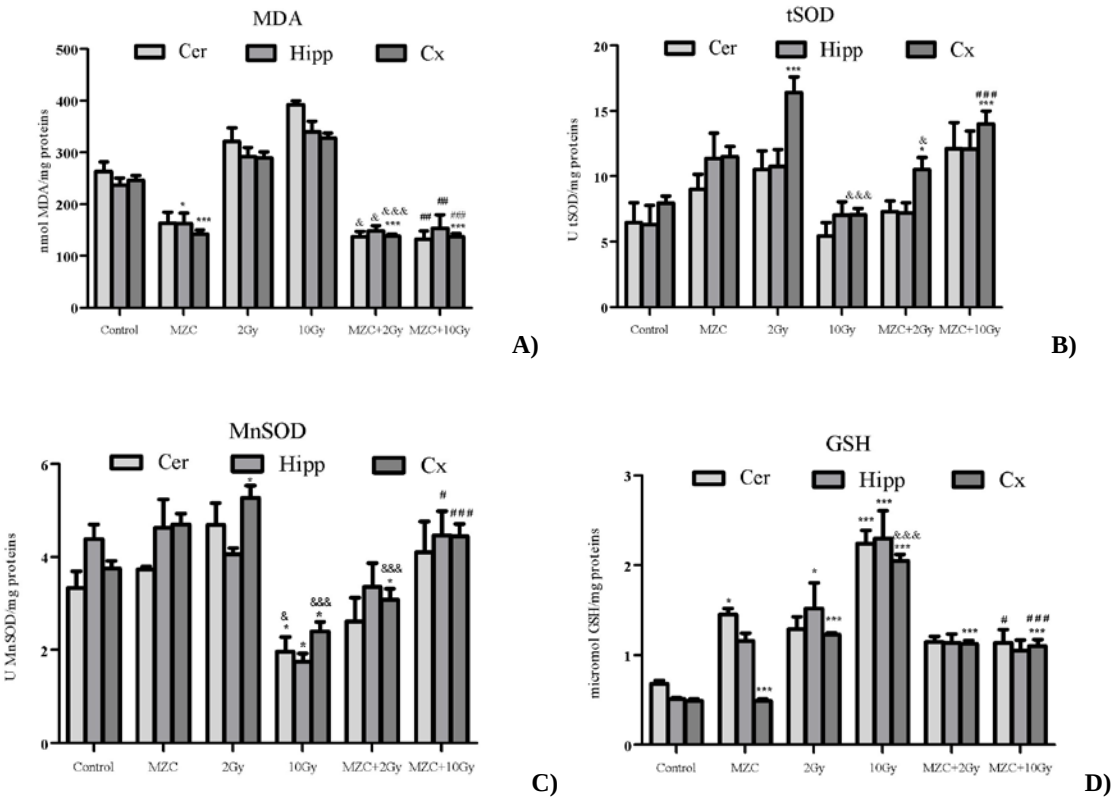

Fig. 1 – Oxidative stress (OS) in selectively vulnerable brain regions of rats on normal and the 5% micronized zeolite (MZC) supplemented diet, lately subjected to single 2 or 10 Gy γ-irradiation. Measured OS parameters in the cerebellum (Cer), hippocampus (Hipp) and cortex (Cx) were: (A) nmol malondialdehyde (MDA)/mg proteins; (B) total superoxide dismutase (tSOD): units of tSOD/mg proteins; (C) manganese superoxide dismutase (MnSOD): units of MnSOD/mg proteins) and (D) nmol glutathione (GSH)/mg proteins. Values are presented as means ± standard deviation (n = 6). Differences were considered statistically significant at: $p < 0.05$ (*, #, &), $p < 0.001$ (, ##, &&) and $p < 0.0001$ (***, ###, &&&). Labeling: * – compared to control, # – compared to 10Gy group and & – compared to 2Gy group). Kruskal-Wallis and *post hoc* Dunn's tests were used for statistical analysis.**

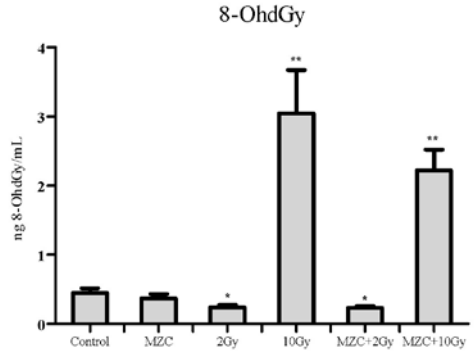

Fig. 2 – The plasma 8-hydroxyguanosine (8-OH-dGy) of rats on the four-week normal and the 5% micronized zeolite (MZC) supplemented diet, lately subjected to single 2 or 10 Gy γ-irradiation. The concentration of 8-OH-dGy was expressed as ng 8-OH-dGy/mL plasma. The values are presented as means ± standard deviation (n = 6). Differences were considered statistically significant at $p < 0.05$ (*) and $p < 0.01$ (**) compared to control. Kruskal-Wallis and *post hoc* Dunn's tests were used for statistical analysis.

the diet type (MZC + 10Gy, 10Gy) ($p < 0.001$), while the decrease was documented in the MZC + 2Gy and 2Gy groups ($p < 0.05$) compared to the C group (Figure 2).

Spearman's nonparametric correlation data analysis for the tested brain regions and OS parameters is presented in tabular form (Table 1).

Table 1

Spearman nonparametric correlations of the tested oxidative stress parameters across the hippocampus (Hipp), cerebellum (Cer), and cortex (Cx) of rats on normal and the 5% micronized zeolite (MZC) supplemented diet, lately subjected to single 2 Gy or 10 Gy γ-irradiation

Group	Parameter/Structure	*r*	*p*
MZC + 10Gy	MDA/Hipp vs. MDA/Cer	+ 0.943*	0.017
	MDA/Hipp vs. MDA/Cx	+ 0.886*	0.033
	GSH/Cx vs. GSH/Cer	+ 0.886*	0.033
10Gy	tSOD/Hip vs. tSOD/Cer	+1.000**	0.003
2Gy	MDA/Cx vs. MDA/Cer	+ 0.886*	0.033
	MnSOD/Cx vs. MnSOD/Cer	–0.943*	0.017

Wistar rats on four-week normal and diet supplemented with 5% MZC, lately subjected to the single 2 or 10 Gy γ-irradiation. Nonirradiated groups (n = 6) were C and MZC. Radiated groups (n = 6) were 2Gy, 10Gy, MZC + 2Gy, and MZC + 10Gy. Oxidative stress parameters [malondialdehyde (MDA), manganese superoxide dismutase (MnSOD), total superoxide dismutase (tSOD), and glutathione (GSH)] were correlated across the Hipp, Cer, Cx. Spearman's correlation coefficient (r) > ± 0.70 was the criterion for the segregation of the results. Differences were considered statistically significant for $p < 0.05$.

Discussion

We showed that a four-week diet supplemented with 5% MZC *per se* resulted in a significant systemic shift of redox homeostasis towards a reductive potential in the tested brain regions susceptible to OS, based on the decreased MDA (in the hippocampus and cortex) and elevated GSH (in the cerebellum, hippocampus, and cortex). The diet supplemented with MZS did not realize protection against DNA oxidation in plasma. Regarding the radioprotective effect of the applied diet supplemented with zeolite in the rats, partial results were achieved in the tested brain regions, referring to suppressed SOD activity at 2 Gy and reduced brain MDA levels at 2 Gy and 10 Gy. The zeolite supplemented diet achieved no radioprotection of DNA against oxidation (Figures 1 and 2).

Assumingly, a decline of OS in the brain tested regions occurred due to reduced bioavailability of some nutrients by zeolite, not because of boosted antioxidant defense system: a) zeolite binds metals and gases (by adsorption or through ion/exchange reactions) from food and remains within the alimentary tract after oral intake; b) transition metals may induce ROS overproduction *via* Fenton reactions, thus, zeolite significantly reduced the yield of this reaction; c) some essential metals are constituents of many antioxidative metalloenzymes, so, zeolite may decrease the availability of the certain metals to be incorporated in the enzymes and make them functional; d) some gasses (nitrogen monoxide and oxygen) liberated from food within the intestine, may contribute to the formation of ROS or reactive nitrogen species [4].

An irradiated body surface and the applied dose of γ-ray irradiation are equally important and influential factors for the induced postirradiated effects. Short term outcomes (such as an acute radiation syndrome) depend on the exposure dose, while low doses are found to be associated with possible late somatic and long-term genetic effects, unlike large doses of radiation with immediate somatic effects on the body [23]. The human body can probably absorb up to 200 rads (2 Gy) acutely without a fatality. Also, the human population can be exposed to 1–10 Gy of γ-irradiation during radiation therapy treatment or radiation accidents or nuclear/radiological terrorism [23, 24]. Upon absorption of ionizing radiation, many chemico-biological changes occur in the living cells, including direct structural disruption or indirectly, through interaction with products of water radiolysis [3]. Often, 30 postirradiated days are taken to determine lethality in mice or rats. Hence, we selected oxidative damage in the brain and plasma DNA, as the end-points provoked by the irradiation imposed a prerequisite – which was to have the rats alive; therefore, 5 postirradiated days were concluded to be the appropriate period [23].

Reduced lipid peroxidation was documented by lower brain MDA levels in the tested brain regions in irradiated MZC pretreated rats, compared to irradiated rats on a normal diet (Figure 1 A). Our previous results have indicated that MZC treatment decreased the levels of $O_2^{\bullet -}$ and nitrates in the brain [25]. One-electron reduction reactions between free radicals and unsaturated fatty acids resulted in cell membrane degradation and elevated production of MDA. Though, $O_2^{\bullet -}$ can initiate lipid peroxidation in its protonated form. However, lipid peroxidation can be triggered more easily by $HO^{\bullet}$, which is the most potent free radical [easily generated by homolytic cleavage of hydrogen peroxide (H_2O_2) (the product of SOD catalyzed reactions) or generated through Fenton-like reactions that occur between transition metals (in their reduced form) and H_2O_2] [4]. According to Hill and Switzer [26], certain brain regions, such as the cortex, striatum, and hippocampus, are highly enriched with nonheme iron, which is catalytically involved in the production of ROS [26]. Water radiolysis occurs within cells during radiation [1]. In such circumstances (radiation disease), the overproduction of H_2O_2 occurs. In Fenton-like reactions, H_2O_2 easily reacts

with low valent transition metals, such as iron-Fe^{+2}, copper-Cu^{+1}, manganese-Mn^{+2}, etc. to form $OH^{\bullet}$, which spontaneously triggers free radical chain reactions with all kinds of biomolecules in a body. Assumingly, lowered lipid peroxidation in the group of the rats on a 5% MZC diet is related to the lower bioavailability of transition metals.

Cellular antioxidant defense system, including antioxidative metalloenzymes [tSOD (CuZnSOD, MnSOD), catalase, GSH peroxidase, GSH reductase, etc.] is responsible for free radicals scavenging/neutralization [27, 28]. The first-line antioxidant enzyme, SOD [catalyzes $O_2^{\bullet-}$ dismutation into H_2O_2 and molecular oxygen] involves cytosolic and extracellular (CuZn-SOD) and mitochondrial (MnSOD) isoforms [29]. The fraction of MnSOD in tSOD is extremely small, thus observed changes in tSOD are mainly due to CuZn-SOD.

The 5% MZC 4-week diet, *per se*, did not affect the activity of MnSOD and tSOD (i.e., CuZn-SOD). Herein, we showed that the activity of SOD isoforms was not affected by zeolite subacute intake, but lipid peroxidation in rats.

We showed that the activities of t-SOD were significantly elevated only in the cortex (not in the cerebellum and the hippocampus) of the rats on normal diet at both doses of γ-ray irradiation, while the activity of MnSOD was significantly elevated only in the cortex at the dose of 2 Gy, and lowered in all three tested brain regions at 10 Gy, compared to the controls. Our results are similar to those of the study of Lee et al. [30], who observed no changes for SOD and catalase, but GSH in mouse spleen induced by doses of γ-ray irradiation from 0.02 to 0.2 Gy. The disparities in the content of SOD-isoenzymes across the tested brain regions may explain their unequal responses associated with oxidative stress against applied treatments in the rats. Also, brain regions are not equally susceptible to a variety of neuronal injuries associated with oxidative stress, for the same reason (Table 1).

Different distribution of the SOD isoforms within the brain and spinal cord tissues and cells is confirmed by confocal laser scanning microscopy and digital photoimaging according to the study reported by Lindenau et al. [31]. Cu/Zn-SOD is predominantly localized in astrocytes of the CNS and the motor neurons of the spinal cord (much more than in brain neurons). In lower amounts, Cu/Zn-SOD is present in the nucleus sparing the nucleolus, neuronal perikarya, and in the structures of the neutrophil.

Mitochondrial MnSOD is more abundant in the brain and the spinal cord neurons than in astroglial cells. The higher susceptibility of the cortex to OS in the rats observed in our study (the activity of SOD isoforms) is in accordance with the study of Melov et al. [32], who demonstrated that transgenic MnSOD knockout mice easily develop neuronal phenotype and a spongiform degeneration of the cortex and specific brain stem nuclei. Additionally, the cerebral cortex and striatum are more prone to oxidative damage due to a higher oxygen consumption rate in those regions [33]. The phenomenon of selective neuronal vulnerability is related to the difference in susceptibility of neuronal populations in the CNS to different kinds of stressors (including oxidative and nitrosative stress) that induce neurodegeneration. This phenomenon is not limited to cross-regional differences in the brain, as within a single brain region – such as the hippocampus or the entorhinal cortex – it also manifests in internal, subregional differences in relative susceptibility to OS (as it was confirmed by the correlation analysis, Table 1). While most brain neurons can tolerate OS, some of them (small pyramidal neurons and the third layer of the cerebral cortex and striatum, the hippocampal CA1 region and cerebellar granule cell layer) are particularly vulnerable to OS [11, 34, 35]. The susceptibility of the cerebellar neurons to OS and NS might play an important role in the significant loss of these neurons in the aging process [36].

Activities of both SOD isoforms were significantly higher in rats on the normal diet after radiation by 2 Gy than in those on zeolite supplemented diet and the opposite for 10 Gy. The type of initiated antioxidant defense mechanistic pathway depends on the dose of γ-irradiation, which was confirmed by our results for the 2Gy and 10Gy groups [37]. Additionally, $O_2^{\bullet-}$ can act both as an initiator and a terminator of free radicals chain reaction mediated [3].

For 2 Gy radiation, only in the cortex, the activity of tSOD and MnSOD was higher in the rats on the normal diet than in the control ($p < 0.001$ and $p < 0.05$, respectively) and those on the zeolite supplemented diet ($p < 0.05$ and $p < 0.001$, respectively) (Figures 1B and 1C). These results follow the Pathak et al. [38] study, showing that lower doses of γ-radiation induce the activity of SOD in various organs in rats. According to our results, the dose of 2 Gy of radiation increases the activity of tSOD (in the cortex statistically significant), which implies the increased production of H_2O_2 as well, and which may consequently explain higher MDA levels (Figure 1B).

Contrary to that, for 10 Gy radiation, the activity of MnSOD were lower in the rats on normal diet compared the control (in all tested brain regions, $p < 0.05$) and those on the zeolite supplemented diet (in hippocampus, $p < 0.05$ and in cortex, $p < 0.001$), while the activity of tSOD did not differ from the controls and was lower from the rats on the zeolite supplemented diet only in the cortex ($p < 0.001$) (Figures 1B and 1C).

The *in vitro* study of Wu and Navrotsky [39] showed that zeolite binds metals in the following descending order Mn > Zn > Mg > F > Cu. Also, the *in vitro* study of Jacobs and Waite [40] confirmed the strong zeolite affinity for Mn. Our results indicate that zeolite indirectly affects the activity of SOD by binding essential metals (Mn, Cu, and Zn) that are cofactors and constituents of metalloenzymes.

Brain GSH is a confirmed neuromodulator, neurotransmitter, and neurohormone. The sulfhydryl group of cysteine of the tripeptide GSH is responsible for its antioxidant (donor of reducing equivalents) and metal-binding abilities. We showed significantly elevated brain GSH levels after exposure to γ-ray irradiation at both doses (Figure 1D), which is in agreement with Ballatori et al. [41] study. These results adhere to the literature, showing that lower doses of radiation cause an increase of GSH levels in many organs, including of GSH synthesis-related proteins via the de novo synthesis [42]. According to Kawakita et al.[43], it is known that changed cellular redox signalisation leads to

phosphorylation of various serine/threonine mitogen-activated protein (MAP) kinases that further activate different redox-sensitive transcription factors like nuclear factor-κB (NF-κB) and activating protein-1 (AP-1), resulting in the gene expression of various antioxidant defense proteins (enzymatic and no-enzymatic) to overcome the effect of OS-mediated cellular damage.

Yamaoka et al. [44] reported that persistent radiation increases SOD activity in the rat liver and spleen up to 8–12 weeks after exposure. Additionally, the increased induction of GSH by low-dose γ-rays appears to activate immune function, according to Kojima et al. [42]. Seven decades ago, Avti et al. [45] and Patt et al. [46] reported that cysteine (the key amino acid in tripeptide GSH) administered to rats before 800 R of X-rays, significantly increased survival. However, Teshima et al. [47] found that the increase of intracellular GSH induced by low-dose gamma-radiation occurs because of higher expression of mRNA for γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme of the *de novo* GSH synthesis pathway, rather than because of glutathione reductase. The study of Lee et al. [30] confirmed that the elevation of GSH at low-dose γ-ray irradiation (0.02 and 0.2 Gy) is accompanied with the elevated expression of glutamate-cysteine ligase modifier (not catalytic) subunit, emphasizing that no changes in the expression of thioredoxin occurred in de novo GSH synthesis.

We showed that10 Gy dose of radiation causes oxidative DNA damage in plasma no matter what diet the rats were on (Figure 2). Prooxidants such as $HO^{\bullet}$, excited oxygen, photosensitizers, or $ONOO^{-}$, produce 8-OH-dG in the reaction with DNA. The study of Floyd and Carney [48] underlined that iron and reactive oxygen-free radical intermediates are involved in the oxidative damage of proteins and DNA. Very high plasma 8-OH-dG concentrations obtained in the rats subjected to 10 Gy (regardless of the diet) are in accordance with Cuttler and Pollycove [49] study, affirming that damaging or lethal cellular effects are observed following high radiation doses, while cellular stimulatory effects happened following low-dose-short-term exposures in the range 0.01–0.50 Gy.

Based on our results, the applied 5% MZC diet in rats appeared to have no radioprotective effect against oxidative damage of DNA in plasma (Figure 2).

The correlation analysis confirmed differences across the hippocampus, cerebellum, and cortex responses to applied treatments in rats (Table 1). Correlation of the data related to lipid peroxidation (MDA) and GSH changes, within the MZC + 10 Gy group, showed that both the hippocampus/cerebellum and hippocampus/cortex, and cortex/cerebellum similarly responded, respectively. Also, changes of tSOD activity upon radiation of 10 Gy were similar in the hippocampus/cerebellum, while the 2 Gy radiation caused similar changes in MDA in the cortex/cerebellum, but the MnSOD activities in the cortex/cerebellum. Anatomical and physiological characteristics of the tested brain regions (such as localization of antioxidant enzymes, an abundance of transition metals, richness with polyunsaturated free fatty acids, etc.) dictate a profile of OS response to zeolite diet and against gamma radiation, in rats [31, 33–35].

Conclusion

Gamma-ray irradiation of 2 and 10 Gy changes brain redox homeostasis and causes oxidative alternations of plasma DNA at higher doses, in rats. Subacute MZC pretreatment accomplished partial radioprotective effect in rats based on reduced brain MDA and activity of SOD, compared to the rats on a normal diet. The cortex appears to be the most susceptible to OS induced by γ-ray irradiation.

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Conflict of interest

The authors declare no conflict of interest.

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Predictors of renal outcome in ANCA-associated glomerulonephritis

Prediktori bubrežnog ishoda kod ANCA-udruženih glomerulonefritisa

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Abstract

Background/Aim. Primary anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitis are chronic multisystemic autoimmune diseases which include microscopic polyangitis (MPA), granulomatosis with polyangitis (WG), eosinophilic granulomatosis with polyangitis (EPGA; churg-strauss syndrome – CSS), and also a localized forms of illness. In our research, we studied clinical and serological parameters in patients, in order to find out which of them would be the best predictor of renal outcome in ANCA-associated vasculitis. **Methods.** Data from 42 patients with diagnose of MPA (9), WG (17), EPGA (0), CSS (0), and also idiopathic rapidly progressive glomerulonephritis (ROEB) without immune deposits (renal-limited vasculitis – 16) were analyzed. Cockroft formula was used for calculating the glomerular filtration in the moment of presenting the illness, and also after five year follow-up period. Other factors that were analyzed are: gender, age, type of ANCA antibodies, type of infections, stage of chronic kidney disease, need for heamodialysis and mortality. **Results.** Of a total of 42 patients, 17 (40.48%) were male. The average age of the patients at the time of diagnosis was 57.8 (± 10.44) years. Seventeen patients (40.48%) had a diagnosis of WG, 9 (21.43%) MPA, and 16 (38.09%) iRPGN. The presence of positive anti-proteinase (anti-PR3) antibodies was confirmed in 18 patients, and anti-MPO antibodies in 17 patients. Three patients had both subtypes of ANCA antibodies (anti-PR3 and anti-MPO). Initially, 12 patients required heamodialysis treatment. Twenty nine patients had a complete and 13 patients had partial remission. Out of the total number of patients, 8 patients (19.04%) developed the terminal renal failure stage, and ended up on a chronic dialysis program. During a five-year follow-up period, 12 patients (28.57%) resulted in death. The age of the patient proved to be statistically significant predictor of glomerular filtration rate (GFR) at the moment of presentation of the disease (p = 0.011). GFR t = 0 was statistically significant (p = 0.000) for the evaluation of kidney function outcomes in ANCA-associated glomerulonephritis. **Conclusion.** Kidney function in the moment of illness presentation, determined by GFR t = 0, is the most important significant factor for predicting renal outcome in ANCA-associated vasculitis, and also the mortality in these patients.

Key words:
glomerulonephritis; antibodies, antineutrophil cytoplasmic; glomerular filtration rate; mortality.

Apstrakt

Uvod/Cilj. Primarni anti-neutrofilna citoplazmatska antitela (ANCA)-udruženi vaskulitisi predstavljaju hronično multisistemsko autoimunsko oboljenje u koje se ubrajaju mikroskopski poliangiitis (MPA), granulomatoza sa poliangiitisom (WG), eozionofilna granulomatoza sa poliangiitsom (EPGA; Churg-Stranss sindrom – CSS), kao i lokalizovane forme bolesti. U našem ispitivanju, koristili smo kliničke i serološke parametre kod bolesnika kako bismo pronašli koji od njih bi bili najbolji prediktori bubrežnog ishoda kod ANCA-udruženih glomerulonefritisa. **Metode.** Analizirani su podaci 42 bolesnika sa dijagnozom MPA (9), WG (17), eozionofilna granulomatoza sa poliangiitsom (EPGA; CSS) (0), kao i idiopatski rapido-progresivni glomerulonefritis bez imunskih depozita (16). Cockcroft formula je upotrebljena za izračunavanje glomerulske filtracije u momentu prezentacije bolesti i nakon petogodišnjeg praćenja bolesnika. Ostali faktori koji su analizirani bili su: pol, starost, tip ANCA antitela, tip infekcija, stepen hronične bubrežne insuficijencije (HBI), potreba za hemodijalizom i mortalitet. **Rezultati.** Od ukupno 42 bolesnika, 17 (40,48%) su bili muškog pola. Prosečna starost bolesnika u vreme postavljanja dijagnoze bila je 57,8 ± 10,44 godina. Prisustvo pozitivnih anti-proteinaze 3 (anti-PR3) antitela potvrđeno je kod 18 bolesnika, a anti-MPO antitela kod 17 bolesnika. Pozitivnost anti-PR3 i anti-MPO antitela dokazana je kod tri bolesnika. Inicijalno, hemodijalizni tretman je

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sproveden kod 12 bolesnika. Nakon sprovedene terapije kod 29 bolesnika postignuta je potpuna, a kod 13 bolesnika delimična remisija. Od ukupnog broja bolesnika, osam (19,04%) je razvilo terminalni stadijum slabosti bubrega i nastavilo lečenje hroničnim hemodijalizama. Tokom perioda praćenja od pet godina, 12 bolesnika (28,57%) je umrlo. Starost bolesnika bila je statistički značajan predictor brzine glomerularne filtracije (GFR-a) u momentu prezentacije bolesti (p = 0.011). GFR t = 0 pokazao se statistički značajnim (p = 0.000) za procenu ishoda bubrežne funkcije kod ANCA-udruženih glomerulonefritisa. **Zaključak.** Bubrežna funkcija u momentu prezentovanja bolesti, određena putem GFR t = 0, predstavlja jedini značajni faktor za procenu ishoda bubrežne funkcije kod ANCA-udruženih glomerulonefritisa, kao i mortaliteta kod ovih bolesnika.

Ključne reči:
glomerulonefritis; antitela, antineutrofilna, citoplazmatska; glomerulska filtracija; mortalitet.

Introduction

Primary types of vasculitis that are associated with antineutrophil cytoplasm antibody (ANCA-associated vasculitis; AAV) are chronic multisystemic autoimmune diseases which include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (WG), eosinophilic granulomatosis with polyangiitis (EPGA; Churg-Strauss syndrome – CSS), and also a localized forms of illness. After receiving the corticosteroid and immunosuppressive therapy, most of the patients experience early remission, but patients with ANCA-associated vasculitis continue to be at increased fatal risk compared with a healthy population [1].

Kidney affection is one of the most common manifestation of vasculitis and it has a great impact on the outcome of the disease [2, 3]. Renal vasculitis is the most common severe manifestation of ANCA-associated vasculitis (AAV) and it is typically presented with rapidly progressive glomerulonephritis (GN). During the diagnostic phase of AAV, dialysis is often needed, however renal recovery and withdrawal from dialysis after the treatment is possible, in more than 50% patients [4]. Renal impairment as prognosis is also a predictor of poor renal outcome [5–8] and also of poor patient survival [1, 9].

Treatment of AAV may also cause significant morbidity, and patients with impaired renal function may be particularly prone to treatment-emergent adverse events [4]. Medication based on cyclophosphamide (CYC) and corticosteroids (CS), which have been used since the 1970s [10], changed the prognosis of AAV from lethal to a chronic relapsing disease. Around a half of the patients have a relapse within five years after diagnosis [11, 12].

Mortality of the patients with ANCA-associated vasculitis is high, 10–15% within the first year following treatment initiation. These patients have 2.7-fold increase in mortality compared with the general population. Some of the studies have shown that the mortality of the patients with renal involvement depends on factors such as: older age, side effects of the therapy, lung haemorrhage, high disease activity score based on the Birmingham Vasculitis Activity Score (BVAS), etc [13–18]. In order to control the inflammation, patients are treated with immunosuppressive and/or cytostatic therapy.

In our study, we used clinical and serological parameters in 42 patients, in order to find out which of them would be the best predictor of renal outcome in ANCA-associated vasculitis.

Methods

Forty two patients, with diagnose of WG, MPA, CSS, idiopathic rapidoprogressive glomerulonephritis (iRPGN) were enroled in this study. Disease diagnose was based on the Chapel Hill Consensus Conference criteria for ANC-associated vasculitis [10]. Inclusion criteria were: positive anti-myeloperioxidase-antineutrophilic antibodies (anti-MPO-ANCA) or anti-proteinase 3- antineutrophilic antibodies (anti-PR3-ANCA); kidney damage; rapid increase of serum creatinine. Glomerular filtration rate (GFR) was calculated by Cockroft formula and it was used as a marker of kidney function [11]. GFR was determined in the moment of kidney biopsy (t = 0), and after a five year follow-up period. In order to do analysis in a less complicated manner, CSS and iRPGN were marked as renal-limited form of vasculitis, because separated, those data would be statistically insignificant. Patients with secondary vasculitis, including lupus nephritis, were excluded from the study. Induction therapeutic approach was consistent as follows: methylprednisolone and cyclophosphamide; methylprednisolone, cyclophosphamide and therapeutic plasma exchange; methylprednisolone; cyclophosphamide. After achieving remission, the therapy was: azathioprin *peroral* 2 mg/kg/24 h; mycophenolat mofetil 2–3 g/24 h; combination of corticosteroid therapy and azathyoprin in patients whose condition went worse after stopping corticosteroids.

Statistical data processing was performed in IBM SPSS Statistics v.23. Categorical variables are represented by absolute and relative frequencies. The central tendency of the continuous variables is represented by the arithmetic mean, the deviation with the standard deviation, the minimum and the maximum. Multivariate linear regression model were studied by the predictors of the renal function of the patient at the time of presentation of the disease and after the follow-up period. The stability of the 95% predictor confidence interval was confirmed by the bootstrap resampling method with 1,000 samples and the Mersenne Twister random number generator (bootstrapping confirms that predictive models remain the same on a larger sample, that is, not to get different results when the sample would be larger).

Results

Characteristics of patients included in the study are given in Table 1. Of a total of 42 patients, 17 (40.48%) were male. The average age of the patients at the time of diagnosis

was 57.8 (± 10.44) years. Seventeen patients (40.48%) had a diagnosis of GW, 9 (21.43%) MPA, and 16 iRPGN (38.09%). None of the patients had CSS. The presence of positive anti-PR3 antibodies was confirmed in 18 patients, and anti-MPO antibodies in 17 patients. Three patients had both subtypes of ANCA antibodies (anti-PR3 and anti-MPO). At the time of diagnosis, the mean value of the glomerular filtration volume (eGFR) was 52.71 mL/min / 1.73 m^2 (eGFR values ranged from 4 to 156 mL/min /1.73 m^2). Nine patients had preserved kidney function, five of them had stage 1 of chronic kidney disease (CKD), five had stage 2 of CKD, five had stage 3 of CKD, six had stage 4 of CKD, and twelve stage 5 of CKD. In 19 patients, the presence of pulmonary lesions was established. Therapeutic protocols involved the following options: 25 patients received a combination of methylprednisolone and cyclophosphamide (14 patients with GW; 7 patients with MPA; 4 patients with iRPGN), 13 patients methylprednisolone, cyclophosphamide and plasma therapeutic modification (4 patients with WG; 8 patients with MPA; 1 patient with iRPGN), 3 patients methylprednisolone as a monotherapy because of the neutropenia (2 patients with WG, 1 patient with iRPGN), 1 patient cyclophosphamide due to unregulated diabetes (patient with MPA). Initial doses of corticosteroid therapy was 1mg/kg intravenous, and for cyclophosphamide 500–750 mg/m^2 (applied monthly). Plasma exchange therapy was applied in 13 patients, who had severe alveolar heamorrhage and end-stage renal disease (ESRD) in the moment of disease presentation. The number of plasma therapeutic modification

Table 1

Clinical characteristics of patients at the moment of the disease presentation (t = 0)

Parameters	Values
Gender, n (%)	
male	17 (40.48)
female	25 (59.50)
Age (years), min-max; mean ± SD	30-83; 57.77 ± 10.44
Anti-neutrophil cytoplasmatic antibody (ANCA) subtype, n (%)	
antiMPO	17 (41)
antiPR3	18 (43)
antiPR3 + antiMPO	3 (7)
undifferentiated	4 (10)
Diagnosis, n (%)	
iRPGN	16 (38.09)
MPA	9 (21.43)
GPA	17 (40.48)
Affection of other organs, n (%)	
skin	5 (18.52)
lung	19 (70.37)
ORL	3 (11.11)
in total	27 (100)
Induction therapy, n (%)	
CYP	1 (2.4)
CS	3 (9.60)
CS+CYP	25 (59.50)
CS+CYP+PF	13 (31)
GFR (mL/min), min-max; mean ± SD	4-156; 52.71 (42.46)
Kidney function at the moment of disease presentation, n (%)	
preserved	9 (21.42)
CKD grade 1	5 (11.90)
CKD grade 2	5(11.90)
CKD grade 3	5 (11.90)
CKD grade 4	6 (14.29)
CKD grade 5	12 (28.57)
Hemodialysis, n (%)	
iRPGN	2 (4.76)
MPA	6 (14.29))
GPA	4 (9.52)

Anti-MPO – anti-myeloperoxydase-antineutrophic antibodies;
anti-PR3 – anti-proteinase 3; iRPGN – idiopathic rapidly progressive glomerulonephritis;
MPA – microscopic polyangiitis; GPA – granulomatosis with polyangiitis;
CYP – cyclophosphamide; CS – corticosteroids; PF – physical therapy;
ORL – otorhinolaryngology; GFR – glomerular filtration rate;
CKD – chronic kidney disease; SD – standard deviation.

was: 5 procedures in patients with WG, 5 procedures in patients with MPA, 3 procedures in patient with iRPGN. Initially, 12 patients required heamodialysis treatment (2 patients with iRPGN, 6 patients MPA, 4 patients with WG). Twenty nine patients had a complete and 13 patients had partial remission. Table 1 presents the clinical characteristics of patients in the moment of disease presentation. The most common cause of hospitalization of patients with ANCA vasculitis were infections: urinary tract infections (in 11 patients), then lower respiratory tract infections (in 6 patients), and upper respiratory tract infections (ear, throat and nose) (in 6 patients). After five-year follow up period, 14 patients did not have kidney weakness, and in other patients the most frequent was the grade 2 renal failure. Out of the total number of patients, 8 patients (19.04%) developed the terminal renal failure stage, and ended up on a chronic dialysis program (2 patients with iRPGN, 4 patients with MPA, 2 patients with WG). Six of these patients were initially on haemodialysis, and two of them had partially remission after initial treatment and were corticosteroid dependent. During a five-year follow-up period, 12 patients (28.57%) resulted in death (1 patient with iRPGN, 7 patients with MPA, 4 patients with WG). Seven of these patients were initially on haemodialysis, and cause of death was alveolar heamorrhage in 4 patients, and severe infections in 8 patients. Table 2 presents the clinical characteristics of patients after five-year follow-up period.

Based on the results of the general significance test [$F(1.40) = 7.155$, $p = 0.011$], one can conclude that the predictive GFR model in t = 0 is statistically significant. According to $R^2 = 0.152$ the model explains 15.2% variation of the dependent variable.

The age of the patient proved to be statistically significant predictor of GFR at the moment of presentation of the disease. Estimated glomerular filtration decreased with the age of patients with a factor of 0.390 (Table 3).

Based on the results of the general significance test [$F(4.37) = 16.633$, $p = 0.000$], it was concluded that the predictive GFR model in t = 5 was statistically significant. The cor-

Table 2

Clinical characteristics of patients after five-year follow-up period (t = 5)

Parameter	Values	
Infections	1	4.76
encephalitis	6	28.57
RTI	11	52.38
UTI	1	4.76
nediastinitis	6	28.57
ORL	21	100
in total		
GFR (mL/ min), min–max; mean ± SD	4–148; 58.21 ± 37.54	
Kidney function, n (%)		
preserved	14	34.10
CKD grade 1	1	2.40
CKD grade 2	11	26.80
CKD grade 3	7	17.10
CKD grade 4	1	2.40
CKD grade 5	8	17.10
Haemodialysis, n (%)	34	82.90
no	8	17.10
yes		
Mortality, n (%)	30	71.40
no	12	28.57
yes		

RTI – respiratory tract infections; UTI – urinary tract infections; CKD – chronic kidney disease; ORL – otorhinolaryngology; GFR – glomerular filtration rate.

Table 3

Predictive model for glomerular filtration rate at the moment of the disease presentation (t = 0)

Predictors	Coefficient β	*t*	*p*	95% CI	
Constant		4.143	0.000	72.039	240.803
Age	-0.390	-2.675	0.011	-3.147	-0.372

CI – confidence interval.

rected determination coefficient showed that the model explains 60.4% of variation of the dependent variable.

Of all potential renal outcome predictors, only GFR t = 0 was statistically significant, which was directly proportional to the factor 0.818 (Table 4).

Table 4

Predictive model for glomerular filtration rate (GFR) after five-year follow-up period (t = 5)

Predictors	Coefficient β	*t*	*p*	95% CI	
Constant		0.532	0.598	-23.131	36.839
GFR	0.818	7.682	0.000	0.509	0.957
Therapy	0.073	0.672	0.506	-12.143	22.556
Infections	0.106	1.051	0.300	-1.798	6.520
ANCA subtype	0.026	0.228	0.821	-7.036	7.789

ANCA – anti-neutrophil cytoplasm antibody; CI – confidence interval.

Discussion

This retrospective study was done with a purpose to identify the best predictors of renal outcome in AAV. Renal dysfunction is known risk factor for mortality in patients with AAV [1], and for that reason, the accent on providing the better outcome should be focused on the treatment of renal vasculitis [19, 20]. Better understanding of the factors that are associated with the prognosis of AAV can help to choose the right therapeutic approach in patient with this diagnose. Despite the significant kidney damage, in our study, 34 patients were not dialysis-dependent. End-stage kidney disease was developed in 8 patients, and 12 (28.57%) patients had lethal outcome due to complications of the disease itself, or of the therapy. Our results are similar to the multicentric clinical research of Walsch et al. [21], and prospective multicentric clinical study of de Lind van Wijngaarden et al. [22] (21%) and Titeca-Beauport et al. [23] (36.61%). In our research, 13 patients had additional plasma exchange therapy. There was no statistically significant impact of the plasma exchange therapy on the outcome of the patients. These results are different from the multinational randomized controlled study (MEPEX) study [24], in which the patients who received plasma exchange therapy had better outcome of renal function. The data on antibody subtypes and prognosis of renal function are different. Recent studies have shown that MPO ANCA-positive patients have significantly more expressed chronic changes in kidney biopsies than patients with PR3 ANCA [25]. Other histological research did not prove the difference between antiPR3 and antiMPO antibodies [26]. In our study, we noticed that the subtypes of ANCA antibodies affected the prognosis. Average GFR t = 0 was significantly higher in patients with antiPR3 antibodies than in patients with antiMPO antibodies. The difference was not verified in patients after the five-year follow-up period (GFR t = 5). Twenty one patients (50%) had renal-limited form of the disease, and in 19 patients (70.37%) lung damage was present. Infection is one of the main problems during the treatment of AAV, and also is the main cause of death in immunosuppressed patients [27–29]. Unlike the study of Kronbichler et al. [30], in our work, the most frequent were urinary tract infections (26.19%). Also, hospitalization of patients with ANCA vasculitis due to infections was less common than in the published studies so far, where cumulative incidence at 1, 2 and 5 years of any infection was 51%, 58% and 65%, respectively [1, 31–33].

Conclusion

The renal function at the moment of presentation of the disease, determined by GFR t = 0, is the most important independent factor for assessing the outcome of renal function in ANCA-associated glomerulonephritis, as well as the mortality of these patients.

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Therapeutic role of selective preoperative embolization in patients with paragangliomas of head and neck

Terapijska uloga selektivne preoperativne embolizacije kod bolesnika sa paragangliomima glave i vrata

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Abstract

Background/Aim. Paragongliomas are rare hypervascular neoplasms. The aim of this study was to present the experience in the treatment of paragangliomas using preoperative embolization. **Methods.** This retrospective cross-sectional study included 10 patients (7 women and 3 men; median age 55 years) with paragangliomas that were embolized before surgery. **Results.** Three patients had tympanicum paragangliomas, two carotid bodies, three jugular and two jugulare-tympanicum paragangliomas. During the operation, only one out of 10 patients had bleeding which required blood transfusion. This patient received 1,130 mL of blood transfusion due to surgical complication. **Conclusion.** Adequate preoperative selective embolization of paragangliomas is essential in the preoperative preparation of these patients, because this strategy is feasible with low complication rates.

Key words:
blood transfusion; embolization, therapeutic; head and neck, neoplasms; paraganglioma; preoperative period; treatment, outcome.

Apstrakt

Uvod/Cilj. Paragangliomi su retke hipervaskularne neoplazme. Cilj rada je bio da se prikaže iskustvo u lečenju paraganglioma preoperativnom embolizacijom. **Metode.** U retrospektivnu studiju preseka bilo je uključeno10 bolesnika (7 žena i 3 muškaraca; medijana godina starosti iznosila je 55) sa paragangliomima koji su bili embolisani pre operacije. **Rezultati.** Tri bolesnika su imala timpanične paragangliome, dva karotidne, tri jugularne i dva jugularno-timpanične paragangliome. Tokom operacije, samo jedan od 10 bolesnika imao je krvarenje koje je zahtevalo nadoknadu krvi. Ovaj bolesnik je primio 1 130 mL krvi zbog hirurške kompilikacije. **Zaključak.** Adekvatna preoperativna selektivna embolizacija paraganglioma je osnovna tehnika u preoperativnoj pripremi ovih bolesnika, budući da se radi o izvodljivoj proceduri sa niskom stopom komplikacija.

Ključne reči:
transfuzija krvi; embolizacija, terapijska; glava i vrat, neoplazme; paragangliom; preoperativni period; lečenje, ishod.

Introduction

Paragangliomas (glomus tumors or chemodectoma) are rare hypervascular neoplasms. They arise from paraganglionic cells located in the walls of blood vessels or in specific nerves [1]. They can be located in the carotid body (*glomus caroticum*) as the most common site [1], tympanic plexus (*glomus tympanicum)*, located within the adventitia layer of the jugular bulb wall (*glomus jugulare*) and vagal nerve typically near the jugular foramen. The rare locations are trachea, larynx and nose cavity [2]. In most cases these tumors are benign, slow-growing and locally destructive neoplasms, and a small percentage of tumors produce catecholamines [1, 3]. The incidence of the paragangliomas is one per million people [3],

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where head and neck paragangliomas comprise 3% of all paragangliomas [1]. The majority of these tumors manifest in the fifth and sixth decade of life, predominantly in women [1, 3, 4].

Symptoms depend on the localization and type of paraganglioma. *Glomus tympanicum* causes conductive hearing loss, pulsatile tinnitus which is synchronized with the heart beat, and, on rare occasions, otorrhea hemorrhage. *Glomus jugulare* tumors can cause jugular foramen syndrome (paresis of cranial nerve IX and X), which is pathognomonic for this type of tumor. Paragangliomas may also present as hypertension and tachycardia if they are functional catecholamine–producing tumors (dopamine, norepinephrine, somatostatin), and rarely producing vasoactive intestinal polypeptide, calcitonin [2, 4].

Otoscopic examination shows characteristic reddish-blue pulsatile mass, localized behind the tympanic membrane [2]. Classical radiography (X-ray) of the skull base can show widening of the foramen jugulare. Clinical diagnosis is confirmed by imaging methods – ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI). Images with bone window are used for better visualization of the bone resorption caused by the *glomus tympanicum*. MRI is important for assessing the soft tissue involvement. T1 and T2 sequences after gadolinium enhancement are mainly used, and sometimes fat-suppression sequences [1]. Combination of CT and MRI is a gold standard for the diagnoses of these tumors [2]. Digital subtraction angiography (DSA) is very important and is used for the identification of tumor feeding arteries during embolization planning [1, 3, 4].

Therapy for the paragangliomas is total surgical resection, but because of its rich vascularization and high risk of hemorrhage, preoperative embolization is beneficial for reducing blood loss in the surgical field [4–8].

The aim of this study was to present the experience in the treatment of paragangliomas with preoperative embolization.

Methods

This was a retrospective cross-sectional study of 10 patients with paragangliomas that were embolized before surgery. We included all patients who were embolized before the surgery of paragangliomas during the seven-year period, from 2012–2018. Patients with paragangliomas were treated with preoperative embolization using a sclerosing agent (smaller particles from 100-300 μ) in the Institute of Radiology, Military Medical Academy, Belgrade, Serbia, and a total surgical resection with a function preserving intent in Surgical Clinics Group at the Military Medical Academy. We retrospectively analyzed the patients' medical and imaging records.

After clinical examination as a part of the diagnostics, a multislice CT (MSCT) examination was performed (64- and 128-slices MSCT, Aquilion system, Toshiba®; field of view 20 cm, section thickness 1 mm, contrast material volume 80 mL (iohexol), contrast material injection rate 3 mL/s). The MSCT examination was used to assess tumor size, relationship to surrounding anatomical structures and tumor vascularization.

After clinical diagnosis, patients underwent imaging diagnosis with US (Figures 1A and 1B) and CT (Figures 2A,

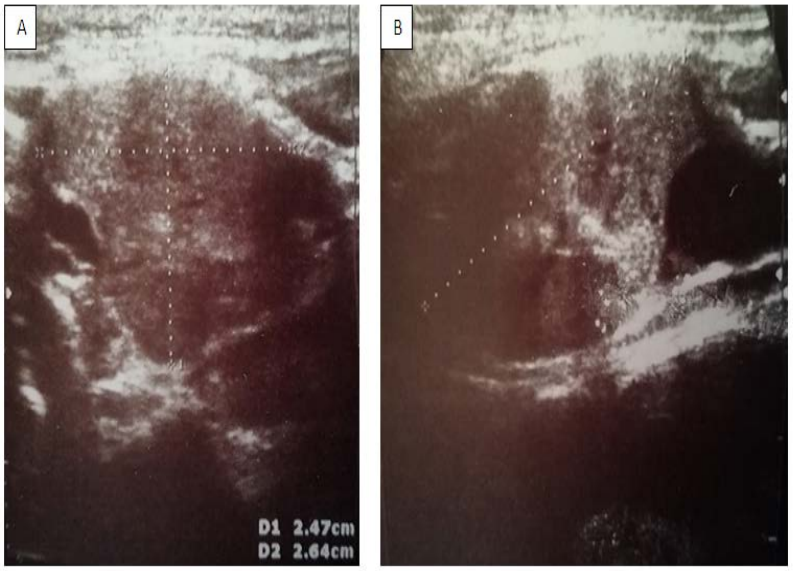

Fig. 1 – Ultrasonic finding: heteroechoic, dominant hyperechoic change, relatively clearly limited.
A) anteroposterior x craniocaudal diameter about 25 × 27 mm;
B) lateral-lateral diameter about 19 mm.

2B and 2C) and preoperative endovascular embolization. Preoperative embolization started with a diagnostic DSA *via* a transfemoral Seldinger approach. Right transfemoral access was obtained using a 6 Fr sheath-guiding catheter (Merit Medical). A guide wire (length 150 mm and 0.035 In) and 5 Fr diagnostic angiographic catheter (SIM II or JB Terumo® or Optitorque®) was then advanced into the common carotid artery on the side the tumor resided. After that, the injection of radiocontrast iohexol (Omnipaque®, GE Healthcare Ireland limited, Ireland) by Avanti avast® pump (rate flow 6 mL/s) was used to visualize vascularization of tumor DSA (Figure 3A). Angiography was performed *via* the diagnostic catheter, placed into common carotid artery and other blood vessels, to determine the arterial feeders of the tumor.

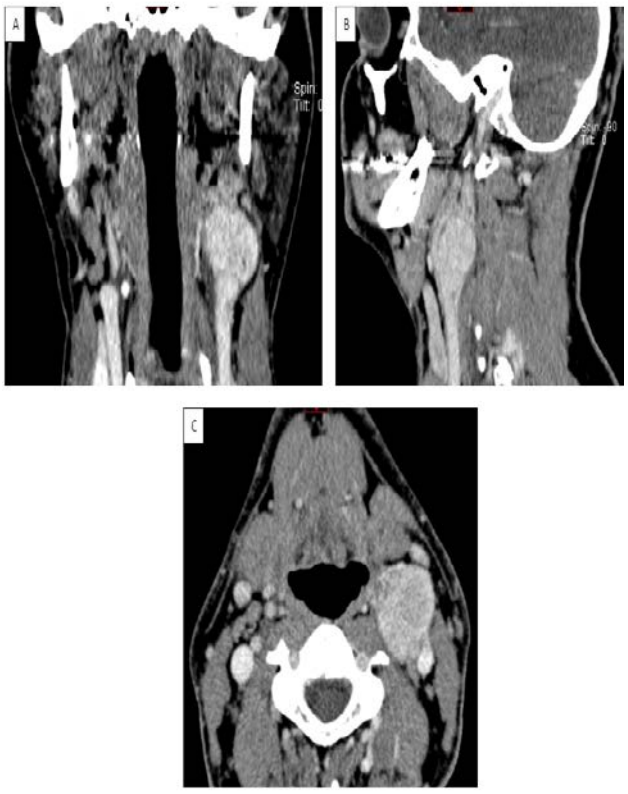

Fig. 2 – Multislice computed tomography findings of the neck: hypervascular expansive lesions at the level of bifurcation of common carotid artery which move the external and internal carotid arteries – carotid glomus. A) coronal; B) sagittal; C) axial slices.

After completion of the diagnostic angiogram, a 2.7 Fr microcatheter (Progreat®, Terumo interventional systems) was placed through the diagnostic catheter and advanced into selective branches of arteries where selective arteriograms could be performed for improved tumor visualization (Figure 3B). Smaller particles (100–300 μ, Bead Block™, BTG International Ltd, UK) were used for embolization. Beads were placed in one mL syringes which were attached to the microcatheter and injected in pulses that were synchronized with systolic heartbeat monitored by radioscopy (Figure 3C). If the flow to the tumor was not diminished, larger bead sizes were incrementally selected until there was the cessation of flow or reflux of the contrast along the microcatheter. This technique was repeated for all branches that were large enough to accommodate the microcatheter. A final angiogram from the common carotid artery was performed to evaluate the degree of embolization and ensure patency of the internal carotid artery circulation (Figure 3D).

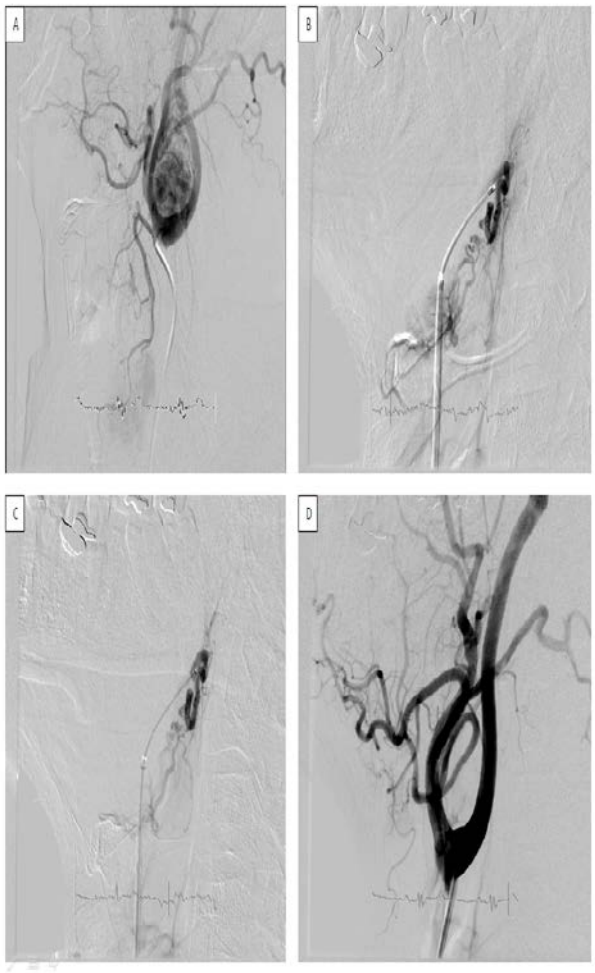

Fig. 3 – A) angiography at the level of bifurcation of common carotid artery by 5 Fr diagnostic angiographic catheter (Terumo®) showed hypervascular tumor change, which moves the external and internal carotid arteries: carotid glomus; the tumor is dominantly vascularized from the pronounced, tortuous ascending pharyngeal artery; B) selective catheterization of external carotid arteries through the diagnostic catheter and supraselective catheterization of ascending pharyngeal artery by Progreat (Terumo®) microcatheter; C) control angiography through microcatheter after the application of embolization agent (Bead Block™) size 100–300 μm; D) control angiography through the diagnostic catheter in the common carotid artery after embolization with complete tumor devascularization.

After embolization, paragangliomas were resected [6]. A retroauricular tympanic access route with canaloplasty was used for paragangliomas in the middle ear. Depending on the location and size of the paragangliomas, an endaural ap-

proach to the middle ear with additional mastoidectomy and myringoplasty was performed. Ossicular reconstruction was performed if required.

The surgical technique for carotid body paragangliomas included precise anatomic dissection and vascular control prior to the attempted tumor excision [6]. The dissection to remove the carotid body paraganglioma was carried out along the arterial subadventitial plane to allow for complete local tumor excision, as well as the preservation of critical vascular structures (Figure 4). Postoperative care included close pharmacologic control of systolic blood pressure and postoperative clinical neurologic evaluation.

Complete statistical analysis of data was performed using the statistical software package, PASW Statistics 18® [SPSS (Hong Kong) Ltd., Hong Kong]. All variables were presented as frequency of certain categories. χ^2 test was used for analyzing the significance of differences of categorical variables. Continuous variables were presented as median and interquartile range (IQR) and were compared using non-parametric Mann-Whitney *U* test. Distribution normality was tested using the Shapiro-Wilk Normality test (number of subjects was < 50). All analyses were estimated at $p < 0.05$ level of statistical significance.

All procedures performed in our study with human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Declaration of Helsinki or comparable ethical standards.

Results

We analyzed the data from 10 patients with paragangliomas (7 women and 3 men), median age 55.0 years, and with IQR from 49.75 to 61.25 years, who were treated with embolization before surgery (Table 1). Female patients were statistically significantly older in comparison to males (Mann-Whitney test; $p = 0.017$) (median age of males and females was 49.0 and 59.0 years, respectively).

Three patients had *glomus tympanicum*, two with carotid body, three with *jugulare* and two with *jugulare-tympanicum glomus* tumor (Table 1). Seven patients had

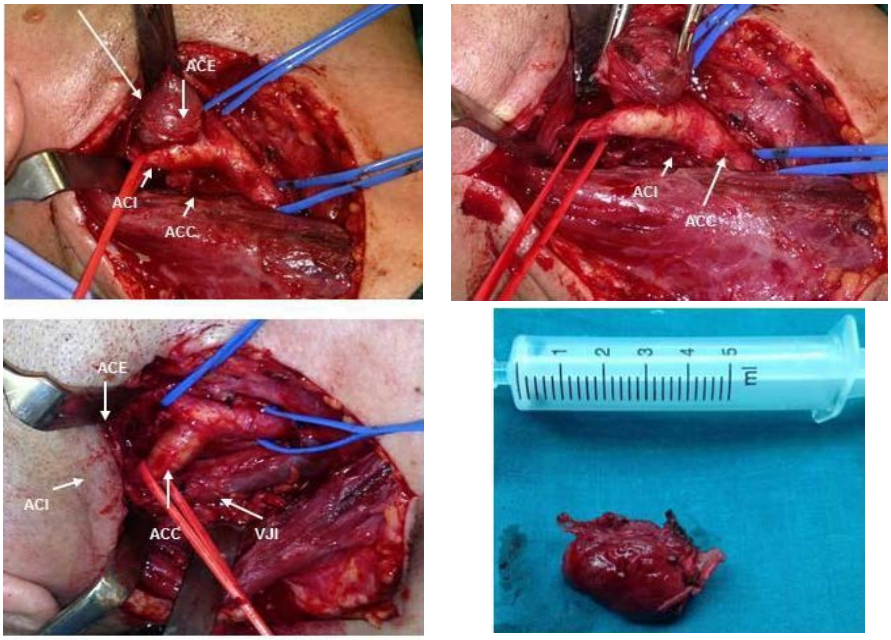

Fig. 4 – Surgical resection of carotid body tumor.
ACI – internal carotid artery; ACE – external carotid artery; ACC – common carotid artery.

Table 1

Demographic and clinical characteristics of patients with paragangliomas

Patient	Gender	Age (years)	*Glomus*	Side	Tumor diameter (mm)	Blood transfusion (mL)
1	male	50	*tympanicum*	left	26	0
2	female	61	carotid body	right	30	0
3	female	65	*jugulare*	both	39	0
4	male	46	*tympanicum*	left	32	0
5	female	59	*tympanicum*	right	12	0
6	female	52	carotid body	right	24	0
7	female	53	*jugulare*	right	24	0
8	female	62	*jugulare- tympanicum*	right	28	0
9	male	49	*jugulare*	right	30	0
10	female	57	*jugulare- tympanicum*	right	34	1,130

right side paraganglioma, while two patients had left side tumor and one patient had tumors on both sides. The largest tumor diameter median was 29.0 mm with IQR from 24.0–32.5 mm. During the operation, 9 out of 10 patients did not have bleeding which would require blood transfusion. One patient who had *jugulare-tympanicum glomus* tumor received 1,130 mL of blood transfusion because of the hemorrhage from carotid sinus during the surgical intervention.

Table 2 shows the clinical characteristics of patients treated in this study. Most patients with paragangliomas had arterial hypertension (six patients) and hearing impairment (also six patients). Five patients had tinnitus, and six patients had pulsations in the ears. Only three patients had ear pain. On MSCT examination, five patients had osteolysis.

Median number of tumor feeder branches was 1.5 from 1 to 3 (Table 2). Five tumors had one feeder branch, and five tumors had two or three feeder branches. In our patients we showed that the embolization had the extent of 100% devascularization of the paragangliomas in all patients.

Discussion

Paragangliomas are rare hypervascular neoplasms, whose surgical resection is at great risk for intraoperative bleeding. Therefore, preoperative reduction of perfusion of these tumors with embolization is very important for reducing the risk of bleeding, both for the patient and for the operation performed by a surgeon [9].

Our results show that preoperative embolization using small sized beads (100–300 μ) with superselective access to arterial feeders, results in almost complete tumor devascularization by 100% patients.

The results of our study in 10 patients showed that devascularization eliminated the need for intraoperative blood transfusion. An exception was one patient who required significant blood transfusion during the surgery (1,130 mL) due to the hemorrhage from carotid sinus.

Surgical resection of paragangliomas can be complicated due to massive bleeding because of their high vascularity [10]. With the evolution of preoperative planning, surgical techniques, and diagnostic evaluations, the risk of artery injury is minimal [11]. The risk of injury to the carotid artery following the treatment of carotid body tumors is size specific: tumors larger than 5 cm are likely to require carotid reconstruction. Unlike jugulotympanic paragangliomas and carotid body tumors, vagal paragangliomas are not closely associated with the carotid artery, although the internal carotid artery may be involved in its petrous portion in advanced disease. Rarely an injury may occur, even with adequate surgical exposure and microsurgical tech-

Table 2

Clinical symptoms and signs of patients with paragangliomas

Clinical symptom/sign	Patients									
	1	2	3	4	5	6	7	8	9	10
Arterial hypertension	yes	yes	yes	no	no	yes	yes	yes	no	no
Heart arrhythmia	no	no	yes	no	no	yes	no	no	no	no
Hearing impaired	yes	no	no	yes	yes	no	yes	yes	no	yes
Types of hearing loss	conductive	-	-	conductive	mixed	-	sensorineural	sensorineural	-	mixed
Tinnitus	yes	no	no	yes	yes	no	no	yes	no	yes
Pulsation in the ears	yes	no	no	yes	yes	no	yes	yes	no	yes
Bleeding from ear	yes	no	no	no	yes	no	no	yes	no	no
Ear pain	no	no	no	no	no	no	no	yes	yes	yes
Visualization of tympanic membrane	no	no	no	no	yes	no	yes	no	no	no
Color of tympanic membrane	-	-	-	bluish	-	-	-	bluish	-	-
Dizziness	no	no	no	no	no	no	no	no	no	yes
Vomiting	no	no	no	no	no	no	no	no	no	yes
Nistagmus	no	no	no	no	no	no	no	no	no	no
Osteolysis	no	no	no	yes	yes	no	yes	yes	no	yes
Number of feeder branches	1	1	2	1	3	1	2	1	2	3

nique. If the patient is at high risk for a vessel injury within the petrous carotid portion and balloon occlusion testing has been safely and satisfactorily performed, then the surgeon may consider permanent preoperative occlusion of the carotid distal to the tumor.

Preoperative embolization of paragangliomas is a very safe adjuvant therapy before surgical resection [1]. Bead embolization dramatically reduced tumor vascularity. The classic angiographic appearance of a paraganglioma is that of a hypervascular mass with robust feeding arteries and intense tumor blush [12]. Successful embolization hinges upon occlusion of all feeding vessels, based upon DSA [13]. A delay of 1–2 days between embolization and total surgical resection allows time for local edema or inflammation to resolve with minimal time for revascularization or recruitment of feeding arteries [13, 14]. The effectiveness of embolization hinges upon occlusion of the feeding tumoral vessels of paragangliomas. The catheterization technique should be superselective, aiming only for feeding vessel of the paraganglioma [1].

Other studies show reduction of 60% to 68% intraoperative blood loss in patients who underwent preoperative embolization when compared with those who did not [15, 16]. In the study by White et al. [13], it was shown that post-embolization angiography revealed an average decrease in blood flow to tumor of 75%. An 80–90% reduction in tumor vascularity is often obtained [4, 13]. The intraoperative blood loss for each tumor type was 289 mL for carotid body, 243 mL for *glomus vagale*, and 1,018 mL for *glomus jugulare*. Larouere et al. [5] showed that the average blood loss for the embolized patients was 650 mL (range from 500–1,000 mL). In the non-embolized patients, the average blood loss was 1,375 mL (range from 1,200–1,725 mL).

Jackson et al. [17] suggested that preoperative embolization in the patients with paragangliomas leads to a decrease in intraoperative blood loss and operative time. The mean estimated blood loss among the patients with embolization was 0.52 standard deviations lower (0.77 to 0.28 lower) than that of patients without embolization.

Therapy for paragangliomas is total surgical resection, but because of its rich vascularization and high risk of hemorrhage, preoperative embolization is beneficial for reducing blood loss in the surgical field [4, 5]. On the other hand, for small localized *glomus tympanicum* tumors, transcanal endoscopic ear surgery is a favorable surgical method [18]. These tumors present high bleeding risk during resection [18]. Embolization can be curative with surgical resection, palliative or supportive, but the main reason for this procedure is to detect and obliterate the abnormal vascular structures of the tumor. This way it is possible to decrease the vascularity and volume of the tumor, and make it safer for the surgery [4].

Limitations of the study

Unlike other reported studies, there is no control group of non-embolized tumors to compare embolization efficacy as manifested by operative blood loss. We also had a small number of these patients.

Conclusion

Adequate preoperative selective embolization of paragangliomas is essential in the preoperative preparation of these patients, because this strategy is feasible with low complication rates.

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The right of Jehovah's Witnesses to refuse and to accept blood transfusion

Pravo Jehovinih svedoka da prihvate ili odbiju transfuziju

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Key words:
jehovah's witnesses; patient rights; blood transfusion; treatment refusal.

Ključne reči:
jehovini svedoci; bolesnik, prava; transfuzija krvi; bolesnik, odbijanje lečenja.

Introduction

In 1829, James Blundell, an English physician (obstetrician), performed the first successful human-to-human blood transfusion [1]. Contemporary era of transfusion medicine begins with a groundbreaking discovery of an Austrian physician, Karl Landsteiner, who introduced blood types A, B and 0 in 1901. For this, he received the Nobel Prize in medicine and physiology [2].

Transfusion is undoubtedly one of the greatest leaps in medicine, enabling treatments of, by then, incurable diseases. It also enabled performing extensive surgical treatments that often involve recoupment of the entire circulatory volume – the so-called exsanguine transfusion (thoracoabdominal aortic aneurysm – TAAA). Still, a question arises of whether the "complex" surgical interventions can be performed without transfusion of someone else's – allogeneic blood. This issue can be resolved in two ways. The first is preoperative blood donation from an individual who is to undergo surgery, which, of course, is possible only in case of planned, elective surgeries, or in case patient's condition is suitable for preoperative blood donation. The second way is intraoperative blood salvaging and autotransfusion. A study from the Clinic for Vascular and Endovascular Surgery, Clinical Center of Serbia (CCS), showed that intraoperative blood salvaging and autotransfusion reduce the 30-day mortality in patients treated for TAAA rupture [3]. Even though this method significantly reduced the need for allogeneic blood transfusion (donor blood), it cannot be completely eliminated. Is it possible to perform even slightly more complex surgeries in patients who refuse to accept someone else's (allogeneic) blood? This mainly concerns Jehovah's Witnesses. At the CCS, only one TAAA surgery has been performed in a Jehovah's Witness patient, without allogeneic blood transfusion. Due to a high risk, only a small number of medical institutions in the world would accept to perform a surgery on a Jehovah's Witness patient without transfusion of allogeneic blood. One of the largest studies regarding this topic included 144 Jehovah's Witnesses who had undergone complex cardiovascular procedures (aortic surgery, aorto-coronary bypass, valvular surgery, heart transplant) in the period between 1999 and 2014 without allogeneic blood [4].

However, while it is possible to perform elective surgeries without transfusion of allogeneic blood, that is not possible in cases of emergency where patients have already suffered due to excessive blood loss (injuries, TAAA rupture, etc.). The same applies to patients who are planned to undergo elective surgery but have certain hematological diseases.

The aim of this paper is to answer the following questions: whether a Jehovah's Witness patient has the right to refuse transfusion; whether a Jehovah's Witness patient has the right to accept transfusion; is it allowed to administer blood transfusion to a Jehovah's Witnesses if they are in a state of unconsciousness; whether intraoperative blood salvaging can resolve the conflict between patients' rights to refuse a medical treatment based on their religious beliefs and their need to receive an adequate medical treatment. The authors will also attempt to answer the questions on whether a physician may cancel surgical treatment if a patient refuses transfusion due to religious reasons.

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Jehovah's Witnesses Organization

Jehovah's Witnesses are a neo-protestant Christian denomination [5]. The Organization of Jehovah's Witnesses was formed at the end of the 19th century in Pennsylvania, USA [6]. In 1884, a non-profit corporation, called The Watch Tower Bible and Tract Society of Pennsylvania (WTS), was established. This is the central organization of Jehovah's Witnesses on a global level, whereas, there is a number of branches worldwide [7]. This organization has more than eight million active followers [8]. The highest authority in its hierarchy is the Governing Body of Jehovah's Witnesses. The Jehovah's Witnesses consider that "the Governing Body is a small group of mature Christians" invited to direct Jehovah's Witnesses and supervise their actions worldwide [9]. The highest organ in Jehovah's Witnesses hierarchy, based on its interpretation of the Bible, has introduced various bans that followers are obliged to adhere to. Some of the bans are medical bans. In the period from 1921 to 1952, vaccination was banned. The WTS claimed that "Vaccination is a direct violation of the everlasting covenant that God made with Noah after the flood" [10]. The governing body of the Jehovah's Witnesses also put a ban on organ transplant for a certain period of time [10]. Due to medical reasons, leaders of the Jehovah's Witnesses religion even banned the usage of aluminum cookware [11]. Global community of Jehovah's Witnesses calls its followers to accept in obedience any change in doctrine prescribed by the leadership. The majority of Jehovah's Witnesses obediently accepted the change in regard to vaccination and transplantation, without questioning whether the abandoned doctrine lead to health decline and loss of life [12]. The WTS calls its followers to sustain from free thinking [13]. Should an individual, after demonstrating negative options on their organization, fail to express a satisfactory level of repentance, one will be excommunicated. The excommunication entails even a ban on greeting in case one meets the other follower in the street. Even cessation of communication with other family members who remained members of Jehovah's Witnesses organization is expected [14].

Ban on transfusion

Jehovah's Witnesses ruling body introduced a religious ban on transfusion on July 1st, 1945 [15]. The Holy Bible does not allow eating the blood [16]. Rigid interpretation of the Bible promoted by the WTS equalizes accepting blood transfusion and eating the blood [17]. The refusal of transfusion and blood products by Jehovah's Witnesses makes this group a unique medical population [18]. In 1961, the WTS corporation started to meticulously implement their doctrine on blood transfusion among their followers and they introduced the so-called "no-blood card", which is a specific form. Carrying the signed "no-blood card" became a religious duty of every Jehovah's Witness. With time, the form got modified and modernized [19]. The current form that Jehovah's Witnesses have on their person was signed in January 2016 and its official title is Durable Power of Attorney for Health Care [20]. Jehovah's Witnesses organization threatens by sanctioning the followers who accept transfusion. The WTS considers that if a member of the faith wilfully accepts the blood transfusion, it indicates that the member no longer wish to be one of Jehovah's Witnesses. According to the rules of Jehovah's Witnesses, an expelled member or one who left the organization on their own is considered outcast. Other followers ought to avoid such an individual. Shunning by family and friends works as a strong deterrent against leaving the religion and acting against the organization's teachings on blood transfusion [21]. Jehovah's Witnesses organization wants to know whether their followers are receiving medical care in accordance with the principles of their religion. Jehovah's Witnesses established a network of boards that liaise with hospitals (hospital liaison committee). There are more than 1700 of such committees that are active in 110 countries worldwide [22]. Jehovah's Witnesses groups that visit their fellow followers at hospitals are instructed to check whether the medical staff was informed that the patient does not accept transfusion [23]. Jehovah's Witnesses have set up a very well constituted network of scrutinizers, lawyers and even physicians that pay visits to hospitals. Therefore, it is not unusual that even medical staff perform illegal acts in the interest of Jehovah's Witnesses [24]. Obedience to the religious organization signifies more importance to Jehovah's Witnesses than keeping the physician-patient privilege. The WTS tends to suggest to their followers who are health professionals that they should secretly inform the organization about every medical intervention that is not allowed, but accepted by a follower [25]. There are informants among Jehovah's Witnesses who will inform on all the banned activities done by their family members [14]. Excommunication, being a proposed sanction, and denunciation, as a means through which the organization receives information on potential transfusion, add to the effectiveness of the imposed blood transfusion ban. It is hard to believe that an individual, when threatened with such a grave and contingent punishment, has the actual freedom of choice. Free will, being an essential element of the choice on whether to accept or refuse the blood transfusion, is rarely present among Jehovah's Witnesses.

Jehovah's Witnesses right to refuse the blood transfusion

A medical procedure is not allowed without a patient's consent, as freedom of will and personal integrity are above reasons that exist due to medical nature [26]. Every individual that is capable of giving consent in regard to accepting a medical intervention can also refuse it, no matter how dangerous or mindless that might seem. This principle lies on one's right to make their own decisions and choices. Unlike the paternalistic traditional medicine and its main principle that "saving a patient is the ultimate law", modern ethics and the concept of patients' rights along with the modern medicine insist that "patient's will is the ultimate law" [27]. The concept of informed consent was adopted in the Convention for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedi-

cine concluded under the auspices of Council of Europe [28] and in the domestic Law on Patients' Rights ("Official Gazette of the Republic of Serbia" No. 45/13) [29]. An informed patient has the right to accept medical intervention proposed by a physician, or refuse it. Therefore, there is a possibility that a patient opts for the wrong option, makes a choice that is not in their best health interest, and even makes a choice that will put their life in jeopardy [30]. The European Court of Human Rights reiterates that according to its case-law, the physical integrity of a person is covered by the concept of "private life" protected by article 8 of the European Convention for the Protection of Human Rights and Fundamental Freedoms (European Convention on Human Rights) [31]. Standing point on Jehovah's Witnesses rights regarding the refusal of blood transfusion has been shown in the European Court of Human Rights case-law. The European Court considers that freedom to accept or refuse specific medical treatment, or to select an alternative form of treatment, is vital to the principles of self-determination and personal autonomy. However, for this freedom to be meaningful, patients must have the right to make choices that accord with their own beliefs and values, regardless of how irrational, unwise or imprudent such choice may appear to others. In the absence of any indication of the need to protect third persons, the State must abstain from interfering with the individual freedom of choice in the sphere of health care [32]. In accordance with the law, a patient has the right to freely decide on everything concerning their life and health, except in a case where such decision would directly jeopardize life and health of others. Medical procedure that is against patient's will can only be performed in exceptional cases which are explicitly prescribed by law and in accordance with medical ethics [33]. The right of a patient capable of rational thinking to refuse a medical treatment even exists in the case where the treatment would save or preserve one's life [34]. Respecting patient's rights on consent is also a physician's ethical duty [35]. Accepting blood is a particularly sensitive medical topic. This is the reason why the law prescribes special rules for the form in which consent to accept blood is given, withdrawal of the consent, duty to inform patients prior to their consent, as well as the form for the notification of the withdrawal in medical records [36]. The right on informed consent guaranteed by the Convention on Human Rights and Biomedicine and Law on Patients' Rights is not an absolute right. This right may be restricted by the law. The restriction is legitimate only if it is explicitly prescribed by the law and necessary in a democratic society in the interest of public safety, for the prevention of crime, for the protection of public health or the protection of the rights and freedoms of others [37]. For instance, for the purpose of fighting crime, the Criminal Procedure Code ("Official Gazette of the Republic of Serbia", No. 72/11, 101/11, 121/12, 32/13, 45/13 and 55/14) prescribes that certain medical procedures can be performed without one's consent [38]. Obligatory immunization (vaccination) against contagious diseases is an example of compulsory medical treatment lawfully prescribed in public health interest [39]. If every competent patient has the right to refuse medical intervention, why would a Jehovah's Witness not be given the right to refuse a specific medical treatment – blood transfusion? Disregard of such subjective right of a Jehovah's Witness would take a patient's position back to time when the decision on the therapy was solely given to discretion and values of the attending physician [40]. If a patient, capable of rational thinking and of free will, who has been previously informed about considerable risks by a qualified physician, persistently refuses transfusion, then one should not receive it.

The right of Jehovah's Witnesses to accept blood transfusion

Absolute obedience regarding religious bans does not exist in cases of bans that are related to health. It is possible and permitted by the positive law that a follower accepts a medical procedure that is prohibited by religious norms. Certain research has documented that Jehovah's Witnesses could be willing to accept the transfusion [18]. Jehovah's Witnesses dissidents indicate that Jehovah's Witness population has always been divided between those who believe it is wrong to accept the blood transfusion and those who find it right [41]. It is a universal physician's duty to attempt to influence a patient, in order for the latter to act reasonably, being medically inconversant [42]. This duty exists regardless of the patient's religion. Accepting blood transfusion is generally considered reasonable in cases where a surgical procedure is necessary and when it cannot be performed without the transfusion. A physician should attempt, through conversation, medical information and recommendations, to influence a Jehovah's Witness patient to make a choice that is reasonable under general opinion, i.e., to opt for the necessary transfusion. Regardless of the standings of the religious organization, a patient is the one to make a decision. Hospital liaison committees established by the WTS and local elders of the Jehovah's Witnesses community must not be involved in their followers' treatment process. Health professional should not inform them about a patient's decision on potential transfusion, especially not to confirm whether a patient accepted it or not. Treatment is required for a patient as an individual. A physician is to perceive a patient as an individual, not as a "sect". Medical doctor must never consider an individual patient to be the same as religious group to which that patient belongs. Wrong actions might be undertaken whether a patient should be treated merely as a religious follower. A physician with such perception will not provide appropriate treatment to a patient, due to their unfavorable standing on a "religious sect". Reverse scenario might be that a physician who considers that a Jehovah's Witness patient is the same as their religious organization, might involve the WTS in the entire treatment and decision process, due to the appreciation towards the minority religion group and his personal dislike towards traditional church. In that case, a physician will consult the leaders of the Jehovah's Witnesses and their hospital liaison committee members, thus allowing them to make a decision on transfusion.

Patient in a state of unconsciousness

Unconscious patients cannot make a decision for themselves. Medically indicated intervention is in the patient's interest, therefore, it is considered that the patient would agree with the procedure [42, 43]. The Convention on Human Rights and Biomedicine stipulates that a medical intervention may only be carried out on a person who does not have a capacity to consent, for his direct benefit [43]. According to the Convention, a risky medical intervention, such as giving someone else's blood to an unconscious patient, may be undertaken if there is no alternative of comparable effectiveness. Risks which are incurred in that situation should not disproportionate to the potential benefits of the transfusion [44]. Laws on patient's rights also stipulate that an urgent medical procedure can be performed on a patient in a state of unconsciousness without their consent. Such medical procedure is provided based on *consilium medicum*. Immediate family members must be informed about the medical procedure performed without patient's consent, whenever possible [45]. Medical ethics states that a physician is to administer urgent medical procedure to a patient in a state of unconsciousness even without patient's consent [46]. Being given someone else's blood, in case there is no alternative equally efficient, is a medical intervention that can produce real and direct benefit for the recipient's health. *Lex specialis* regulating transfusion permits administering blood transfusion to a patient in a state of unconsciousness, or in other cases when patient is unable to provide consent. Under these circumstances physician who is administering immediate medical care is allowed to opt for the transfusion without patient's consent [47]. Transfusion is regulated by the law and in modern medicine it is accepted medical procedure that directly benefits a patient whose life is in jeopardy. It can be undoubtedly concluded that the transfusion is allowed even in case a patient is in a state of unconsciousness without one's prior consent, if administering the blood is required in order to save patient's life. No-blood card that an unconscious patient carries with themselves can cause dilemma. An answer to a question on whether an unconscious patient with a no-blood card can be administered transfusion can be found in comparative court practice. A Jehovah's Witness patient arrived at hospital in a state of unconsciousness in a town of Pordenone in Italian region of Friuli-Venezia Giulia. He was carrying a filled-out form regarding blood refusal – the no-blood card, but nonetheless received a blood transfusion. After a certain period of time, once he had recovered from the medical intervention, he submitted a lawsuit due to the transfusion without consent. The proceedings were finally concluded by the Supreme Court of Cassation of the Republic of Italy, under the number 23676, dated on 15th September 2008. The Supreme Court has recognized that physicians acted correctly. Italian highest court has assessed that physicians could not presume the real "resistance" against transfusion in sudden life-threatening event, merely on no-blood card [48]. The Convention on Human Rights and Biomedicine stipulates that previously expressed wishes relating to a medical intervention by patients who are not, at the time of intervention, in a state to express their wishes should be taken in account [49]. Filled out forms regarding refusing the transfusion that are carried by a Jehovah's Witness could be treated as a form through which such wishes are expressed. Therefore, the medical staff, when found the no-blood card should not ignore it, neither hide it nor destroy it. The Convention on Human Rights and Biomedicine makes clear difference between previously expressed wishes on the one hand and patient's informed consent or refusal on the other. Previously expressed wishes should be taken into account by physicians, but physicians must obey a patient free and informed consent or refusal. Due to this, physicians must not blindly obey the instructions stated in the form. No-blood card, imposed by the WTS should only be taken into account. Legally, the act of "taking into account" is fulfilled if physicians acknowledge the form, if they assess whether patient's condition has changed since the form was signed, if they potentially consult each other and make a proper notification in a medical record. After all these formalities, physicians should maintain their approach, as they would towards any patient in a state of unconsciousness.

Autologous transfusion

Autologous transfusion in which own blood is being accepted, i.e., in which donor and receiver are the same person, has a number of advantages over allogeneic transfusion, in which a receiver is given someone else's blood. Risk of transmitting contagious diseases is eliminated. Also, shortage of blood supply is one of the reasons for the autologous transfusion. Medical advantages of autologous transfusion are *ratio legis*, due to which this method has the legal priority over allogeneic transfusion. An acting physician has a legal obligation to inform a patient about the possibility of autologous transfusion. The WTS has a different standing on autologous transfusion in comparison to allogeneic transfusion. Accepting someone else's blood, as well as giving own blood for someone else is prohibited. However, transfusion in which the donor and receiver are the same individual may be acceptable for this religious group [12]. The Law does not differentiate between intraoperative blood salvaging and autologous preoperative blood giving that would be used in the perioperative period. The WTS differentiates between autologous transfusion in which the blood is taken (and put back in the body) during the procedure itself and autotransfusion of predeposited blood (blood that was taken before and saved for the operation). Intraoperative blood salvaging is acceptable as per the WTS, if the extracorporeal circulation, circulation of blood outside patient's body, is uninterrupted [50, 51]. Therefore, this method can, in an optimal way, resolve the conflict between the patient's right to refuse a medical treatment that is not in accordance with their religious beliefs and the need for him/her to receive an adequate medical treatment.

The right of a physician to cancel surgical intervention

Patients are obliged to actively participate in the protection, preservation and improvement of their health, having received medical care [52]. The duty of a patient to cooperate with health professionals is in direct relation with one's own health. If patients do not cooperate with a physician, they may bear consequences that would affect their own health [53]. Refusal to accept someone else's blood, in case when it is not possible to perform required medical procedure *lege artis* without allogeneic transfusion, could be considered as a patient's refusal to cooperate. The Law does not prescribe neither legal nor financial penalty for a patient who does not cooperate with health professionals in the process of their own medical treatment. However, a physician is not required to act in the same manner concerning a patient who actively cooperates with a view to his own healing, in contrast to a patient who refuses to cooperate. A physician is permitted by the law to cancel further treatment, should patients fail to fulfil their obligations, including the obligation to cooperate. Cancelling the treatment is to be followed by certain formalities, such as initial warning of the patient by a physician and, afterwards, a written notice by the physician to the director of the medical institution [54]. Article 14 of the European Convention on Human Rights prohibits discrimination, while recognizing rights and freedoms prescribed by the Convention. Along with the European Convention, Protocol No. 12 provides for a general prohibition of discrimination [55]. A physician, upon deciding whether to perform a procedure or not, cannot be influenced by potential discriminatory motives. Difficulties with which bloodless surgery faces should not be an excuse for religious discrimination. Depriving of the right to heal, due to patient's religious affiliation, is a type of discrimination. Denying or restricting the right to heal on the grounds of religion could be considered as violation of equality crime [56]. In particular situations physicians should exercise their discretion to cancel the treatment due to the fact that a patient refuses blood transfusion, not only without any discrimination, but also restrictively. Technical conditions and lack thereof, as well as insufficient expertise of medical staff, could be a fair reason due to which a Jehovah's Witness patient who refuses transfusion would be recalled. Safe and successful performance of a surgical treatment without the application of allogeneic transfusion cannot be performed *lege artis* in every medical institution. A medical institution and its health professionals that manage to perform a complex surgical intervention without allogeneic transfusion deserve the highest praise.

Conclusion

Jehovah's Witnesses' teachings on refusing transfusion, based on a ban to eat blood, seems bizarre in a modern world. Still, patients have the right to make a choice based on their beliefs and values, regardless of whether that choice may seem irrational, unwise and ill-considered. Undoubtedly, a patient has the right to choose whether one will accept the transfusion or not. It is an innate patient's right, not the right of a religious organization to which the individual belongs. Jehovah's Witnesses organization, their hospital liaison committees, as well as their local elders, ought to be excluded from the decision making process regarding the treatment. Jehovah's Witnesses, as per the rules of their organization, have an obligation to carry a signed form on refusing the transfusion, which may cause dilemma if a patient is in a state of unconsciousness. Health professionals must not demonstrate full obedience towards instructions given in the document. It would be enough for physicians to take the wishes expressed in no-blood card into account, to consider the no-blood card, observe it and assess whether patient's health state changed from the moment of the document signing. All these activities should be noted in a patient's medical records. After these formalities, physicians should act as they would with any other unconscious patient. Intraoperative blood salvaging, as one of the methods of autologous transfusion, enable an adequate medical treatment to a Jehovah's Witness patient, still being in accordance with their religious beliefs. Refusal to accept someone else's blood, in case when it is impossible to perform necessary medical intervention *lege artis* without allogeneic transfusion can be qualified as patient's refusal to cooperate. In such cases, the law empowers physicians to cancel the treatment. This possibility should not be applied extensively. A surgeon's decision to recall a surgical procedure on a patient who is a Jehovah's Witness has to be, primarily, based on a justified reason. It should most certainly not be based on religious discrimination.

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Reporting of the *Politika* Belgrade daily newspaper on the epidemic of typhus in Serbia during the First World War

Izveštavanje beogradskog dnevnog lista Politika o epidemiji tifusa u Srbiji za vreme Prvog svetskog rata

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Abstract

Background/Aim. The epidemic of typhus lasted for several months in the Kingdom of Serbia during the First World War, and a vast number of people lost their lives. The objective of the paper was to investigate how the *Politika*, Belgrade daily newspaper, informed the then Serbian public about the epidemic of typhus in the Great War. **Methods.** By using statistical and historical-critical method, the analysis of all texts published in the *Politika* from February 1915, when the Serbian government and the Supreme Command allowed the press to write about the epidemic of typhus, until the outbreak began to calm down at the beginning of May 1915, was performed. **Results.** In the observed period, among the texts dedicated to the typhus epidemic, news about people who died of typhus (obituaries), news about the epidemic itself, as well as affirmative texts about the feats of doctors. There were more texts about allies' military missions than about Serbian Army Medical Corps. The reporting was balanced and highly professional. **Conclusion.** In the period February–May 1915, a number of different articles about the typhus epidemic in Serbia during the Great War were published in the daily newspaper *Politika*. The reporting was well-balanced, ethical and highly professional.

Key words:
world war I; disease outbreaks; typhus, epidemic louse borne; newspaper article; serbia; history of medicine; history, 20th century.

Apstrakt

Uvod/Cilj. Epidemija tifusa trajala je nekoliko meseci tokom Prvog svetskog rata na teritoriji Kraljevine Srbije odnevši veliki broj života. Cilj rada je bio da se istraži kako je beogradski dnevni list Politika izveštavao tadašnju srpsku javnost o epidemiji tifusa u Velikom ratu. **Metode**. Primenom statističkog i istorijsko-kritičkog metoda, izvršena je analiza svih tekstova objavljenih u dnevnom listu Politika u periodu od februara 1915, kada je srpska Vlada i Vrhovna komanda Srpske vojske dozvolila novinsko izveštavanje o epidemiji tifusa, do početka maja kada se epidemija počela smirivati. **Rezultati.** U posmatranom periodu, među tekstovima posvećenim epidemiji tifusa dominirale su vesti o osobama umrlim od tifusa (čitulje), novosti o samoj epidemiji, kao i afirmativni tekstovi o podvizima lekara. Bilo je više napisa o savezničkim vojnim misijama nego o srpskom vojnom sanitetu. Izveštavanje je bilo balansirano i visoko profesionalno. **Zaključak**. U periodu februar–maj 1915, u dnevnom listu Politka objavljivan je veći broj različitih tekstova o epidemiji tifusa u Srbiji tokom Velikog rata. Izveštavanje je bilo dobro balansirano, etično i visoko profesionalno.

Ključne reči:
prvi svetski rat; epidemije; tifus, pegavi; novine; srbija; istorija medicine; istorija, xx vek.

Introduction

Every war, and especially a global war, brings great human, material, and technology casualties. Apart from the losses on the battlefield in the conflict of the two armies, there are also losses resulting from various misfortunes accompanying any major armed conflict. "It is widely known that in addition to military operation adversities, wars bring armies and civilian population sufferings such as starvation, infectious diseases and a dearth of every kind. In 1915, the Serbian army had a high mortality rate additionally contributed by major epidemics and infections (epidemic of typhus, typhoid fever, relapsing fever, dysentery, cholera, diphtheria), resulting in the death of 35,000 Serbian soldiers and about 30,000 Austro-Hungarian prisoners," [1]. The epidemics of typhus, typhoid fever, and relapsing fever lasted for sever

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al months in the Kingdom of Serbia during the First World War, and a vast number of people lost their lives. The epidemic of typhus (especially *typhus exanthematicus*) has been well addressed in the professional and scientific literature, and one of the earliest scientific papers on the subject was published by British Army Medical Corps colonel William Hunter, M.D, entitled: *The Serbian Epidemics of Typhus and Relapsing Fever* in 1915 [2]. The importance of Dr. Hunter's work is reflected in the fact that he was one of the key physicians devoted to preventing the spread of the epidemics and that as a commanding officer of the British Army Medical Corps mission, he provided enormous assistance to the Serbian army and people in the fight against the epidemics. Lieutenant Colonel Vladimir Stanojević stands out among the Serbian authors who wrote on the epidemics following the Great War with his proceedings *The History of Serbian Army Medical Corps and Our Wartime Medical Experience* (1925 edition) [3]. Lieutenant Colonel Stanojević held significant duties in the Serbian Army Medical Corps during the epidemic of typhus, and the significance of his work is reflected in his collection of (in the proceedings) the experiences of other Serbian Army Medical Corps officers on the epidemics of typhus. Among recent research endeavors, Goran Čukić's book *Prevention of Typhus in Serbia in 1915* [4] is worth mentioning. In *the Vojnosanitetski Pregled*, Čukić published a remarkable paper entitled: *Serbian, the first phase of the suppression of epidemics in 1914 and 1915* [5]. The aforementioned professional and scientific papers deal with typhus from the point of view of doctors – medical corps officers who participated in the prevention of the epidemics, and the authors of the texts present the medical aspects of the disease, the methods of treatment, the evaluation of medicine administration success, the methods of depediculation, etc.

Our work aims to investigate, analyze, and systematically present how the oldest daily newspaper in the Balkans, the *Belgrade Politika*, reported on the epidemic of typhus in Serbia during the First World War. Although much has been written about the epidemic of typhus, it is evident that there are not enough papers dealing with media reporting, or more precisely, reporting in the then daily newspapers on the major epidemics. This is a particularly big challenge if we know that in the first phase of the outbreak, the attitude of the military authorities, but also the Serbian government, was that the truth about the scale of the epidemic should be concealed. This was primarily true of the print media at the time. There was a prevailing fear of severe consequences of the enemy's gaining the impression that the epidemic had weakened the country's military and economic strength or had broken its morale [2]. The military and civilian authorities' ban imposed on the then print media about the reporting on the epidemic of typhus was partly annulled in February 1915, and it is, therefore, essential to analyze the typhus epidemic through the media which even then played a significant role in the education of the population. The significance of the media on the suppression of typhus was best evidenced by the following quotation: "Thus, on March 16, 1915, *Srpske novine* (printed in Niš) published an article entitled *Fighting the Typhus* (from the letter of a battalion commander)", which showed that his battalion was stationed in dugouts in a small, poor and dirty village on the border. In one newspaper, the commander read a lecture given by Dr. Subottić (originally written with double t – author's note) at a doctors' meeting in Niš, where it was said that disinfection furnaces could be built everywhere. He, too, made them in a cave with his soldiers... This feat of the nameless battalion commander showed what an individual's initiative means and can do. He saved his battalion (several hundred soldiers?) from typhus infection" [7].

Methods

By using statistical method, historical-critical method, content analysis method, comparative method, and media discourse analysis [7], the *Politika* analysis was done on a day-by-day basis, covering all issues from February 1915, when the Serbian government and the Supreme Command allowed the press to write about the epidemic, until the outbreak that followed at the beginning of May 1915. It was not necessary to analyze the paper editions before February 1915 as there were no texts on typhus (except for the obituaries which sporadically mentioned the cause of death) due to the strict prohibition of writing about the epidemics imposed by civilian and military authorities.

The *Politika* newspaper was taken as a representative medium which began to be published on January 25, 1904, and was owned by the Ribnikar family. The first issue was printed in 2,450 copies. Before the First World War, the newspaper was rated as a reputable, objective, and media-balanced daily information and political newspaper [8]. Some of our most celebrated and most prominent journalists and editors who worked before and after World War I wrote in the *Politika*. At the onset of the war, the founders of the *Politika* themselves, the Ribnikar brothers, lost their lives while performing their military duties: "Immediately after the announcement of the mobilization, the two Ribnikar brothers – Vladislav and Darko, were mobilized as reserve officers – captains. The third brother, Dr. Slobodan, was hired as a military doctor. Unfortunately, the war brought a great tragedy to the founder of the *Politika* and his family. Darko and Vladislav were killed at the very beginning of the war, on August 31 and September 1, 1914." [9].

Results and discussion

The average number of texts in the period we observed are presented in Table 1 as part of the research results analysis.

The descriptive statistics for all variables of this research point to the following elements: there was genre diversity in the *Politika* when it wrote about the epidemic of typhus in Serbia. The newspaper's editorial staff showed great media interest in allies' assistance in military medical missions. The research suggests that when compared with other text categories, the newspaper text structure was dominated by obituaries dedicated to persons who died of typhus.

Table 1

Number of texts on typhus epidemic in Serbia in the Great War published in the *Politik*a in the period February–May 1915

Text category	Total number of texts on the epidemic	Average number of texts on the epidemic*
Feuilleton	2	0.022
News	72	3.08
Report	23	1.13
Coverage	7	1.00
Comment	17	1.12
Obituary	85	7.32
Other	2	1.00
Authorial text	15	1.13
Has statistics	6	1.17
Motivational text	14	1.21
Critical text	18	1.00
Educational text	16	1.06
Text about the death of a doctor	41	1.37
Text with medical advice	3	1.00
Text on a foreign medical corps mission	56	1.70
Text on the Serbian medical corps	22	1.09
Affirmative text on doctor feats	19	1.11

***per single issue of the *Politika* printed in the observed period.**

The *Politika* wrote more about foreign military missions than about the Serbian Army Medical Corps, which can be interpreted as gratitude of journalists and the entire Serbian people to allies' military doctors. Serbian journalists and editors thus paid tribute to allies' military missions – doctors and medical corps staff who, risking their own lives, came to a small Balkan country to help its army and people to fight the epidemics of typhus.

The *Politika* had the highest number of texts affirming the feats of doctors, but there was also a relatively large number of critical texts stating editorial staff objections to the ineffective work of individual government bodies on combating the epidemics. One example of critical texts is the following text in which the editorial staff of the *Politika* highlighted the problem of lack of hygiene in Serbian trains, which was one of the reasons for the rapid spread of the typhus epidemics: "A few years ago, our Railway Directorate purchased a wagon and train disinfection device for 85,000 Serbian dinars. Now the wagons and trains are so contaminated and infected with insects and dirt, filth and germs of terrible diseases, that a man shudders and risks his life when getting on a train. It is about time to disinfect wagons. But they cannot find this expensive and much-needed device!" [10]. On the other hand, it should be noted that there were very few educational texts on the epidemic that would give medical advice as to how to protect oneself from the epidemics.

It was especially important to investigate, in the paper, the media presentation of affirmative texts on the feats of doctors during the outbreak and duration of the epidemic of typhus, typhoid fever, relapsing fever in Serbia during the First World War. The *Politika* made doctors, who sacrificed themselves and gave their lives to fight the typhus epidemic, the media heroes, and an excellent example of a media initiative was the acknowledgments the newspaper published. We believe that this is a fair and ethically acceptable approach in the media. The *Politika* published acknowledgments from ordinary citizens-readers who were rescued or had a member of their family healed by the doctors. An example is: "To Mr. L. Coyen – Medical Corps Major and Head of Surgical Department at Palanka Reserve Hospital" [11].

Based on the obtained research results, we can conclude that the *Politika* daily reported on the epidemic of typhus in Serbia during the First World War in a balanced, ethically-based, and, in terms of the media, highly professional manner.

In the first phase of the epidemic, due to the strict State prohibition on writing about typhus, it was not possible to publish articles on that topic. In the next phase (since February 1915) it was allowed to write about typhus, and journalists and editors were supposed to publish more educational texts about the epidemic. It was necessary to publish specialized texts, written by doctors, in the *Politika* and they were published every day. Journalists and editors could interview domestic and alliance military doctors who would give advice on how to stop the epidemic. Confidential data were obtained from the Chief of Medical Corps of the Supreme Command and other high medical officers. Although a significant part of the population was illiterate at the time, this medical action for typhus suppression was effective because the literate soldiers and citizens would read to those who could not read. Hence, a significant portion of the population "consumed" those articles. The target audience for those educational articles was the whole population of the Kingdom of Serbia. It would raise awareness and enhance sanitary measures and prevention in order to suppress the epidemic.

Based on the research, we can conclude that at the time of the typhus epidemic, the *Politika* newspaper wrote more about allies' military missions (56 units of analysis in the corpus) than about Serbian Army Medical Corps (22 units of analysis in the corpus).

As to the genre structure of the *Politika*, it was dominated by the news on the epidemic of typhus (72 units of

analysis in the corpus) and had the least feuilletons (2 units of analysis in the corpus).

As to the evaluation text structure in the *Politika*, it was dominated by affirmative texts on the feats of doctors (19 units of analysis in the corpus), which makes them the most represented category of evaluation texts in the newspaper. On the other hand, there are fewer educational texts (16 units of analysis in the corpus). Also, the research results indicate that in the *Politika* there were more articles about the deaths of Serbian military doctors.

In the professional and general public of the observed period, there were differing opinions about the origin of the typhus epidemics in the Kingdom of Serbia during the Great War. There are discussions in scientific papers as to whether the epidemic of typhus was brought by Austro-Hungarian prisoners of war from Galicia or there were other sources of infection in the country itself. Lieutenant Colonel Vladimir Stanojević, a war doctor at the Combined Division Polish Hospital and the head of the Moravian Military Hospital in Niš, wrote about the occurrence of typhus: “Apart from this, the first cases, even before the epidemic typhus on the battlefield, were officially recorded in southern Serbia. These were reported by the Chief of Medical Corps of the Supreme Command at Ministry of Defence on October 10, 1914, with LO No. 368, reporting that there were only three cases of epidemic typhus in Serbia, 1 in Debar Hospital and 2 in Mitrovica, one of which died ” [6].

As far as the source of epidemic is concerned, it should be noted that a competent source, such as the Chief of Medical Corps of the Supreme Command Colonel Dr. Lazar Genčić, emphasized that there were several sources of outbreak, both imported and domestic. Southern Serbia (the territory of today’s Northern Macedonia) was identified as one of the places, and it was assumed that the epidemic started in the Western Serbia. Colonel Dr. Genčić wrote: “Our victorious army, marching and fighting the enemy, occasionally carried the typhus germ, but also, in the places where Austro-Hungarian army retreated, came across many more sources and was exposed to a typhus contagion on a large scale” [12, 13].

Although there was no news of typhus, the first news of cholera in the Austro-Hungarian Army was published already in 3,839th issue of the *Politika* dated 03/10/1914: "Vienna, September 29. Today, three cases of cholera are registered in Vienna, one in Lower Austria, Styria and Silesia, four in Galicia. Therefore, in most military centers of the northern battlefield" [13]. It was the beginning of the war, but also the announcement of severe typhus epidemics that stroke the Serbian Army and civilians.

In the *Politika*, we find evidence that the Serbian *Army* also suspected the biological war being waged by the Austro-Hungarian troops, as on the cover of the edition dated 15/01/1915, the text Danube Hunting reported on soldiers taking barrels of wine and rum out of the river: "But our officers thought it was also possible that the Austrians poisoned the wine with cholera or some other bacilli, and ordered the barrels to be immediately thrown back into the Danube" [14].

Although the military and civilian authorities imposed a ban on writing about the typhus epidemics, as early as in January 1915, the cause of death of some distinguished citizens could be clearly read in the obituaries: "Dr. Milutin Perišić, a well-known Belgrade doctor who has been the head of one of the hospitals in Skopje since the beginning of this war, as a reserve medical corps major, died in that city. A few weeks ago, Dr. Perišić lost his wife in Skopje after her long and severe illness, and now he too has succumbed to typhus" [15].

A major problem was the inability of Serbian doctors to mark lice as the main carrier, about which the *Politika* wrote in the article *"On Epidemic of Typhus – Interview with a Regiment Doctor"*: "When asked how come so many doctors got infected with typhus, this doctor said: I do not believe that the typhus was passed on to doctors by lice; all of them are people who could and wanted to protect themselves from the plague" [16]. It should be emphasized that typhus caused huge losses not only to the Serbian Army but also to the civilian population since, according to competent authors’ estimates, Serbia lost between 100,000 and 200,000 civilians in the epidemics [6].

All of the above elements should be kept in mind when reaching the conclusion of the *Politika* daily writing on the epidemic of typhus in Serbia in 1915.

This research is the first research on media coverage of typhus epidemic in the World War I in Serbia, and we hope it will be useful for future authors writing on the topic relevant to the history of Serbian medicine and military medical corps.

Conclusion

In the period February–May 1915, a number of different articles about the typhus epidemic in Serbia during the Great War were published in the daily newspaper Politka. The reporting was well-balanced, ethical and highly professional.

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I N M E M O R I A M

prof. dr sc. med. DUBRAVKO BOKONJIĆ
redovni profesor u penziji

(1950–2021)

U Beogradu je 27. maja ove godine iznenada preminuo profesor u penziji, dr Dubravko Bokonjić, redovni profesor farmakologije i toksikologije na Medicinskom fakultetu Vojnomedicinske akademije (VMA) Univerziteta odbrane u Beogradu. Vest o njegovoj smrti duboko je potresla brojne kolege, saradnike, prijatelje i generacije bivših studenata kojima je godinama nesebično prenosio svoje ogromno znanje i ljubav prema medicinskoj struci i nauci.

Profesor Bokonjić rođen je 6. decembra 1950. godine u Zagrebu. Osmogodišnje školovanje započeo je u Beogradu i nastavio ga u Sarajevu, u kome je kasnije završio i gimnaziju. Medicinski fakultet upisao je u Beogradu i na njemu diplomirao 1976. godine. Posle obavljenog lekarskog staža, 1979. godine zasniva radni odnos u Medicinskom odeljenju Sektora za atomsko-biološko-hemijsku zaštitu Vojnotehničkog instituta (VTI) u Beogradu. U svojstvu istraživača saradnika započinje svoju naučnu karijeru u Laboratoriji za bihevioralnu farmakologiju i toksikologiju, koju je nešto ranije oformio jedan od velikana jugoslovanske i srpske toksikologije prof. dr Nedeljko Rosić. Ova Laboratorija, pod njihovim rukovodstvom, postala je mesto gde su svoja prva znanja i veštine u ovoj oblasti sticali mnogi, kasnije istaknuti srpski farmakolozi i toksikolozi.

U vreme dolaska prof. Bokonjića u VTI, vojni toksikolozi iz Srbije bili su među vodećim naučnicima u toj oblasti na svetskom nivou, što je sigurno doprinelo i njegovom razvoju i afirmaciji kao vrhunskog istraživača. O tome svedoče brojni visokocitirani radovi proistekli iz njegovog magistarskog („Uticaj centralnih holinergika i oksima na promene uslovljenog ponašanja izazvane neletalnim koncentracijama somana“) i doktorskog rada („Antikonvulzivni i zaštitni efekti diazepama i midazolama u životinja tretiranih visokotoksičnim organofosfornim jedinjenjima“), odbranjenim 1983, odnosno 1995. godine na VMA.

Po prelasku Medicinskog odeljnja VTI-a u Nacionalni centar za kontrolu trovanja (NCKT) VMA 1998. godine, prof. Bokonjić biva postavljen na mesto načelnika Odseka za radiobiologiju Odeljenja za eksperimentalnu toksikologiju i farmakologiju NCKT VMA, a 2005. godine postaje načelnik tog Odeljenja. Dve godine kasnije, 2007. godine, postaje načelnik Instituta za toksikologiju i farmakologiju NCKT VMA i na toj funkciji ostaje do odlaska u penziju 2015. godine.

Od samih početaka rada u VTI, prof. Bokonjić je bio uključen i u nastavni proces na VMA, kao saradnik u nastavi iz oblasti toksikologije na poslediplomskim studijama na VMA i u Školi rezervnih oficira sanitetske službe. Za docenta iz oblasti farmakologije i toksikologije na VMA biran je 1995. godine, za vanrednog profesora 2002, a za redovnog profesora 2007. godine. Osim na VMA, a kasnije na Medicinskom fakultetu VMA, držao je predavanja i vežbe po pozivu i studentima na Biološkom fakultetu u Beogradu, studentima Medicinskog fakulteta u Foči (Republika Srpska, BiH), a od osnivanja doktorskih studija na Medicinskom fakultetu u Banjaluci, postao je stalni nastavnik na predmetu Statistika u biomedicini. Angažovanje na mestu nastavnika statistike ne treba da čudi jer je prof. Bokonjić, još početkom 80-ih godina prošlog veka, među prvima savladao veštine

rada na računaru i osnove programiranja, što mu je kasnije omogućilo da vrlo lako uđe u problematiku statističke obrade kompleksnih podataka proisteklih iz biomedicinskih istraživanja. U tome je bio pravi virtuoz i nesebično je pomagao brojnim kolegama koji nisu bili vični u statističkoj obradi podataka. I ne samo to, prof. Bokonjić je, kao vrsni istraživač, lako uočavao i prednosti i mane pojedinih istraživanja i pomagao kolegama da svoje idejne projekte, a kasnije i rezultate proistekle iz njih, ispravno postave i komentarišu. Bio je mentor/komentor ili član komisija za odbranu niza magistarskih i doktorskih radova i ime profesora Bokonjića sigurno je jedno od najviše pominjanih u zahvalnicama tih radova odbranjenih na fakultetima iz medicinskog naučnog polja u zemlji i regionu. Osim toga, bio je jedan od najomiljenijih nastavnika na fakultetu, koji je uvek dobijao najviše ocene od strane studenata.

Profesor Bokonjić je bio izuzetno cenjen među kolegama, zbog čega je i bio biran dva puta uzastopno, u periodu od 2009–2014, za šefa Katedre za farmakološke nauke Medicinskog fakulteta VMA.

Iz naučnih projekata kojima je rukovodio ili bio saradnik na njima proistekao je velik broj radova koji su objavljeni u najprestižnijim biomedicinskim časopisam širom sveta i koji su citirani više od 900 puta od strane drugih autora.

Bio je recenzent u više naučnih časopisa. Posebno je bilo dragoceno njegovo angažovanje kao recenzenta za oblast statistike u časopisu „Vojnosanitetski pregled“ (VSP), što je značajno doprinelo podizanju kvaliteta radova objavljenih u njemu, a time i kvaliteta samog časopisa što je rezultiralo njegovim uključenjem u svetski poznate baze naučne publicistike.

Koautor je u dva udžbenika: „Farmakološki priručnik sa recepturom“ (objavljen 2012, drugo izdanje 2015) za predmet Farmakologija na Medicinskom fakultetu VMA i „Odabrani metodi statističke analize za biomedicinska istraživanja“, objavljen 2018. godine i odobren kao udžbenik za doktorske studije na Medicinskom fakultetu u Banjaluci (Republika Srpska, BiH). Bio je i stručni redaktor 2. dopunjenog izdanja kapitalnog udžbenika iz dermatologije na našem jeziku (Đ. Karadaglić, urednik) koji je objavljen 2016. godine.

Profesor Bokonjić bio je aktivni član Srpskog lekarskog društva, Srpskog farmakološkog društva, Udruženja toksikologa Srbije, a od međunarodnih udruženja, Evropskog udruženja za farmakologiju, Internacionalne unije farmakoloških društava i Evropskog udruženja toksikologa.

Za svoj rad više puta je nagrađivan i pohvaljivan.

O profesoru Bokonjiću, našem Bokiju, kako smo ga mi, njegove kolege i saradnici od milošte zvali, mogle bi da se ispišu čitave stranice jer je bio čovek u pravom smislu te reči, koji je uvek imao razumevanja i davao nam podršku za sve naše, ne samo profesionalne probleme. U trajnom sećanju ostaće nam njegova dobrota, optimizam, disciplina i odgovornost. Zato je teško prihvatiti činjenicu da nije više među nama, tim pre što je do zadnjeg dana bio aktivan i što je još mnogo toga mogao da pruži u naučnoistraživačkom radu na ovim prostorima. Nedostajaće svima, a nadasve njegovoj porodici, supruzi Dragici, ćerki Dubravki i sinu Borisu.

Za sve što je učinio za razvoj nauke i naučnog podmlatka u Srbiji, neka mu je večna slava i hvala!

prof. dr Silva Dobrić

C O R R I G E N D U M

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I. In the Case report by ***Saša Vojinov, Mladen Popov, Ivan Levakov, Aleksandra Levakov Fejsa, Dimitrije Jeremić, Dragan Grbić***: **Adenocarcinoma of the prostate with small cell component and low levels of prostate specific antigen** (Adenokarcinom prostate sa mikrocelularnom komponentom i niskom vrednosti prostata specifičnog antigena). **Vojnosanit Pregl 2020; 77(10):1101-3. (https://doi.org/10.2298/VSP181212029V),**

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1. Vojinov S, Popov M, Lavakov I, Levakov Fejsa A, Jeremić D, Grbić D. **Adenocarcinoma of the prostate with small cell component and low levels of prostate specific antigen. Vojnosanit Pregl 2020; 77(10):1101-3. (https://doi.org/10.2298/VSP181212029V).**
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[1]Online First July, 2021.

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

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

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

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

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

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

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

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