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Every November 10, World Science Day (WSD) is celebrated, thus highlighting the importance of science in the everyday life of all the inhabitants of our planet. The theme of WSD 2022 is "Basic Sciences for Sustainable Development". Possessing capacities in the basic sciences is essential for progress in all areas of human life and work and in the interest of all countries worldwide. WSD is an opportunity to reflect once again on all the scientific discoveries that shaped the development of our civilization.

Svakog 10. novembra obeležava se Svetski dan nauke (SDN) kojim se ističe važnost nauke u svakodnevnom životu svih stanovnika naše planete. Tema SDN 2022. godine je „Osnovne nauke za održivi razvoj“. Posedovanje kapaciteta u osnovnim naukama je od vitalnog značaja za napredak u svim oblastima ljudskog života i rada i u interesu svih država sveta. SDN je prilika da se još jednom podsetimo svih naučnih otkrića koja su oblikovala razvoj naše civiliziacije.

ORIGINAL ARTICLES

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Pathogenic *TP53* mutations influence chemotherapy response and survival rate of patients with HPV-negative oral carcinomas

Uticaj patogenih *TP53* mutacija na preživljavanje i odgovor na hemioterapiju bolesnika sa HPV-negativnim oralnim karcinomima

Saša Jović*†, Ružica Kozomara*†, Srboljub Stošić*†, Stevo Jovandić‡, Katarina Zeljić§, Gordana Šupić†‡

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Abstract

Background/Aim. Oral squamous cell carcinoma (OSCC) is the most common tumor type of head and neck carcinomas, characterized by a high recurrence rate and patients' poor survival. Further elucidation of the function and regulation of the *TP53*, a pivotal tumor suppressor gene, would provide advances in predicting the clinical behavior, prognosis, and chemotherapy response of OSCC patients. Thus, we investigated the association of *TP53* gene mutations with survival and response to cisplatin chemotherapy in human papilloma virus (HPV)-negative OSCC patients. **Methods.** The potential clinical relevance of *TP53* mutations was analyzed in 82 patients with HPV-negative OSCC. All patients underwent radiotherapy, and 25 patients received cisplatin chemotherapy. A negative HPV status was determined by type-specific polymerase chain reaction (PCR) for high-risk HPV 16, 18, 31, and 33. Targeted sequencing of *TP53* exons 4–8 was assessed by Sanger sequencing. **Results.** Of 82 HPV-negative OSCC patients, 49 (59.79%) had *TP53* mutations, and 26 patients (31.7%) carried pathogenic *TP53* mutations. Patients with pathogenic *TP53* mutations had significantly reduced overall survival ($p = 0.009$). Recurrence status, but not *TP53* mutations, was an independent marker of poor survival in our cohort [hazard ratio (HR) = 4.733, 95% confidence interval (95% CI): 2.027–11.053, $p = 0.0001$]. In the subcohort of patients who underwent cisplatin-based chemotherapy, pathogenic *TP53* mutations were predictors of poor response to chemotherapy ($p = 0.026$). **Conclusion.** Our findings indicate that pathogenic *TP53* mutations in HPV-negative OSCC tumors could be a prognostic marker of patients' reduced overall survival. In addition, pathogenic *TP53* mutations in HPV-negative OSCC could be a marker of poor chemotherapy response of OSCC patients.

Key words:
carcinoma, squamous cell; drug therapy; genes, tumor suppressor; head and neck neoplasms; mutation; prognosis; radiotherapy; survival rate.

Apstrakt

Uvod/Cilj. Oralni planocelularni karcinom (OPCK) je najčešći tip karcinoma glave i vrata, koji se odlikuje visokom stopom recidiva i lošim preživljavanjem bolesnika. Dalje razjašnjenje uloge i regulacije *TP53*, ključnog tumor supresorskog gena, omogućilo bi napredak u predviđanju toka, prognoze i hemioterapijskog odgovora obolelih od OPCK. Zbog toga smo istražili povezanost mutacija gena *TP53* sa preživljavanjem i odgovorom na hemioterapiju cisplatinom bolesnika sa HPV-negativnim OPCK. **Metode.** Potencijalni klinički značaj mutacija *TP53* analiziran je kod 82 bolesnika sa HPV-negativnim OPCK. Svi bolesnici su bili podvrgnuti radioterapiji, a 25 bolesnika primilo je hemioterapiju cisplatinom. Negativni HPV status utvrđen je tip-specifičnom metodom *polymerase chain reaction* (PCR), za visoko rizične HPV 16, 18, 31 i 33. Ciljno sekvenciranje *TP53* egzona 4–8 rađeno je Sanger kapilarnim sekvenciranjem. **Rezultati.** Od 82 HPV-negativnih OPCK bolesnika, njih 49 (59,79%) imalo je *TP53* mutaciju, a 26 (31,7%) bolesnika je imalo patogene *TP53* mutacije. Bolesnici sa patogenim mutacijama *TP53* imali su značajno smanjeno celokupno preživljavanje ($p = 0,009$). Status recidiva, ali ne i *TP53* mutacije, bio je nezavisni marker lošeg preživljavanja bolesnika u našoj studiji [*hazard ratio* (HR) = 4,733, 95% *confidence interval* (95% CI): 2,027–11,053; $p = 0,0001$]. U

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podgrupi bolesnika koji su bili podvrgnuti hemioterapiji cisplatinom, patogene *TP53* mutacije bile su prediktori slabog odgovora na hemioterapiju (p = 0,026). **Zaključak.** Naši nalazi ukazuju da bi patogene *TP53* mutacije u HPV-negativnim OPCK tumorima mogle biti prognostički marker smanjenog ukupnog preživljavanja bolesnika. Pored toga, patogene *TP53* mutacije u HPV-negativnom OPCK mogu biti marker lošeg odgovora tih bolesnika na hemioterapiju.

Ključne reči:
karcinom, planocelularni; lečenje lekovima; geni, tumor-supresori; glava i vrat, neoplazme; mutacija; prognoza; radioterapija; preživljavanje, stepen.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common tumor type of head and neck carcinomas, characterized by a high recurrence rate and patients'poor survival. This malignancy is the sixth most common cancer worldwide in men and eighth in women in developed countries, while in developing countries, it is the third most common cancer in men and fourth in women, which affects approximately 600,000 new patients every year worldwide [1]. Oral carcinogenesis is a multi-step process that encompasses an accumulation of genetic and epigenetic changes which lead to the disruption of the various signaling pathways controlling the cell cycle, proliferation, apoptosis, senescence, and DNA [2]. Genetic changes are progressively accumulated, and the inactivation of tumor suppressor genes by point mutations, deletions, and gene rearrangement is one of the key changes for malignant transformation. Known etiological factors for developing OSCC are predominantly smoking, alcohol intake, and poor oral hygiene. Approximately 20–30% of OSCC cases can be associated with tobacco smoking and 7–19% with heavy alcohol drinking, which increases the risk of oral cavity cancer 2–3 times [3]. One of the most important advances in oral carcinogenesis in recent decades is the evidence of an association between oral cancer and some types of human papillomavirus (HPV) infection, predominantly HPV16 [4].

Gene coding for protein p53 (*TP53*) is one of the most prominent tumor suppressor genes located on the short arm of chromosome 17 (17p13.1) [5]. Protein p53 is a key factor in a signaling pathway that helps the cell to recover from DNA damage [6]. Upon DNA damage, the wild type (WT) p53 arrests the cell cycle in the G1 phase prohibiting transition to the S phase until the damage is repaired. Additionally, throughout the retinoblastoma tumor suppressor pathway, p53 can direct cells to a state of permanent cell cycle arrest or induce pro-apoptotic genes cellular senescence [7]. *TP53* is one of the most frequently mutated human genes in more than 50% of cancers. Germline *TP53* mutations cause Li-Fraumeni syndrome, a rare autosomal, hereditary disorder predisposing to sarcoma, breast cancer, leukemia, and adrenal gland carcinoma [8].

TP53 mutations are early alterations during oral carcinogenesis, and more than 25,000 mutations have been discovered so far [9]. Most of them (70%) are missense mutations in the coding regions [10], where approximately 30% of mutations occur in exon 7 and exon 8, known as mutation hot spots. These exons code for the DNA binding domain, preventing the p53 binding to the promoter of target genes [11]. Common *TP53* codon 72 gene polymorphism in this domain produces two functional variants of p53 − p53P (proline) and p53A (arginine), which could reduce its ability to mediate apoptosis and cell cycle arrest and could, therefore, affect the survival and chemotherapy response [12]. A number of studies reported inconsistent findings regarding whether the *TP53* mutations and codon 72 polymorphism influence survival and chemotherapy response in OSCC patients [13].

Inactivation of WT p53 can also be achieved throughout HPV E6 protein [11]. HPV-positive oropharyngeal cancer cells have a different molecular profile from HPV-negative oropharyngeal cancer cells, where HPV-negative oropharyngeal cancers have a more frequent loss of heterozygosity of 3p, 9p, or 17p chromosomal regions [14]. HPV-negative oropharyngeal cancers have at least two times more mutations compared to HPV-positive tumors [15, 16], and worse outcome [17], indicating the necessity of molecular characterization of p53 in HPV-negative OSCC.

Further elucidation of the function and regulation of p53 in HPV-negative OSCC would provide advances in predicting the clinical behavior, prognosis, and patients' chemotherapy response. Finding the potential markers that could predict tumor response to chemotherapy, developing new strategies with therapeutics targeting different pathways that will override the resistance, and tumor molecular profiling would provide an individualized approach to the treatment modalities of OSCC patients.

Methods

The current study was approved by the Ethics Committee of the Military Medical Academy (No 162/2019, from December 26, 2019), according to the Helsinki Declaration (1964). The study group included 82 OSCC patients, Caucasians of the same ethnicity. All patients were diagnosed and subsequently operated on at the Clinic for Maxillofacial Surgery, Military Medical Academy, Belgrade, Serbia, between 2012 and 2020. All of them were operated on and received radiotherapy (60 Gy in 2 Gy dose per day), and 25 of the patients received cisplatin therapy in a dosage of 100 mg/m^2 of body surface area in one-week cycles. The face-to-face interviews were conducted to obtain demographic data. The evaluation of lymph node status and the tumor, node, metastasis (TMN) classification were determined by an experienced pathologist in accordance with the classification of the American Head and Neck Society and American Joint Committee on Cancer (AJCC Cancer Staging Manual 8th Edition, 2018). Of 82 OSCC patients, 35 (42.7%) were under 58 years of age, 58 (70.7%) were male,

57 (69.5%) had a history of alcohol abuse, 19 (23.2%) had stage II OSCC while 63 (76.8%) had advanced-stage tumors.

DNA isolation and HPV analysis

OSCC tissue samples were stored at -20 ˚C until DNA extraction. Genomic DNA was isolated by the TRI Reagent® (Sigma-Aldrich, USA) according to the manufacturer's protocol. DNA samples were stored at -20 ˚C until further analysis. Type-specific polymerase chain reaction (PCR) was assessed for high-risk HPV types 16, 18, 31, and 33.

TP53 Sanger sequencing

Targeted sequencing of p53 exons 4-8 was assessed by Sanger sequencing on ABI 3130 automated sequencer (Applied Biosystems, USA). The primers flanking exons 4-8 were retrieved from the IARC *TP53* database. PCR reactions were performed using the Platinum Taq DNA Polymerase PCR kit (Life Technologies). Amplicons were sequenced using the BigDye terminator cycle sequencing kit. Sequencing traces were analyzed with GeneScreen (http://dna.leeds.ac.uk/genescreen/) followed by visual inspection, with reference to the human genome reference sequence build hg19/GRCh37 (http://genome.ucsc.edu).

TP53 mutation classification according to its clinical significance

To provide information on pathogenic *TP53* mutations, genetic variants with clinical significance, we assessed the ClinVar database of the NCBI (National Center for Biotechnology Information) and a web server application Simple ClinVar [18].

TP53 mutations were classified as pathogenic and non-pathogenic mutations according to the ClinVar database, Simple ClinVar [18], and previous studies on head and neck carcinoma [19, 20]. Missense, stop-gain, in-frame insertions/deletions, frameshift, and splice site *TP53* mutations with pathogenic and likely pathogenic significance, and criteria provided by multiple or single submitters, reviewed by expert panels or given in practice guidelines, were classified as pathogenic mutations. On the other hand, likely benign, protective, or with uncertain significance were classified as non-pathogenic mutations.

Statistical analysis

Obtained data were analyzed by SPSS 20.0 software (IBM Inc., Chicago, IL, USA). Contingency tables were assessed by χ^2-test or Fisher's exact test. Overall survival was calculated from the date of diagnosis until death from any cause. Kaplan-Meier survival curves were compared using the log-rank test. Cox proportional hazard regression analysis was performed to estimate the hazard ratios (HR) with a 95% confidence interval (95% CI). Variables found significant in the univariate analysis, including those with a significance level below 20%, were subsequently analyzed in multivariate Cox's regression. The Cox model was performed using the forward stepwise method, which removed variables with $p < 0.1$. The associations were considered as significant when *p*-values were less than 0.05.

Results

Association of p53 gene mutations, pathogenic p53 mutations, and polymorphism p72 with demographic and clinicopathological features of HPV-negative OSCC patients

Eighty-two HPV-negative OSCC samples were screened for *TP53* mutations in exons 4-8, and mutations were found in a total of 49 (59.8%) patients. *TP53* mutations were classified as pathogenic and non-pathogenic, as previously suggested [19, 20]. Pathogenic *TP53* mutations were detected in 26 of 82 (31.7%) OSCC patients.

The association of *TP53* gene mutations, pathogenic *TP53* mutations, and polymorphism p72 with demographic and clinicopathological features of OSCC patients are presented in Table 1. No association was found between *TP53* mutations or pathogenic *TP53* mutations and sex or smoking. Pathogenic *TP53* mutations were significantly associated with age ($p = 0.005$) and high alcohol intake ($p = 0.009$). Locally advanced tumors did not have a statistically higher *TP53* mutation rate or pathogenic *TP53* mutations compared to early-stage OSCC. Mutations in exon 4 of the p53 gene were significantly associated with histological and nuclear grade ($p = 0.012$ and $p = 0.032$, respectively), while mutations in exon 7 were associated with smoking status ($p = 0.017$).

Association of TP53 gene mutations, pathogenic TP53 mutations, and polymorphism p72 with overall survival of OSCC patients

Overall survival (OS) curves were assessed by the Kaplan-Meier analysis and compared by the log-rank test**.** HPV-negative OSCC patients with mutated *TP53* tended to have worse survival ($p = 0.085$) as opposed to patients without *TP53* mutations. However, OSCC patients with pathogenic mutations in *TP53* had significantly reduced OS ($p = 0.009$, Figure 1). Non-pathogenic *TP53* mutations were not associated with OS of OSCC patients ($p = 0.785$, log-rank test). No significant difference was observed in OS between OSCC patients with different genotypes of p72 polymorphism ($p = 0.905$, log-rank test).

In the subgroup of 25 patients who received chemotherapy in our cohort, when all *TP53* mutations were taken into account, *TP53* mutation status was not associated with chemotherapy response ($p = 0.641$, Figure 2A). However, OS in patients who had received cisplatin chemotherapy was significantly shorter for those with pathogenic p53 mutations compared to patients with WT *P53* ($p = 0.026$, Figure 2B). Non-pathogenic *TP53* mutations in patients who had received cisplatin chemotherapy were not related to OS in our cohort

Table 1

Association of p53 gene mutations and polymorphism p72 with demographic and clinicopathological features of OSCC patients

Variables	Total (n)	All *TP53* Mutations		E4		E5		E6		E7		E8		p72 rs1042522			Pathogenic *TP53* mutations	
		+	-	+	-	+	-	+	-	+	-	+	-	WT	het	mut	+	-
Sex																		
male	58	35	23	30	28	6	52	16	42	2	56	16	42	35	22	1	16	42
female	24	14	10	13	11	1	23	10	14	0	24	4	20	11	11	2	10	14
p		0.866		0.840		0.362		0.357		0.295		0.479			0.231		0.231	
Age (years), median																		
< 58	35	23	12	17	18	4	31	17	18	1	34	9	26	17	15	3	17	18
≥ 58	47	21	26	26	21	3	44	9	38	1	46	11	36	29	18	0	9	36
p		0.342		0.545		0.419		0.832		0.810		0.563			0.093		**0.005**	
Smoking																		
never	25	15	10	15	10	1	24	7	18	0	25	7	18	13	12	0	7	18
ever	57	34	23	28	29	6	51	19	38	2	55	13	44	25	32	0	19	38
p		0.976		0.364		0.330		0.343		0.614		**0.017**			0.375		0.798	
High alcohol intake																		
no	57	33	24	30	27	5	52	13	44	1	56	14	43	33	23	1	13	44
yes	25	16	9	13	12	2	23	13	12	1	24	6	19	13	10	2	13	12
p		0.604		0.958		0.908		0.544		0.957		0.419			0.375		**0.009**	
Histological grade																		
1	61	34	27	27	34	6	55	16	45	1	60	15	46	39	19	3	16	45
2/3	21	15	6	16	5	1	20	10	11	1	20	5	16	7	14	0	10	11
p		0.206		**0.012**		0.473		0.424		0.943		0.342			**0.014**		0.069	
Nucleus grade																		
1	58	32	26	26	32	6	52	17	41	1	57	15	43	36	19	3	17	41
2/3	24	17	7	17	7	1	23	9	15	1	23	5	19	10	14	0	9	15
p		0.188		**0.032**		0.362		0.514		0.629		0.220			0.072		0.468	
Nodal status																		
-	19	14	5	10	9	2	17	7	12	0	19	5	14	9	10	0	7	12
+	63	35	28	29	34	5	58	19	44	2	61	15	48	37	23	3	19	44
p		0.158		0.614		0.723		0.432		0.824		0.865			0.336		0.583	
Tumor size																		
T1/2	60	37	23	29	31	6	54	21	39	2	58	15	45	31	26	3	21	39
T3/4	22	12	10	14	8	1	21	5	17	0	22	5	17	19	7	0	5	17
p		0.560		0.219		0.434		0.386		0.832		0.137			0.299		0.290	
Tumor stage																		
II	19	13	6	8	11	3	16	5	14	1	18	4	15	8	10	1	5	14
III	63	36	27	35	28	4	59	21	42	1	62	16	47	38	23	2	21	42
p		0.380		0.303		0.197		0.363		0.699		0.387			0.372		0.564	

OSCC – oral squamous cell carcinoma; n – total number of patients; E – exon; WT – wild type; het – heterozygosity; mut – mutation.
Statistically significant values are bolded.

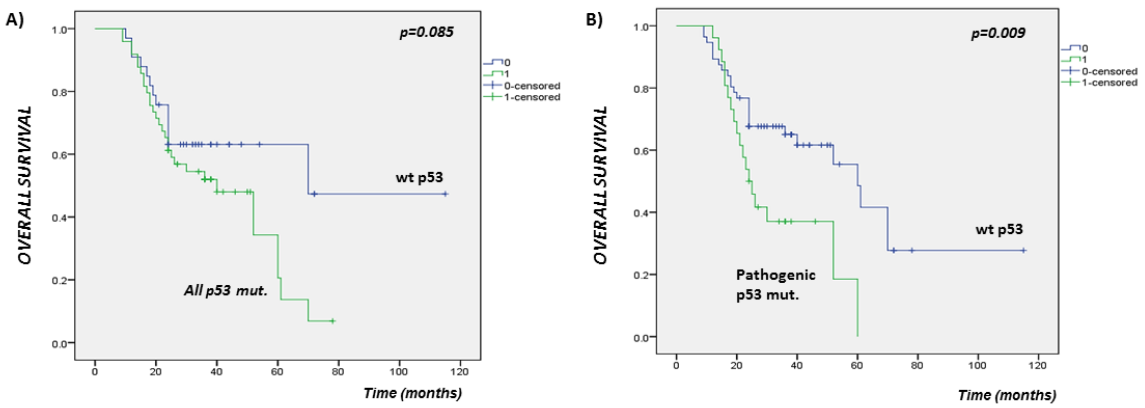

Fig. 1 – Kaplan-Meier curves for overall survival based on the *TP53* mutation status in a total cohort of 82 HPV-negative OSCC patients: A) Survival comparison between all *TP53* mutations (mut.) and wild type (WT) *TP53*; B) Comparison of pathogenic *TP53* mutations and WT *TP53*.
OSCC – oral squamous cell carcinoma.

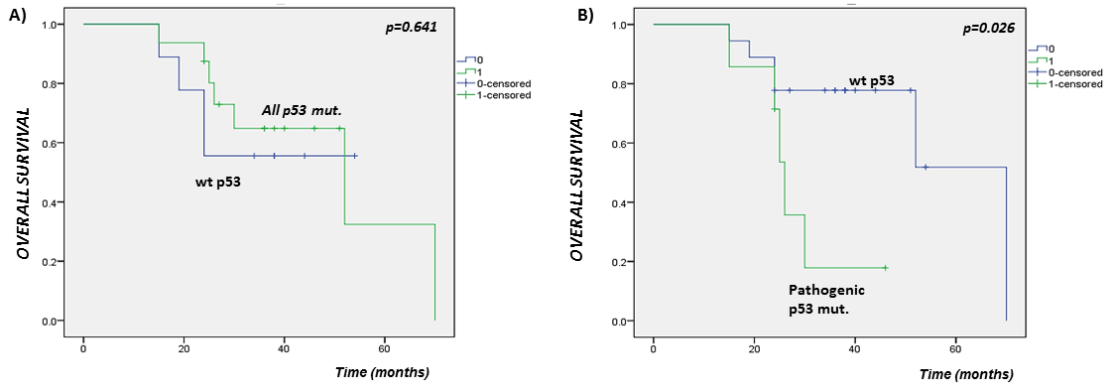

Fig. 2 – Associations between *TP53* mutations and survival outcome of the subgroup of 25 OSCC patients who received platinum-based chemotherapy: A) Survival comparison between all *TP53* mutations (mut.) and wild type (WT) *TP53*; B) Comparison of pathogenic *TP53* mutations and WT *TP53*.
OSCC – oral squamous cell carcinoma.

(p = 0.453, log-rank test). These findings indicate that the response to chemotherapy was associated with the type of p53 mutation and that the patients with pathogenic *TP53* mutations were resistant to platinum-based chemotherapy, as opposed to the patients with WT *TP53*.

The Cox regression analysis demonstrated that high alcohol intake, stage, tumor size, nodal status, and recurrences are highly associated with hazard risk (Table 2). While patients with *TP53* mutations had increased but insignificant hazard risk [HR=1.747, 95% CI (0.907–3.366), p = 0.096], patients with pathogenic *TP53* mutations had significantly increased risk of poor survival [HR = 2.230, 95% CI (1.186–4.194), p = 0.013]. Variables found to be statistically significant, according to the univariate analysis, including the variables with a significance level below 20%, were subsequently analyzed in multivariate Cox hazards regression analysis. The multivariate analysis revealed that the recurrences persisted as an independent prognostic factor in our cohort [HR = 4.733, 95% CI (2.027–11.053), p = 0.0001] (Table 2). The list of detected *TP53* mutations and their classification according to clinical significance, assessed by the ClinVar database and Simple ClinVar web server, is given in Table 3.

Table 2
Cox proportional hazards regression analysis, according to overall survival of OSCC patients

Cox regression analysis	Demographic or pathological features	Overall survival	
		HR (95% CI)	*p*
Univariate analysis	Sex	0.660 (0.335–1.300)	0.230
	Age	0.605 (0.332–1.102)	0.100
	Smoking	1.682 (0.804–3.519)	0.167
	High alcohol intake	2.938 (1.610–5.360)	**0.0001**
	Nuclear grade	1.245 (0.900–1.721)	0.186
	Histological grade	1.270 (0.914–1.764)	0.155
	Stage	3.898 (1.388–10.947)	**0.010**
	Tumor size	1.654 (1.189–2.302)	**0.003**
	Nodal status	3.055 (1.199–7.786)	**0.019**
	Recurrences	4.727 (2.108–10.597)	**0.0001**
	All *TP53* mutations	1.747 (0.907–3.366)	0.096
	Pathogenic *TP53* mutations	2.230 (1.186–4.194)	**0.013**
	p72 SNP	1.065 (0.645–1.759)	0.806
Multivariate analysis	Recurrences	4.733 (2.027–11.053)	**0.0001**

OSCC – oral squamous cell carcinoma; HR – hazard ratio; CI – confidence interval; SNP – single nucleotide polymorphism.
Statistically significant values are bolded.

Table 3

Data on *TP53* mutations, assessed by the ClinVar database of the NCBI and a web server application Simple ClinVar[18]

TP53 mutation name	Clinical significance (Last reviewed)	Condition(s)
NM_000546.5(TP53):c.215= (p.Pro72=)	Benign	Not specified
NM_000546.5(TP53):c.273G>A (p.Trp91Ter)	Pathogenic	Hereditary cancer-predisposing syndrome; Neoplasm of the ovary; Li-Fraumeni syndrome; Li-Fraumeni syndrome 1
NM_001126114.2(TP53):c.560-11_560-8dup	Likely benign	Li-Fraumeni syndrome
NM_000546.5(TP53):c.820_821delGT (p.Val274Leufs)	Pathogenic	Li-Fraumeni syndrome
NM_000546.5(TP53):c.896_909del (p.Leu299Hisfs)	Pathogenic	Not provided
NM_000546.5(TP53):c.273G>A (p.Trp91Ter)	Pathogenic	Li-Fraumeni syndrome; Not provided; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.380C>G (p.Ser127Cys)	Uncertain significance	Li-Fraumeni syndrome
NM_000546.5(TP53):c.384T>C (p.Pro128=)	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.389T>C (p.Leu130Pro)	Likely pathogenic	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.401T>G (p.Phe134Cys)	Likely pathogenic	Not provided; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.411G>C (p.Leu137=)	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.421T>A (p.Cys141Ser)	Likely pathogenic	Multiple myeloma; Squamous cell carcinoma of the head and neck; Lung adenocarcinoma; Squamous cell lung carcinoma; Acute myeloid leukemia; Renal cell carcinoma, papillary; Neoplasm of the brain; Neoplasm of the breast; Pancreatic adenocarcinoma; Neoplasm of the large intestine; Colorectal neoplasms; Malignant neoplasm of body of uterus; Adenocarcinoma of the prostate
NM_000546.5(TP53):c.428T>G (p.Val143Gly)	Uncertain significance	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.517G>T (p.Val173Leu)	Pathogenic	Liver cancer; Malignant melanoma of skin; Squamous cell carcinoma of the head and neck; Small cell lung cancer; Lung adenocarcinoma; Li-Fraumeni syndrome; Neoplasm of the brain; Neoplasm of the breast; Hepatocellular carcinoma; Pancreatic adenocarcinoma; Brainstem glioma; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal neoplasms; Adrenocortical carcinoma; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Malignant neoplasm of body of the uterus
NM_000546.5(TP53):c.560-15A>C	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.578A>G (p.His193Arg)	Likely pathogenic	Liver cancer; Chronic lymphocytic leukemia; Squamous cell carcinoma of the head and neck; Small cell lung cancer; Lung adenocarcinoma; Li-Fraumeni syndrome; Squamous cell lung carcinoma; Acute myeloid leukemia; Not provided; Neoplasm of the brain, Neoplasm of the breast; Hepatocellular carcinoma; Hereditary cancer-predisposing syndrome; Pancreatic adenocarcinoma; Transitional cell carcinoma of the bladder; Brainstem glioma; Neoplasm of the large intestine, Carcinoma of the esophagus; Colorectal neoplasms; Papillary renal cell carcinoma, sporadic; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Malignant neoplasm of body of uterus; Adenocarcinoma of the prostate; Uterine carcinosarcoma
NM_000546.5(TP53):c.591G>A (p.Val197=)	Uncertain significance	Li-Fraumeni syndrome
NM_000546.5(TP53):c.592delG (p.Glu198Lysfs)	Pathogenic	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.599delA (p.Asn200Ilefs)	Pathogenic	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.642T>C (p.His214=)	Likely benign	Li-Fraumeni syndrome
NM_000546.5(TP53):c.678C>A (p.Gly226=)	Likely benign	Not specified
NM_000546.5(TP53):c.698A>G (p.His233Arg)	Uncertain significance	Not specified; Hereditary cancer-predisposing syndrome

Table 3 (continued)

NM_000546.5(TP53):c.700T>C (p.Tyr234His)	Pathogenic	Liver cancer; Squamous cell carcinoma of the head and neck; Small cell lung cancer; Li-Fraumeni syndrome; Squamous cell lung carcinoma; Neoplasm of the breast, Glioblastoma; Hepatocellular carcinoma; Hereditary cancer-predisposing syndrome; Pancreatic adenocarcinoma; Transitional cell carcinoma of the bladder; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal neoplasms; Adrenocortical carcinoma; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Adenocarcinoma of the prostate
NM_001126113.2(TP53):c.710T>A (p.Met237Lys)	Likely pathogenic	Squamous cell carcinoma of the head and neck; Lung adenocarcinoma; Squamous cell lung carcinoma; Neoplasm of the brain; Neoplasm of the breast; Pancreatic adenocarcinoma; Brainstem glioma; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal neoplasms; Adenocarcinoma of the stomach, Ovarian serous cystadenocarcinoma; Malignant neoplasm of body of the uterus
NM_000546.5(TP53):c.712T>C (p.Cys238Arg)	Pathogenic	Liver cancer; Chronic lymphocytic leukemia; Multiple myeloma; Squamous cell carcinoma of the head and neck; Lung adenocarcinoma; Neoplasm of the brain; Neoplasm of the breast; Glioblastoma; Hepatocellular carcinoma; Hereditary cancer-predisposing syndrome; Pancreatic adenocarcinoma; Transitional cell carcinoma of the bladder; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal Neoplasms; Uterine cervical neoplasms; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Malignant neoplasm of body of uterus; Uterine carcinosarcoma
NM_000546.5(TP53):c.718A>G (p.Ser240Gly)	Likely pathogenic	Li-Fraumeni syndrome
NM_000546.5(TP53):c.724T>C (p.Cys242Arg)	Likely pathogenic	Not provided
NM_000546.5(TP53):c.727A>C (p.Met243Leu)	Uncertain significance	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.728T>C (p.Met243Thr)	Conflicting interpretations of pathogenicity	Li-Fraumeni syndrome; Not specified; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.730G>A (p.Gly244Ser)	Pathogenic/Likely pathogenic	Liver cancer; Squamous cell carcinoma of the head and neck; Small cell lung cancer; Lung adenocarcinoma; Li-Fraumeni syndrome; Squamous cell lung carcinoma; Neoplasm of the brain; Glioblastoma; Hepatocellular carcinoma; Hereditary cancer-predisposing syndrome; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal Neoplasms; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma, Malignant neoplasm of body of uterus; Uterine carcinosarcoma
NM_000546.5(TP53):c.734G>C (p.Gly245Ala)	Likely pathogenic	Liver cancer; Squamous cell carcinoma of the head and neck; Lung adenocarcinoma; Squamous cell lung carcinoma; Not provided; Neoplasm of the brain; Neoplasm of the breast; Glioblastoma; Hepatocellular carcinoma; Pancreatic adenocarcinoma; Transitional cell carcinoma of the bladder; Brainstem glioma; Neoplasm of the large intestine; Carcinoma of the esophagus; Colorectal neoplasms; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Adenocarcinoma of the prostate; Uterine carcinosarcoma
NM_000546.5(TP53):c.737T>G (p.Met246Arg)	Pathogenic	Not provided
NM_000546.5(TP53):c.770T>A (p.Leu257Gln)	Uncertain significance	Li-Fraumeni-like syndrome; Li-Fraumeni syndrome
NM_000546.5(TP53):c.782+12C>T	Benign/Likely benign	Li-Fraumeni syndrome 1; Not specified; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.782+14T>G	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.783-1G>A	Pathogenic/Likely pathogenic	Li-Fraumeni syndrome; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.785delG (p.Gly262Valfs)	Pathogenic	Not provided; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.789T>C (p.Asn263=)	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.794T>C (p.Leu265Pro)	Pathogenic/Likely pathogenic	Li-Fraumeni syndrome 1; Li-Fraumeni syndrome; Not provided; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.401T>G (p.Phe134Cys)	Likely pathogenic	Hereditary cancer-predisposing syndrome; Not provided
NM_000546.5(TP53):c.798A>T (p.Gly266=)	Likely benign	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.814G>T (p.Val272Leu)	Likely pathogenic	Medulloblastoma; Multiple myeloma; Squamous cell carcinoma of the head and neck; Li-Fraumeni syndrome; Lung adenocarcinoma; Renal cell carcinoma; papillary; Neoplasm of the breast; Hereditary cancer-predisposing syndrome; Pancreatic adenocarcinoma; Squamous cell carcinoma of the skin; Transitional cell carcinoma of the bladder; Neoplasm of the large intestine; Colorectal neoplasms; Adenocarcinoma of the stomach; Ovarian serous cystadenocarcinoma; Malignant neoplasm of body of the uterus
NM_000546.5(TP53):c.829T>C (p.Cys277Arg)	Likely pathogenic	Not provided
NM_000546.5(TP53):c.869G>A (p.Arg290His)	Uncertain significance	Li-Fraumeni syndrome; Li-Fraumeni syndrome not specified; Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.890A>G (p.His297Arg)	Uncertain significance	Hereditary cancer-predisposing syndrome
NM_000546.5(TP53):c.904G>C (p.Gly302Arg)	Uncertain significance	Li-Fraumeni syndrome
NM_000546.5(TP53):c.907A>G (p.Ser303Gly)	Uncertain significance	Li-Fraumeni syndrome; Hereditary cancer-predisposing syndrome

Discussion

As already stated, OSCC is the most common tumor type of head and neck carcinomas, characterized by a high recurrence rate and poor survival of those patients. While oropharyngeal carcinomas are predominantly HPV-positive, basal-type oral cancers are mostly HPV-negative [21]. HPV-negative oral cancer patients have a significantly reduced OS [17], as opposed to patients with HPV-positive cancer [22]. Further elucidation of the function and regulation of the *TP53*, a pivotal tumor suppressor gene, would provide advances in predicting the clinical behavior, prognosis, and chemotherapy response of HPV-negative oral cancers.

Our findings indicate that HPV-negative OSCC patients with pathogenic *TP53* mutations had a significantly lower survival rate. In the subcohort of patients who underwent cisplatin-based chemotherapy, OS was significantly shorter for those with pathogenic *TP53* mutations than those with WT *TP53*. In contrast, when all *TP53* mutations were taken into account, *TP53* mutation status was not associated with OS. These findings indicate that the OS and the resistance to platinum-based chemotherapy in OSCC could be associated with the type of *TP53* mutation and that pathogenic *TP53* mutations are a significant predictor of poor OS as opposed to benign or likely-benign mutations.

Based on the p53 mechanism of action, as one of the key cell cycle regulators after DNA damage, a number of trials investigate the association between *TP53* mutation and survival, as well as radio and chemotherapy response. Our findings of the high incidence of *TP53* mutations in HPV-negative OSCC are in accordance with previous studies, where *TP53* is mutated in approximately 50% of head and neck squamous cell carcinoma (HNSCC) cases [1]. Mutations in the DNA-binding domain of *TP53* may influence individual responsiveness to chemotherapy via its ability to mediate apoptosis and cell cycle arrest [12]. The most frequent genetic change in our study was *TP53* codon 72 polymorphism, but it was not associated with prognosis or chemotherapy response.

Multiple studies are demonstrating a divergent prognosis based on HPV status in OSCC patients. HPV-negative OSCCs have diverse pathological and clinical features compared to HPV-positive tumors [23]. HPV-negative oral cancers are poorly differentiated tumors, and these patients had worse rates of OS compared to the HPV-positive cancers [24]. HPV-positive HNSCCs are commonly associated with a favorable prognosis in a number of studies [24–27]. HPV-positive head and neck tumors are predominantly driven by HPV infection, as opposed to HPV-negative tumors, which are driven by genetic mutations in *TP53* or other tumor suppressor genes, and are, therefore, characterized as tumors with poorer prognosis [26]. The key transcriptional factors that differentiate HPV-positive and HPV-negative oral cancers are p53, AP-1, NF-kappaB, and STAT3 [23]. In HPV-positive HNSCC, p53 protein is generally WT, but its low levels are attributed to the HPV E6 protein activity, which targets p53 and induces its ubiquitination and degradation [28]. This feature of HPV-positive tumors could lead to greater sensitivity to radiotherapy and radiation-induced apoptosis [29, 30]. Clinical studies have demonstrated that patients with HPV-negative tumors have decreased survival as opposed to HPV-positive OSCC patients [31].

In line with our results, *TP53* mutation status associates with resistance to chemotherapy in HNSCC patients [32–34]. The loss of function due to *TP53* mutation was associated with a low remission rate and suboptimal response to cisplatin-based neoadjuvant chemotherapy in patients with OSCC [34]. Although HPV infection is not a predictor for surgery or the response to radiotherapy of oropharyngeal cancers [35], cisplatin, a standard chemotherapy regimen in head and neck cancers, is more effective in HPV-negative cells [36]. Results of the TAX 324 (WU) trial for locally-advanced oropharyngeal cancer suggested that high-risk OSCC patients are HPV-negative and show elevated expression of βT- II or at least 2 out of 3 of the other adverse markers: GST-π, p53, and low Bcl-2. These patients have significantly decreased survival time compared to moderate-risk HPV-positive patients, who are HPV-negative but do not fulfill other criteria [37]. The commonly recommended treatment regimen for postoperative high-risk OSCC includes the administration of cisplatin in a dosage of 100 mg/m^2. Cisplatin induces DNA damage and those cells should be directed to apoptosis, and p53 proapoptotic pathway is carried out through flice-like inhibitory protein (FLIP), direct binding, and inhibition of the antiapoptotic function of Bcl–xL, enhanced expression of PTEN and AMPK inhibition [38].

Mutated *TP53* was previously associated with shorter OS and poor radio and chemotherapy response, which indicates its potential as a marker for a clinical course in OSCC patients. In the study of locally advanced oral cancer patients, who received cisplatin chemotherapy, patients carrying the high-risk *TP53* mutations had reduced cisplatin sensitivity and a ten times greater risk for residual disease compared to patients with low-risk mutations [39].

Lower response to cisplatin-based chemotherapy in patients with *TP53*-mutated tumors [32] suggested the potential clinical use of p53-based therapeutics in restoring the p53 function. As a result of p53 adenoviral monotherapy or the combination with radio and chemotherapy, tumor regression was observed [40]. OSCC patients carrying *TP53* mutations had a 2.7 times higher risk for cisplatin and 5-fluorouracil (5-FU)-based therapy resistance compared to patients with functional p53 [33]. In addition, a strong connection between nonfunctional p53 and a low response rate to cisplatin-based neoadjuvant chemotherapy was demonstrated in OSCC patients [34]. Another potentially promising approach is treatment with small molecules that reactivate mutated p53, using PRIMA-1 (p53 Reactivation and Induction of Massive Apoptosis) as a single agent and in combination with standard chemotherapy [41]. PRIMA-1 therapy is more active in cell lines containing mutant p53 than WT p53 cells and results in the increased expression of p53-target genes *p21, Bax, Puma,* and *Noxa* [41]. Another p53 reactivating molecule RITA (Reactivation of the p53 and Induction of Tumor cell Apoptosis) induces p53 accumulation and reactivation,

promotes apoptosis via p21, BAX, and caspase-3 upregulation, and induces growth inhibition in OSCC cells *in vitro* and *in vivo* [42].

Conclusion

Our findings indicate that pathogenic *TP53* mutations in HPV-negative OSCC tumors could be a prognostic marker of patients' reduced OS. In addition, HPV-negative OSCC patients with the pathogenic *TP53* mutation who received cisplatin chemotherapy have a significantly lower survival rate, indicating that the pathogenic *TP53* mutations might be a marker of chemotherapy resistance in those patients. Further elucidation of the function and regulation of *TP53* and novel therapeutic approach with small molecules that reactivate mutated *TP53* would significantly advance oral cancer therapy.

Conflict of interest

The authors declare no conflict of interest.

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Heart transplant rejection pathology

Patologija odbacivanja transplantata srca

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Abstract

Background/Aim. Heart transplantation is the most effective way to treat patients in the terminal stage of heart failure. Endomyocardial biopsy has proven to be a safe and appropriate technique, with little sampling error, and remains, to this day, one of the most commonly used methods for diagnosing acute rejection. In 1990, the International Society of Heart and Lung Transplantation defined a standardized system for grading the severity of acute transplant rejection regarding endomyocardial sampling histopathological analysis. The aim of the study was to assess the morphological, immunohistochemical, and immunofluorescent markers of cell- and antibody-mediated rejection of heart transplants in patients monitored during 2020. **Methods.** From 31 patients transplanted at the Clinic for Cardiac Surgery of the University Clinical Center of Serbia, endomyocardial biopsy material was obtained, then processed and analyzed at the Institute of Pathology of the Faculty of Medicine, University of Belgrade. **Results.** The average Transplant Rejection Score (TRS) value was 0.42. The Spearman's correlation test did not show a statistically significant relationship between the TRS value and the difference between the ejection fraction values three and twelve months after transplantation. **Conclusion.** The mean TRS value obtained in this study suggests dominant cell-mediated graft rejection.

Key words:
biopsy; heart; heart function tests; heart transplantation; histological techniques; immunohistochemistry; organ dysfunction scores.

Apstrakt

Uvod/Cilj. Transplantacija srca predstavlja najefikasniji način lečenja bolesnika u terminalnom stadijumu srčane insuficijencije. Endomiokardna biopsija se pokazala kao bezbedna i prikladna tehnika, sa malom greškom pri uzorkovanju i do danas ostaje jedna od najčešće korišćenih metoda za dijagnostiku akutnog odbacivanja transplantata. Internacionalno društvo za transplantaciju srca i pluća je 1990. godine definisalo standardizovani sistem za gradiranje težine akutnog odbacivanja transplantata korišćenjem patohistološke analize uzoraka endomiokarda. Cilj rada bio je da se analiziraju morfološki, imunohistohemijski i imunofluorescentni pokazatelji ćelijama- i antitelima-posredovanog odbacivanja transplantata srca kod bolesnika praćenih tokom 2020. godine. **Metode.** Od 31 bolesnika lečenih na Klinici za kardiohirurgiju Univerzitetskog kliničkog centra Srbije uzet je uzorak endomiokardne biopsije, a zatim obrađen i analiziran na Institutu za patologiju Medicinskog fakulteta Univerziteta u Beogradu. **Rezultati.** Prosečna vrednost stepena odbacivanja transplantata (*Transplant Rejection Score* – TRS) iznosila je 0,42. Spirmanovim testom korelacije nije pokazana statistički značajna veza između vrednosti TRS i razlike vrednosti ejekcione frakcije, 3 i 12 meseci posle transplantacije. **Zaključak.** Prosečna vrednost TRS, dobijena u ovom istraživanju, upućuje na dominantno ćelijama-posredovano odbacivanje transplantata srca.

Ključne reči:
biopsija; srce; srce, funkcijski testovi; transplantacija srca; histološke tehnike; imunohistohemija; skorovi, disfunkcija organa.

Introduction

Heart transplantation is the most effective way to treat patients in the terminal stage of heart failure [1, 2]. The International Society for Heart and Lung Transplantation (ISHLT) has suggested guidance for identifying potential candidates for heart transplantation [1, 3]. Unlike improvement and enhanced final result, transplant rejection still represents the heart transplant's Achilles heel [1–4]. Heart transplant rejection (HTR) can be manifested interoperatively, early or a few years after the

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transplantation [1–6]. In the posthospital discharge period, even a few years, the postoperative HTR time is essential for determining the etiology of transplant rejection and its diagnosis. With the development of heart transplantation, heart endomyocardial biopsies (EMBs) have an important role in diagnosing complications and grade of graft rejection [1]. EMB has been shown to be a safe and appropriate technique, with little sampling error, and remains, to this day, the most commonly used method for diagnosing acute rejection [2]. In 1990, the ISHLT defined a standardized system for acute HTR grading. The aim of this system was to enable grading which was easy, reproducible and could be extrapolated to other systems. Regardless of the grading system, the factors that are assessed are: the nature, intensity, and distribution of infiltrates of inflammatory cells; presence or absence of edema; presence or absence of cardiomyocyte damage [2, 3]. The ISHLT system is defined by four grades: grade 0R (no rejection, inflammatory cell infiltration, and cardiomyocyte damage); grade 1R (mild rejection, interstitial or perivascular inflammatory infiltrate with or without the focus of cardiomyocyte damage); grade 2R (moderate rejection, ≥ 2 foci of infiltration by inflammatory cells with cardiomyocyte damage); grade 3R (severe rejection, diffuse inflammatory infiltrate with multifocal cardiomyocyte damage, edema, vasculitis, and interstitial hemorrhage). Granulation connective tissue with inflammatory cell infiltrate is present at the sites of previous EMBs, and it is necessary to distinguish them from histological indicators of acute rejection [2, 7]. Examples of patients with hemodynamic and echocardiographic indicators of graft dysfunction but without histological evidence of cellular rejection are presented in the literature. Such cases represent humoral or antibody-mediated rejection (AMR), which is histologically presented by immune cell infiltrates, interstitial edema, hemorrhage, damaged capillaries, venules, and arterioles as well as cardiomyocyte necrosis [2, 8, 9]. Based on histological and immunocytochemical characteristics, Hammond et al. [8–10] classified AMR into five degrees: negative (without AMR), ambiguous evidence of AMR (endothelial cell activation, edema, damage with or without hemorrhage, and without inflammation or thrombosis), mild AMR (leukocytoclastic vasculitis), moderate AMR (arteriolitis, interstitial edema, fibrin accumulation) – identical to severe acute graft rejection.

The aim of this study was to examine the pathophysiological parameters of HTR, such as inflammatory cell infiltration, cardiomyocyte damage, damaged capillaries, venules and arterioles, and interstitial edema in patients monitored at the Cardiac Surgery Clinic of the University Clinical Center of Serbia in the period from January to December 2020.

Methods

Patients studied

Biopsy material was obtained from 31 patients treated at the Clinic for Cardiac Surgery of the University Clinical Center of Serbia as a clinical routine scheduled for monitoring transplanted patients. Biopsy samples were taken according to the following scheme: the first five biopsy samples were taken every 15 days from the day of transplantation. The next three biopsy samples were taken monthly, and then for a period of 2 years, an endomyocardial biopsy sample was taken every 3 months. During the third and fourth year after transplantation, samples were taken every four months, while biopsies were not routinely performed from the fifth year after transplantation. Obtained biological material was processed and reviewed at the Institute of Pathology of the Faculty of Medicine, University of Belgrade. The clinical and morphological data analyzed were: gender, age, age at the moment of the transplantation, heart disease diagnosis, the Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) profile, the New York Heart Association (NYHA) classification, and the ejection fraction (EF). For the purpose of calculating Transplant Rejection Score (TRS) ACR was graded as follows according to the ISHLT: 0R = 0, 1R = 1, 2R = 2, 3R = 3. AMR was absent in all samples from all 31 monitored patients, thus grade for AMR was 0. The TRS was calculated as the quotient of the total number of rejections and the number of biopsies during a year. Since there were no AMR rejection, calculated TRS presented dominant cellular type of rejection.

Histological sample analysis

The EMB sample was submerged in 4% buffered formalin for 12–24 hrs, then rinsed with water and dehydrated in growing concentrations of alcohol (from 70% up to absolute alcohol) over 24 hrs. The samples were lyophilized using xylol and molded in paraffin. The resulting molds were cut using a microtome into 3–5 μm clips which were then contrasted using the standard hematoxylin-eosin (HE) method.

Immunohistochemical methods

The resulting paraffin sections (3–4 μm thick) were dried at 56 ℃ for 16 hrs and deparaffinized in xylol, 100% ethanol, 96% ethanol, and distilled water, successively. Antigen unmasking was performed by transferring the deparaffinized samples into a plastic cuvette with 250 mL of citric buffer solution (10 mmol/L; pH 6.0), then cooked in a microwave oven two times successively for 5 min at maximum temperature. It was then cooled for 30 min at room temperature in a citric buffer. Endogenous peroxidase activity was blocked by submerging the samples in 3% hydrogen peroxide solution dissolved in distilled water for 5 min, after which they rinsed with distilled water and covered with phosphate buffer (0.02 mol/L; pH 7.0) successively three times for 2 min. The immunohistochemical procedure was done following the manufacturer's instructions using a commercially available kit (labeled streptavidin-biotin (LSAB) method, DAKO, Denmark). The samples were contrasted using the Mayer hematoxylin. Four antibodies (CD3, CD20, CD68, and C4d) were used in a 1:75 ratio, for which there is an external and internal positive control. Specific binding of

antibodies to certain antigens manifests in brown color, while hematoxylin nonspecifically binds nuclei and other cellular structures, coloring them blue. HE and immunohistochemical colored sample analysis was done using an optical microscope (Bx50F4, Olympus Optical, Japan).

Statistical analysis

Statistical data processing was done using the IBM SPSS Statistics computer program (SPSSInc., Chicago, IL, USA). The degree of correlation between clinical and morphological parameters and the TRS was calculated using the Spearman correlation test. Differences between variables with a significance value of ≤ 0.05 were deemed statistically significant.

Results

The following results were obtained by analyzing the data on the examined patients: the average age of the examinees was 45.94 years; the average age during transplantation was 42.72 years; 90.6% of patients were male and 9.4% female.

The prevalence of the most commonly diagnosed heart diseases among the respondents is shown in Table 1.

Table 1

Prevalence of most commonly diagnosed heart diseases among respondents

Diagnosis	% of respondents
Dilated cardiomyopathy	59.4
Viral myocarditis	21.9
Ischemic heart disease	12.5
Non-compaction cardiomyopathy	3.1

INTERMACS profiles are shown in Table 2.

Table 2

Interagency Registry for Mechanically Assisted Circulatory Support (INTERMACS) profiles among the examined patients

INTERMACS profile	% of respondents
1	4.2
2	20.8
3	8.3
4	54.2
5	12.5

NYHA scores are shown in Table 3.

Table 3

New York Heart Association (NYHA) scores among the examined patients

NYHA score	% of respondents
1	3.1
2	0
3	21.9
4	75

The average values of the EF after three, six, and twelve months after transplantation are shown in Table 4.

The Spearman's correlation test showed a negative correlation that was not statistically significant (r = -0.065; $p > 0.05$) between the TRS value and the EF difference value three, six, and twelve months after transplantation.

Table 4

Middle value of ejection fraction (EF) before heart transplantation, and after three, six, and twelve months after heart transplantation

EF	Middle value of EF (%)
Before transplantation	18.81
After transplantation (months)	
3	68.68
6	68.64
12	67.96

The average TRS was 0.42. The TRS was calculated as the quotient of the total number of rejections and the number of biopsies during a year. The histopathological presentations of grades 1R and 2R are shown in Figure 1 and Figures 2 and 3, respectively.

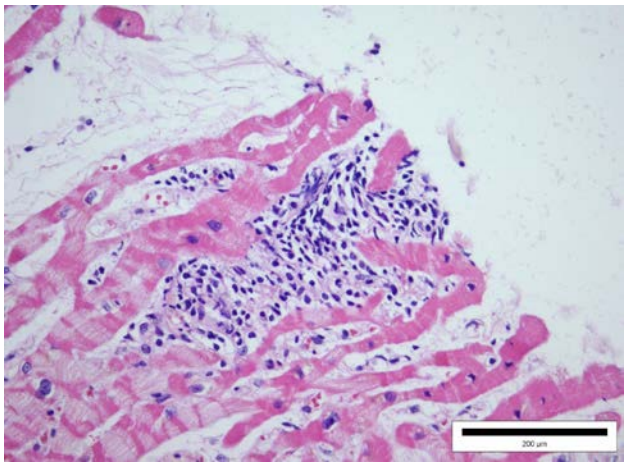

Fig. 1 – Grade 1R: perivascular lymphocyte infiltrate with a single focus of cardiomyocyte damage (hematoxylin and eosin staining, magnification ×40).

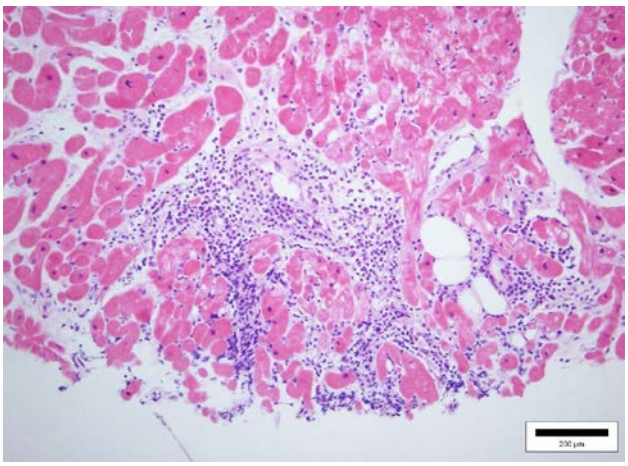

Fig. 2 – Grade 2R: the focus of interstitial infiltration with a large number of lymphocytes with a focus on cardiomyocyte damage (hematoxylin and eosin staining, magnification ×20).

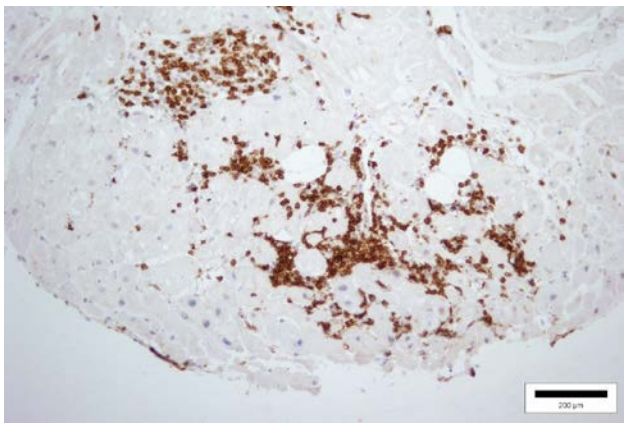

Fig. 3 – Grade 2R: the focus of interstitial infiltration with a large number of lymphocytes with a focus on cardiomyocyte damage (immunohistochemical staining for CD3, magnification ×20).

Immunohistochemical analysis showed the presence of C4d deposits in more than 50% of analyzed capillaries (Figure 4).

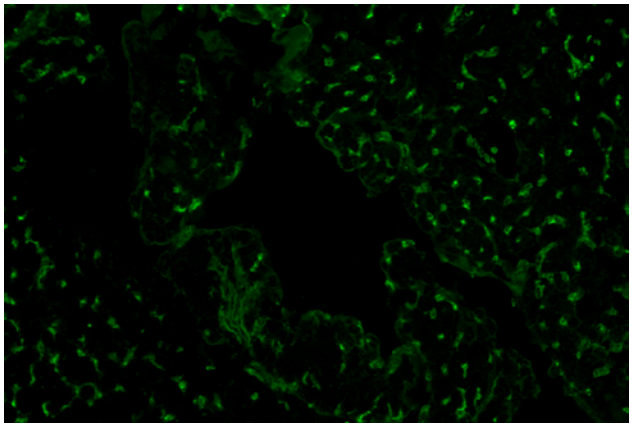

Fig. 4 – Antibody-mediated rejection: C4d deposits in more than 50% of capillaries in the endometrial biopsy sample (immunofluorescence microscopic analysis, magnification ×20).

AMR was observed in only one subject (Figure 5).

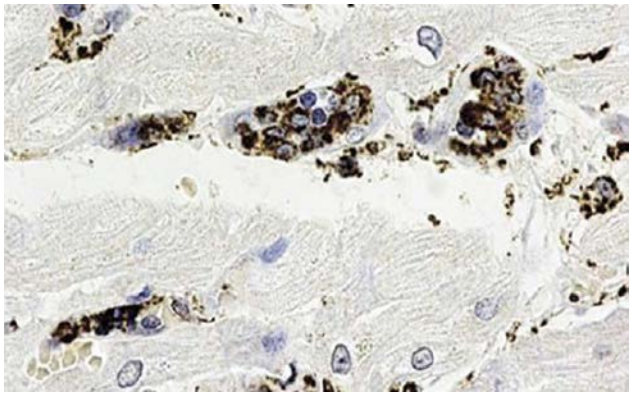

Fig. 5 – Antibody-mediated rejection: the presence of intravascular CD68-positive macrophages in more than 10% analyzed capillaries (immunohistochemical staining for CD68, magnification ×60).

Discussion

Despite the success of heart transplantation as a therapy for end-stage heart failure, acute rejection continues to reduce long-term survival in transplant recipients. Acute rejection develops as a consequence of recognizing histocompatibility antigens and the immune response to allogeneic heart muscle leading to progressive dysfunction and graft loss. EMB is a part of the current standard for monitoring complications and graft rejection and is based on the guidelines of the ISHLT. The highest incidence of HTR occurs in the first year after transplantation. Patients experiencing HTR grades of 2R and 3R in this time frame show poorer five-year survival. Several studies have drawn attention to the discrepancy between histological grade and graft function, especially in settings where declining cardiac function and increased mortality persist independently of grade [9–11].

The age distribution of the respondents in our study correlates with the literature data, which shows that the mean age of the patients is 50.5 years. Literature data show that 68% of patients with heart transplants are male. Dilated cardiomyopathy and ischemic heart damage are the most common causes that lead to the need for transplantation worldwide, which corresponds to the results of our research [12].

Clinical criteria that define eligibility for heart transplantation are partly the subjective discomfort of patients and partly defined on the basis of hemodynamic parameters at rest, and correlated with the NYHA classification. The NYHA classification, as a measure of functional capacity, is a subjective and often non-reproducible index, which varies day to day depending on various factors [13]. Our research showed that the largest number of subjects has an NYHA score of 4. NYHA score 4 and NYHA classification do not determine the best therapeutic approach (medical and pacing therapies, mechanical circulatory support, or heart transplantation); therefore, INTERMACS classification is used. Based on the competence of the prescribed medical therapy, hemodynamic and laboratory parameters, patient outcomes, and risk-benefit ratio, seven profiles have been defined for the INTERMACS. In our study, the majority (54.2%) of patients belong to profile 4 according to the INTERMACS classification [14, 15].

In our population of heart transplant patients and in accordance with the current immunosuppressive therapy regimen, allograph rejection is experienced by about 50% of patients at least once during the first year after transplantation. Monitoring a patient after a heart transplant is essential for the long-term survival of these patients. The gold standard for diagnosing rejection is EMB.

However, EMB is an expensive and invasive procedure that is partly limited by sampling error, as well as the existence of pathologist variability in assessing the degree of rejection. With significant advances in biotechnology, we are now able to explore the relationship between recipient and allography at multiple levels (genomic, epigenetic, transcriptional, proteomic, metabolic, immunophenotypic).

With all this in mind, we can expect that, in the coming period, a detailed description and determination of the meaning of genetic variants will lead to the development of numerous biomarkers (but also multimedia tests, such as genetic and epigenetic analysis) that can assess the patient's risk of continued transplant rejection. All of the above, with carefully balanced immunosuppressive therapy and adequate patient monitoring, can contribute to the better survival of heart transplant patients.

Conclusion

The average TRS obtained in this study is 0.42 and indicates a dominant cell-mediated graft rejection.

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Effects of intraoperative hypothermia on stress hormone response in surgical patients

Uticaj intraoperativne hipotermije na hormonski odgovor na stres kod hirurških bolesnika

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Abstract

Background/Aim. Surgical stress itself, as well as hypothermia induced by general anesthesia and low ambient temperature, activates stress hormone response with changes in catecholamines and counter-regulatory hormones. The aim of this study was to investigate the acute hormone stress response in patients who underwent major surgical procedures and the efficiency of external and internal warming methods in alleviating these changes. **Methods.** The study included a total of 60 patients who underwent major open abdominal surgical procedures and were randomly divided into 4 groups: control non-warmed (C), externally warmed using forced-air warming mattress (W), internally warmed using intravenous amino acids (A), and warmed with a combination of external and internal method (A+W). Esophageal temperature was used as a measure of core temperature. Concentrations of epinephrine, norepinephrine, cortisol, prolactin, and testosterone were measured. Blood samples for hormone measurement were obtained at two time points for catecholamines – 90 min before and 120 min after finishing the surgery, and at additional two-time points for cortisol, prolactin, and testosterone (24 and 48 hrs after surgery). **Results**. In the W and A+W groups, the temperatures did not significantly differ between time points but constantly decreased in the C and A groups, with a statistically significant difference between the anesthesia induction and the 120th min (35.61 ± 042 vs. 33.86 ± 0.71 °C; $p < 0.000$ and 35.81 ± 0.54 vs. 34.45 ± 0.41 °C; $p < 0.000$, respectively). Catecholamine concentrations in all groups showed a significant increase during surgery, with the highest values recorded in the non-warmed group (777.07 ± 800.08 after vs. 106.13 ± 89.63 pg/mL before surgery for epinephrine and 1,349.67 ± 984.16 vs. 580.53 ± 465.38 for norepinephrine, $p < 0.000$). Concentrations of cortisol and prolactin also showed a significant increase at the same time point, with a tendency to normalize after 48 hrs. On the contrary, testosterone concentrations showed a decrease after 120 min without normalization throughout the entire period of observation. Except for testosterone, changes in all stress hormones were attenuated in warmed groups compared to controls. **Conclusions**. Regarding both features of surgical stress investigated in this study (hypothermia and stress hormone response), the combination of endogenous amino acid-induced thermogenesis and external air warming mattress is most effective in its prevention.

Key words:
anesthesia, general; body temperature; hormones; hypothermia; intraoperative complications; stress, physiological; surgical procedures, operative.

Apstrakt

Uvod/Cilj. Hirurški stress sam po sebi, kao i u kombinaciji sa hipotermijom izazvanom dejstvom opšte anestezije, pokreće hormonski odgovor na stres koji karakterišu promene u nivoima kateholamina i drugih regulatornih hormona. Cilj rada bio je da se utvrdi akutni hormonski odgovor na stres kod bolesnika podvrgnutih dugotrajnim hirurškim intervencijama, kao i da se ispita efikasnost spoljašnjeg i unutrašnjeg zagrevanja u ublažavanju tih promena. **Metode**. Istraživanjem je obuhvaćeno ukupno 60 bolesnika podvrgnutih velikim hirurškim intervencijama na otvorenom abdomenu, koji su nasumično podeljeni u 4 grupe: kontrolnu koja nije dodatno zagrevana (C), grupu koja je zagrevana madracem sa toplim vazduhom (W), grupu koja je zagrevana infuzijom aminokiselina (A) i grupu koja je zagrevana kombinacijom te dve metode (A+W). Unutrašnja temperatura merena je ezofagealnom sondom. Ispitivana je koncentracija adrenalina, noradreanlina, kortizola, prolaktina i testosterona. Uzorci krvi uzimani su 90 min pre i 120 min posle završetka hirurške procedure (za kateholamine), a u dodatna dva termina za ostale hormone (24 i 48 sata nakon završetka operacije). **Rezultati.** U W i A+W grupi temperatura se nije razlikovala tokom perioda praćenja, dok se u C i A grupi konstantno snižavala, sa statistički značajnom razlikom između momenta uvođenja u anesteziju i 120 min

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posle operacije (35,61 ± 0,42 vs. 33,86 ± 0,71 °C; $p < 0,000$, odnosno 35,81 ± 0,54 vs. 34,45 ± 0,41 °C; $p < 0,000$). Koncentracija kateholamina je u svim grupama značajno porasla tokom operacije, a najviše vrednosti izmerene su u nezagrevanoj grupi (777,07 ± 800,08 vs. 106,13 ± 89,63 pg/mL za adrenalin i 1349,67 ± 984,16 vs. 580,53 ± 465,38 za noradrenalin, $p < 0,000$). Koncentracije kortizola i prolaktina takođe su porasle u istim intervalima, sa tendencijom normalizacije nakon 48 sati. Naprotiv, koncentracije testosterona značajno su se snižavale posle 120 min i niske vrednosti su se održavale kroz ceo period praćenja (48 sati). Osim u slučaju testosterona, promene svih ostalih hormona ublažene su kod zagrevanih bolesnika u poređenju sa kontrolnom grupom. **Zaključak**. Posmatranjem obe ispitivane karakteristike hirurškog stresa (hipotermija i hormonski odgovor na stres), utvrđeno je da je u njegovoj prevenciji najefikasnija kombinacija endogenog zagrevanja aminokiselinama i spoljašnjeg zagrevanja madracem sa toplim vazduhom.

Ključne reči:
anestezija, opšta; telesna temperatura; hormoni; hipotermija; intraoperativne komplikacije; stres, fiziološki; hirurgija, operativne procedure.

Introduction

Low ambient temperature in the operation theatre and prolonged effects of general anesthesia may lead to intra- and postoperative hypothermia in surgical patients. Complications of hypothermia in these patients are recognized as cardiovascular and respiratory dysfunction, impairment of the coagulation system, and coagulopathy [1]. Hypothermia during major surgical interventions may be prevented using several external warming methods, as well as internal administration of warm fluids with various compositions. The result of such interventions in literature are rather controversial, which may be contributed to the type of anesthesia, type of surgery, patient's age, and comorbidity. In our previous studies, the efficacy of two methods for preventing intraoperative hypothermia was investigated [2, 3]. The results indicated that both external warming (using air-forced warming mattress) and internal warming (administration of amino acid solutions) attenuate perioperative hypothermia.

Response to surgical stress is related to cellular and tissue injuries and nociceptive stimulation which influence hormonal and metabolic processes by activating the hypothalamic-pituitary-adrenal axis and secretion of stress hormones. In addition to surgical stress, the secretion of catecholamines is also induced by hypothermia in order to increase metabolic thermogenesis [4]. High cortisol and prolactin concentrations are common immediately after and up to 4–6 days following surgical procedures. On the other hand, concentrations of testosterone decrease after surgery, and low levels may be sustained for several days, which may delay anabolic processes [5].

Considering the importance of stress response in surgical patients, as well as the impairment of anabolic hormonal activity, the aim of our study was to investigate whether maintaining intraoperative normothermia using external and/or internal warming methods could influence stress response in patients who underwent major open abdominal surgical procedures.

Methods

The study population comprises 60 patients who underwent major open abdominal surgical procedures (between 2 and 4 hrs). The investigation was conducted at the Military Medical Academy in Belgrade, Serbia designed as a single-center prospective controlled interventional study according to ethical principles for investigations in biomedical science. Each participant signed informed consent. The study included adult patients with American Society of Anesthesiologists (ASA) score I or II who underwent elective colorectal surgical procedures for malignancy. Of 124 patients initially considered for enrolment, 64 were excluded according to criteria as follows: other indication than colorectal malignancy, ASA score III or IV, duration of intervention less than 2 hrs or more than 4 hrs, and administration of blood transfusion during surgery.

In all patients, the same method of general balanced anesthesia (GBA) was used. For premedication, 10 mg of diazepam (intramuscular – IM injection) was administered one h before anesthesia induction. Midazolam [0.05–0.15 mg/kg of body weight (BW)], fentanyl (2–6 μg/kg BW), propofol (1–2.5 mg/kg BW), and rocuronium (0.6-1mg/kg BW) were used for induction of GBA. Anesthesia and analgesia were maintained with 2–4 vol% of volatile esthetic sevoflurane (respiratory volume of 6–8 mL/kg BW) with an intermittent bolus of 25–50 μg of fentanyl. Neuromuscular blockade was maintained with an intermittent bolus of 0.15 mg/kg BW of rocuronium. No difference was registered between groups regarding hemodynamic parameters or volume loading, regardless of the warming method. Every patient was treated identically, according to contemporary guidelines.

A detailed description of methods of body temperature measurements (esophageal and skin temperatures) is presented in our previous study [3]. Patients were randomly divided into 4 groups: control group (C) consisted of 15 non-warmed patients, while in the other 3 groups, the same number of patients were either warmed externally (W group) using forced-air warming mattresses as described in the previous study [3], either received iv amino acids intraoperatively (A group), or combination of amino acids and external warming (A + W group). In the latter 2 groups, a commercial solution of 18 amino acids (Aminosol® 15%, Hemofarm AD, Serbia) was administered *via* a central venous catheter at a rate of 125 mL/h immediately after anesthesia induction in order to provide internal thermogenesis [2].

Blood samples for hormone measurement were obtained from each patient at two-time points for epinephrine and norepinephrine: 90 min before and 120 min after finishing the surgery, and at four-time points for cortisol, prolactin, and testosterone: 90 min before and 120 min, 24 hrs, and 48 hrs after surgery. Mean core (oesophageal) tempera-

tures were recorded at the moment of anesthesia induction and after 30, 60, 90, and 120 min.

Concentrations of epinephrine and norepinephrine were measured by competitive ELISA tests (Labor Diagnostika Nord), while concentrations of cortisol, prolactin, and testosterone were measured by the ECLIA method (Elecsys® 2010, Roche).

Statistical analysis

After being tested for normality (by Kolmogorov-Smirnov test), data were presented as mean ± standard deviation (SD) for continuous data, or median followed by interquartile range. The significance of differences between groups and between time points was tested using the *t*-test or Mann-Whitney *U* test (comparison of two groups), ANOVA of Kruskal-Wallis test, *post hoc* Mann-Whitney or Tukey test (multigroup comparison). The statistical significance was accepted at $p < 0.05$. Complete statistical analysis was performed using SPSS 18 package (Chicago, USA). The sample size was calculated using a test power of 0.8 and a Type I (alpha) error of 0.05, which revealed that a sample size of 15 per group could detect the statistically significant differences between independent groups (GPower 3.1).

Results

The baseline characteristics of the patients in all groups, as well as the environmental conditions in the operation theatre, are presented in Table 1. There were no significant differences between the groups.

There was a statistically significant difference between average intraoperative esophageal temperatures regarding time points, and between groups. In the W and A+W groups, the temperatures did not significantly differ between time points but constantly decreased in the C and A groups, with a statistically significant difference between the anesthesia induction and the 120th min (35.61 ± 0.42 vs. 33.86 ± 0.71 °C; $p < 0.000$ and 35.81 ± 0.54 vs. 34.45 ± 0.41 °C; $p < 0.000$, respectively) (Figure 1).

Temperatures in these two groups have had slower recovery after surgery, i.e., postoperative esophageal temperatures were significantly lower compared to the other two groups in all time points (Figure 2). At the 90th min average temperatures were 34.38 ± 1.17 (C) and 35.41 ± 0.79 °C (A) compared to 36.07 ± 0.86 (W) and 36.4 ± 0.66 °C (A+W); $p < 0.000$.

Average concentrations in all groups at four-time points are presented in Table 2.

Statistical analysis by Wilcoxon Signed Ranks revealed a highly significant difference between epinephrine concentrations pre- and postoperatively in all 4 groups: ($Z = -3.408$, $p < 0.01$ in the C group; $Z = -3.181$, $p < 0.01$ in the A group; $Z = -3.351$, $p < 0.01$ in the W group, and $Z = -3.408$, $p < 0.01$ in the A+W group). Despite the highest postoperative values recorded in the control group, the differences were not statistically significant (Table 2).

Table 1

Characteristics of patients and environments

Characteristic	Control group (C)	Amino acid group (A)	Warming mattress group (W)	Combined warming group (A+W)
Age (years)	65.19 ± 4.89	63.89 ± 4.34	63.25 ± 5.8	62.23 ± 3.87
Males	60	40	53.33	46.66
Females	40	60	46.66	53.33
Body weight (kg)	74.28 ± 5.16	73.89 ± 5.65	72.9 ± 6.12	73.24 ± 5.87
Duration of anesthesia (min)	141.28 ± 16.13	139.55 ± 13.59	133.46 ± 12.02	137.21 ± 14.12
Temperature in operation theatre (ºC)	21.25 ± 1.52	21.22 ± 1.47	21.14 ± 1.64	21.18 ± 1.56
Relative humidity (%)	57.13 ± 6.28	56.35 ± 5.59	55.6 ± 5.55	56.25 ± 5.84
Wind speed (m/s)	0.21 ± 0.04	0.21 ± 0.02	0.22 ± 0.04	0.22 ± 0.02

Results are presented as mean ± standard deviation or percentage of patients.

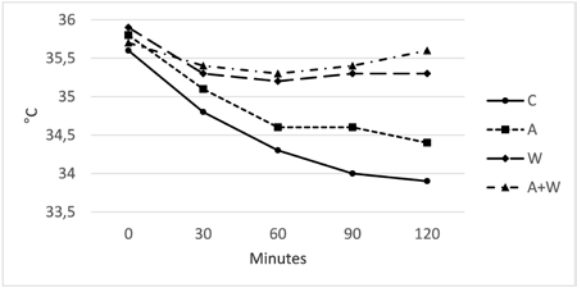

Fig. 1 – Intraoperative esophageal temperatures in all groups. C – control group (non-warmed); W – group warmed with a forced-air mattress; A – group warmed with an infusion of amino acids; A+W – combined warmed group.

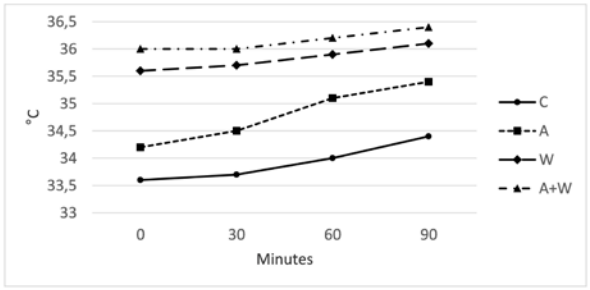

Fig. 2 – Postoperative esophageal temperatures in all groups. C – control group (non-warmed); W – group warmed with a forced-air mattress; A – group warmed with an infusion of amino acids; A+W – combined warmed group.

A similar trend was noticed in concentrations of norepinephrine (Table 2). Basic levels of norepinephrine were similar in all 4 groups, i.e., there were no statistically significant differences between groups. In all 4 groups, a highly significant increase was recorded in postoperative values compared to preoperative (Z = -3.181, $p < 0.01$ in the C group; Z = -2.556, $p < 0.01$ in the A group; Z = -3.237, $p < 0.01$ in the W group, and Z = -2.358, $p < 0.01$ in the A+W group). These values did not differ between groups.

Peak values of cortisol concentrations were recorded 120 min after surgery in all groups. After that, the values decreased toward basic levels in the following 2 days (Table 2). There was a highly significant difference between 4-time points (Wilks Lambda = 0.338; F = 35.323, Partial Eta Squared = 0.662; $p < 0.01$) and between 4 groups (F = 6.002, Partial Eta Squared = 0.243; $p < 0.01$).

Multiple comparison analyses (Tukey test) revealed a highly significant difference between internal warming (A group) compared to the C and W groups (Table 3).

Basic levels of prolactin showed no statistically significant differences between groups. Levels of prolactin were highest 120 minutes postoperatively in all groups (Table 2). There was a statistically significant difference between four-time points (Wilks Lambda = 0.604; F = 11.795, Partial Eta Squared = 0.396; $p < 0.01$) and between groups (F = 4.857, Partial Eta Squared = 0.206; $p < 0.01$). Multiple comparisons (Tukey test) revealed a statistically significant difference between the A group and all other groups ($p < 0.05$).

Concentrations of testosterone decreased in all groups during the surgical procedures and remained lower 24 and 48 hrs after surgery (Table 2). The difference was statistically significant in the W and A+W groups. Tukey test: in the W group: 90 min prior to surgery compared to 120 min after surgery, $p = 0.043$; 24 hrs after, $p = 0.016$; 48 hrs after, $p = 0.032$; in the A+W group: 90 min prior to surgery vs. 120 min after, $p = 0.013$; 24 hrs after, $p = 0.008$; 48 hrs after, $p = 0.022$. There was no significant difference between groups (F = 0.992; Partial Eta Squared = 0.051; $p = 0.403$).

Discussion

Intraoperative hypothermia is a multifactorial and complex condition. It is inadvertent and frequent. The induction and maintenance of general anesthesia change the normal protective response to hypothermia. Because of anesthesia-induced vasodilation and impairment of the normal thermoregulation (reduction of thermogenesis, both shivering and non-shivering), there is significant redistribution of heat from the core to the periphery [1]. Effector response to hypothermia is also altered [6]. Heat loss is influenced by environ-

Table 2

Concentrations of hormones at four-time points in all groups

Hormone	Group	Mean ± standard deviation			
		90 min before surgery	120 min after surgery	24 hrs after surgery	48 hrs after surgery
Epinephrine (pg/mL)	C	106.13 ± 89.63	777.07 ± 800 .08	-	-
	A	53.47 ± 52.022	388.27 ± 293.48	-	-
	W	79.60 ± 81.06	442.07 ± 517.83	-	-
	A+W	80.80 ± 60.99	664.33 ± 606.33	-	-
Norepinephrine (pg/mL)	C	580.53 ± 465.38	1349.67 ± 984.16	-	-
	A	588.73 ± 452 ± 93	1265.67 ± 949.13	-	-
	W	313.53 ± 357.68	800.53 ± 738.04	-	-
	A+W	318.80 ± 238.16	937.80 ± 1063.43	-	-
Cortisol (nmol/L)	C	533.57 ± 199.92	1211.23 ± 373.98	654.29 ± 221.49	494.71 ± 165 ± 39
	A	408.98 ± 201.88	542.98 ± 340.96	584.15 ± 339.08	486.57 ± 418 ± 59
	W	573.22 ± 188.70	1351.59 ± 482.12	643.31 ± 211.01	514.16 ± 183.28
	A+W	400.09 ± 191.49	803.64 ± 279.66	631.65 ± 399 ± 79	495.07 ± 373.74
Prolactin (μIU/mL)	C	226.98 ± 138.32	624.29 ± 417.18	230.51 ± 86.97	273.34 ± 140.11
	A	411.64 ± 224.59	1106.74 ± 922.42	707.51 ± 840.59	487.96 ± 280.82
	W	237.36 ± 166.69	638.37 ± 335.53	258.37 ± 172.69	315.56 ± 211.55
	A+W	295.72 ± 257.15	1162.44 ± 11.6359	363.26 ± 373.29	506.52 ± 479.99
Testosterone (nmol/L)	C	8.92 ± 7.16	6.38 ± 6.01	5.67 ± 4.27	5.74 ± 5.14
	A	6.31 ± 7.48	4.20 ± 4.92	5.27 ± 4.00	3.49 ± 3.18
	W	10.32 ± 8.29	6.41 ± 4.79	5.66 ± 4.19	5.33 ± 4.41
	A+W	11.67 ± 7.69	6.24 ± 4.04	6.75 ± 4.18	5.89 ± 3.86

C – Control group (non-warmed); W – Group warmed with a forced-air mattress; A – Group warmed with amino acids; A+W – Combined warmed group.

Table 3

Multiple comparisons of groups (Tukey test)

Group I	Group II	Mean difference (I-II)	*p*
A	C	-217.7777	< 0.01
	W	-264.8993	< 0.01
A+W	W	-187.9583	< 0.05

C – Control group (non-warmed); W – Group warmed with a forced-air mattress; A – Group warmed with amino acids; A+W – Combined warmed group.

mental conditions in the operating theatre. An essential factor in intraoperative heat loss is the surgical procedure itself. We enrolled patients who underwent large and long abdominal surgical procedures with exposed abdominal cavities. In this setting, hypothermia is a significant problem [6].

One of the endpoints of our study was to evaluate the efficacy of external warming, amino acid-induced endogenous thermogenesis, and their combination in patients undergoing major open abdominal surgical procedures. We have noted an increased interest in this topic in recent years. In our investigation, after the first 30 min of surgery, the lowest esophageal temperature was recorded in the C group. At this time point, the highest temperature was in the A+W group, and the same trend was sustained throughout the entire surgical procedure and up to 90 min after surgery.

Intraoperative hypothermia is defined as an esophageal temperature below 36 °C. After 30 min of surgery, the frequency of hypothermia was 100% in the group C and 93% in the groups A and W. Lowest frequency of hypothermia (80%) was in the group A+W. After 120 min of surgery, all patients in the groups C and A were hypothermic. The frequency of hypothermia in the group W was 86.6%. Again, the lowest frequency of hypothermia (66.6%) was in the A+W group.

In our study, it is evident that more than half of the patients remained hypothermic despite air warming mattress and/or amino acid infusion both 30 min and 120 min after anesthesia induction. That is in accordance with the results from a large retrospective study regarding intraoperative core temperature patterns in patients warmed with forced air [7]. The authors demonstrated that more than 60% of the patients were hypothermic 45 min after induction and that 20% continued being hypothermic for more than 120 min. Administration of iv nutrients, such as amino acids, has been investigated in normothermia maintenance settings due to endogenous metabolic heat production as well as the increase of whole-body heat content by 20% [8]. Salem et al. [9] investigated 42 cancer patients who underwent pelvic abdominal surgery, randomized to receive either amino acid infusion or warm Ringer solution infusion 2 hrs before anesthesia induction. Authors concluded that amino acid infusion before anesthesia and surgery restored core body temperature: in the first 120 min in this group, there was no hypothermia [9]. These results are at odds with ours regarding the A group: after 120 min, all patients were hypothermic. Different results are expected due to the difference in the study design – in our study, infusion started immediately after anesthesia induction while in the study by Salem et al. [9], infusion of the amino acid solution was completed two hrs before anesthesia.

Amino acids infusion might be intraoperatively beneficial for surgical patients because it can counteract the disadvantageous fasting metabolism; metabolic fate is two-fold: oxidation for energy production and/or building blocks for protein synthesis. In both cases, large amounts of energy are needed for amino acid metabolism and possibly heat production [10]. A recent systematic review and meta-analysis regarding perioperative amino acid infusion for preventing hypothermia demonstrated that, based on 626 participants from 14 randomized controlled trials, amino acid infusion led to a +0.46 °C increase in temperature. The authors concluded that this minor difference is of clinical significance and that this method of normothermia maintenance has a similar effect as conventional warming systems and may serve as a viable alternative [11].

In several studies, various methods of perioperative prewarming and warming were investigated in surgical procedures other than exclusively major open abdominal ones [12–14]. The authors concluded that active warming is more efficient than passive in hypothermia prevention. Yet, even with active warming, hypothermia persisted in some patients. The authors also emphasized that continued innovation in active warming technology and research in different methods of active warming is necessary. Given that, one would expect an investigation of various methods of intraoperative warming. Yet, interestingly, in the literature available to us, we did not find any studies regarding the combination of external warming and amino acid-induced endogenous thermogenesis in the prevention of inadvertent intraoperative hypothermia. One recent investigation included a combination of forced-air warming and warmed iv fluid consisting of lactated Ringer solution but not amino acids [1]. Our results showed that the mean core temperature was highest in the group A+W at the 120th min after anesthesia induction. At that time point, the lowest frequency of hypothermia was again in the group A+W. It would seem that combination of endogenous amino acid-induced thermogenesis and external warming mattress is most effective in preventing intraoperative hypothermia.

The primary aim of our study was to investigate stress hormone response in patients undergoing major surgery and the effects of various warming methods. Our results show a substantial increase in concentrations of catecholamines in the postoperative period in all groups. The highest values were recorded in non-warmed patients, but we found no significant difference compared to other groups. Moreover, despite the notably higher fundamental values in the groups C and A compared to the other two groups, these differences did not reach statistical significance. Frank et al. [15] indicated that a decrease in core temperature by 1.5 °C is related to higher epinephrine concentration in the early postoperative period. At the same time, maintenance of normothermia shows little effect on epinephrine and norepinephrine concentrations. Initial studies also failed in the attempt to relate the effects of intraoperative hypothermia to catecholamine response, probably due to an insufficient number of participants, confounding influence of age, lack of randomization, and lack of standardized postoperative analgesia [16, 17]. Other published results also reported that mild intraoperative hypothermia does not have an important effect on stress hormone concentrations in hypothermic patients [18, 19]. In the same study, authors simultaneously estimated cortisol response and did not find any intraoperative increase in its concentrations. Frank et al. [15] found that postoperative cortisol concentrations were much higher in patients who underwent general anesthesia compared to patients exposed to combined or regional anesthesia, with the conclusion that cortisol response is determined by anesthesiology technics rather than hypo-

thermia. In our study, peak values of cortisol levels were achieved in all groups 120 min after surgery, with statistically significant differences regarding the warming method. In the group with simulated endogenous thermogenesis by intravenous administration of the amino acid solution, the lowest changes in cortisol levels were recorded intraoperatively and postoperatively compared to other groups. In all groups, cortisol concentrations tend to normalize in the following 48 hrs.

Along with other stress hormones, surgical stress and intraoperative hypothermia also induce prolactin secretion [20], probably *via* a central dopaminergic mechanism. An increase in prolactin concentration in all four groups in our study, with peak values measured 120 min after surgery, presented the statistically significant difference between groups warmed with amino acids and the combination of amino acids and forced-air mattress compared to the other two groups, which supported the conclusion that amino acids were responsible for the observed difference. Interestingly, we recorded somewhat higher basic levels of prolactin in the A group compared to the other three groups, but the difference was not statistically significant. After 48 hrs, prolactin concentrations decreased toward baseline levels, which is in agreement with recently published results [20].

Finally, analyzed testosterone response presented with a decrease in concentrations after surgery in all groups, and lower levels were sustained throughout the entire observation, i.e., the following 48 hrs. The differences between baseline levels and postoperative measurements were statistically significant in the W and A+W groups, but there was no difference between groups at any time point. Lindh et al. [20] recorded maintenance of low concentration of testosterone up to the 16th postoperative day, with inevitable impairment of anabolic processes necessary for recovery. According to our findings, as well as results reported in other studies, we may notice that major surgical trauma and perioperative hypothermia impose rapid, intensive, and long-lasting effects on gonadal activity (reflected by decreased testosterone levels), while the effect on suprarenal activity is rather mild.

There are several limitations in our study: although the sample size was sufficient for correct statistical analysis, reported patterns and trends in stress hormone profiles and methods of hypothermia should be confirmed in a larger population. In addition, our results are obtained in patients undergoing specific surgical intervention in GBA; hence further investigations need to be conducted in other types of surgery and other types of anesthesia.

Conclusion

Prevention of intraoperative hypothermia is of major importance in reducing complications associated with avoidable hypothermia in surgical patients. This study demonstrates the effects of intraoperative hypothermia and three warming methods on stress hormone response in patients who underwent major open abdominal surgical procedures. Both external and internal warming methods were effective in attenuating intraoperative and postoperative hypothermia, and the most effective was forced-air mattresses *per se* or in combination with internal warming. A comparison of catecholamine concentrations measured 90 min before and 120 min after surgical procedures revealed a significant increase during surgery, with the highest values recorded in the non-warmed group. Concentrations of cortisol and prolactin also showed a significant increase at the same time point, with a tendency to normalize after 48 hrs. On the contrary, testosterone concentrations showed a decrease after 120 min without normalization throughout the entire period of observation. Except for testosterone, changes in all stress hormones were attenuated in warmed groups compared to controls, and internal warming (amino acid solution) with or without forced-air mattresses was the most effective. Regarding both features of surgical stress investigated in this study (hypothermia and stress hormone response), the combination of endogenous amino acid-induced thermogenesis and external air warming mattress is most effective in its prevention.

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Correlation between oxidative stress and cognitive impairment in patients with obstructive sleep apnea-hypopnea syndrome

Korelacija između oksidativnog stresa i kognitivnog oštećenja kod bolesnika sa sindromom opstruktivnog poremećaja disanja (apneje i hipopneje) tokom spavanja

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Abstract

Background/Aim. It is necessary to find relevant oxidative stress markers for predicting the severity of obstructive sleep apnea-hypopnea syndrome (OSAHS), a sleep disorder-related respiratory disease. The aim of the study was to investigate if there is a correlation between oxidative stress and cognitive impairment in OSAHS patients. **Methods.** A total of 220 patients were divided into the group of snoring patients, the group with mild to moderate OSAHS, and the group with severe OSAHS according to polysomnography (PSG). Apnea-hypopnea index (AHI), oxygen desaturation index (ODI), and baseline data were monitored. Oxidative stress indices were measured by colorimetry from blood samples taken early in the morning. The patients were then divided into the group with normal cognition and cognitive impairment group based on mini-mental state examination (MMSE) and Montreal cognitive assessment (MoCA). Independent risk factors for cognitive impairment were analyzed by multivariate logistic regression. The correlation between oxidative stress and cognitive impairment was analyzed by Pearson's method. Receiver operating characteristic (ROC) curves made it possible to analyze the efficiency of oxidative stress combined with detection for assessing cognitive impairment in OSAHS patients. **Results.** The snoring group, mild to moderate OSAHS group, and severe OSAHS group had significantly different snoring loudness, body mass index (BMI), AHI, ODI, MoCA, and MMSE scores, and levels of malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) ($p < 0.05$). The cognitive impairment group and group with normal cognition had different BMI, GSH-Px, MDA, SOD, neuroglobin, hypoxia-inducible factor, AHI, and lowest nocturnal oxygen saturation ($p < 0.05$ or $p < 0.01$) levels. BMI, GSH-Px, MDA, SOD, neuroglobin, hypoxia-inducible factor, AHI, and lowest nocturnal oxygen saturation were independent risk factors for cognitive impairment. The MoCA and MMSE scores of cognitive impairment had positive correlations with GSH-Px and SOD but negative correlations with MDA ($p < 0.05$). The area under the ROC curve of GSH-Px, MDA, and SOD and their combination for prediction of cognitive impairement were 0.670, 0.702, 0.705, and 0.836, respectively. **Conclusion.** Oxidative stress may be the biochemical basis of cognitive impairment in OSAHS patients.

Key words:
cognitive dysfunction; oxidative stress; sleep apnea, obstructive; sleep apnea syndromes.

Apstrakt

Uvod/Cilj. Neophodno je pronaći odgovarajuće markere oksidativnog stresa za predviđanje težine sindroma opstruktivne apneje-hipopneje u snu (SOAHS), respiratorne bolesti povezane sa poremećajem spavanja. Cilj rada je bio da se istraži da li postoji korelacija između oksidativnog stresa i kognitivnog oštećenja kod bolesnika sa SOAHS. **Metode**. Ukupno 220 bolesnika podeljeno je na grupu koja hrče, grupu sa blagim do umerenim SOAHS i grupu sa teškim SOAHS, izmereno primenom polisomnografije (PSG). Praćeni su indeks apneje-hipopneje (IAH), indeks desaturacije kiseonikom (IDK) i osnovni podaci. Indeksi oksidativnog stresa mereni su metodom kolorimetrije iz uzoraka krvi uzetih u ranim jutarnjim časovima. Bolesnici su dalje bili podeljeni na osnovu *mini-mental state examination* (MMSE) i *Montreal cognitive assessment* (MoCA) procena u grupu sa normalnom kognicijom i grupu sa kognitivnim oštećenjima. Nezavisni faktori rizika od kognitivnog oštećenja analizirani su multivarijantnom logističkom regresijom. Korelacija između oksidativnog stresa

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i kognitivnih oštećenja analizirana je Pirsonovom metodom. Krive *receiver operating characteristic* (ROC) omogućile su analizu efikasnosti oksidativnog stresa u kombinaciji sa procenom kognitivnog oštećenja kod bolesnika sa SOAHS. **Rezultati**. Grupa koja je hrkala, grupa sa blagim do umerenim oblikom SOAHS i grupa sa teškim oblikom SOAHS imale su značajno različite jačine hrkanja, različite vrednosti indeksa telesne mase (ITM), IAH, IDK, MoCA i MMSE i različite nivoe malondialdehida (MDA), glutation peroksidaze (GSH-Px) i superoksid dismutaze (SOD) ($p < 0,05$). Grupa sa kognitivnim oštećenjem i grupa sa normalnom kognicijom imale su različite vrednosti ITM, GSH-Px, MDA, SOD, neuroglobina, hipoksijom izazvanog faktora, IAH i najnižu noćnu zasićenost kiseonikom ($p < 0,05$ ili $p < 0,01$). Nezavisni faktori rizika od kognitivnog oštećenja bili su ITM, GSH-Px, MDA, SOD, neuroglobin, hipoksijom izazvan faktor, AHI i najniža noćna zasićenost kiseonikom. Rezultati skorova kognitivnog oštećenja MoCA i MMSE pozitivno su korelisali sa GSH-Px i SOD, ali je korelacija sa MDA bila negativna ($p < 0,05$). Površina ispod ROC krive za GSH-Px, MDA, SOD i njihove kombinacije za predviđanje kognitivnog oštećenja iznosila je 0,670, 0,702, 0,705 i 0,836, redom. **Zaključak**. Oksidativni stres može biti biohemijska osnova kognitivnog oštećenja kod bolesnika sa OSAHS.

Ključne reči:
kognicija, poremećaji; stres, oksidativni; apneja u snu, opstruktivna; apneja u snu, sindromi.

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is a type of sleep disorder-related respiratory disease whose influence on sleep quality and mood is well known. It is featured by increased resistance in the upper respiratory tract that occurs repeatedly during sleep, resulting in apnea, hypopnea, and sleep fragmentation at night [1]. OSAHS is prominently associated with the increased risk of death and occurrence of diseases such as cardiovascular (CV) and cerebrovascular (CEV) diseases (CVD and CEVD, respectively), metabolic disorders, and neurocognitive impairment, among which neurocognitive impairment has been paid progressively more attention to by relevant scholars in recent years [2]. Patients with OSAHS suffer from defects in attention span, distraction, sustained attention, working memory, visual space, and executive function. Cognitive impairment has always been a non-negligible clinical manifestation of patients with OSAHS. The underlying mechanisms of neurocognitive impairment related to sleep apnea may include intermittent hypoxemia, sleep fragmentation, neuroinflammation, CEV changes, and ischemic preconditioning [3]. It has been revealed by some research that sleep disorders can induce oxidative stress, and progressively more attention has been paid to the relationship between oxidative stress and nervous system damage [4]. Oxidative stress is induced generally because the production of oxygen free radicals in the body exceeds the body's endogenous antioxidant capacity (such as reduction of sleep time), resulting in the process of tissue damage, which is essential for the development of neurodegenerative diseases. The association of mechanisms of OSAHS-related cognitive impairment with inflammatory response, oxidative stress, and brain injury has been confirmed [5]. However, there are still controversies over a series of criteria of oxidative stress, the role of the new biomarkers in OSAHS patients, and the best biomarker for cognition [6]. Glutathione, superoxide dismutase (SOD), malondialdehyde (MDA), and advanced oxidation protein products are recognized serum biomarkers of oxidative stress [7]. However, not all the results of studies can confirm the presence of oxidative stress damage in patients with OSAHS, which may be related to the efficacy of the oxidative stress index adopted. Hence, it is of important clinical value to search for oxidative stress markers able to predict the severity of sleep apnea.

The purpose of the present study is to evaluate the cognitive function of patients with OSAHS, group the patients according to their cognitive status, and make a comparison to identify the factors affecting cognitive impairment in OSAHS patients. In addition, the correlations between oxidative stress indices and cognitive impairment were evaluated to provide a reliable basis for early detection and follow-up treatment of cognitive impairment in OSAHS patients.

Methods

A total of 220 OSAHS patients diagnosed and treated in our hospital from January 2016 to June 2018 were enrolled. All of them were confirmed by polysomnography (PSG) of 7hrs sleep at night as meeting the diagnostic criteria in the Guidelines for diagnosis and treatment of OSAHS (revised in 2011). According to PSG results the patients were divided into snoring group (n = 60), mild to moderate OSAHS group (n = 80), and severe OSAHS group (n = 80). Among the subjects, there were 150 males and 70 females, aged 20–60 years old. The general information and laboratory test data of the patients were obtained by consulting the relevant case data of patients in our department and treatment sites. This study was reviewed and approved by the Medical Ethics Committee, and all patients and their family members signed the informed consent.

Inclusion criteria were: (1) patients who were first diagnosed with OSAHS and received no OSAHS-related surgery, (2) those whose main complaints were sleep snoring and sleep suffocation, (3) those who meet the diagnostic criteria for OSAHS according to the Guidelines for diagnosis and treatment of OSAHS [8], and (4) those who were able to cooperate and finish the mini-mental state examination (MMSE) and Montreal cognitive assessment (MoCA) (Chinese versions). Exclusion criteria were: (1) patients with a history of CEVD or other severe organ diseases, (2) those with vascular and other dementias, Parkinson's disease, or other neuropsychiatric diseases such as anxiety and depression, or (3) those dependent on alcohol or diazepam drugs, or with communication disorders.

All the patients were asked in detail about their medical history to reconfirm whether they met the inclusion criteria, and general data such as height, weight, neck circumference, and years of education were obtained. Body mass index (BMI) was calculated based on height and weight as follows: BMI = weight (kg)/height^2 (m^2). Epworth sleepiness scale (ESS) was adopted for evaluating daytime sleepiness, which involved a total of 8 scenes, including sitting and reading, watching television, sitting inactively in a public place, riding as a passenger in a car for an hour, lying down to rest in the afternoon when circumstances permit, sitting and talking to someone, sitting quietly after lunch, and sitting in a car while stopping for a few minutes in traffic. A higher score indicated more severe daytime sleepiness.

Alice LE PSG system (Philips Respironics) was applied for monitoring the PSG of the subjects overnight, and the monitoring time was ≥ 7 hrs. The recorded data were automatically analyzed by the G3 software system and reviewed frame by frame by the same professional sleep physician. In addition, two indices were monitored: the apnea-hypopnea index (AHI) – the number of apneas or hypopneas recorded during the study per hour of sleep (generally expressed as the number of events per hour) and oxygen desaturation index (ODI). Desaturation episodes are generally described as a decrease in the mean oxygen saturation of ≥ 4% (over the last 120 sec) that last for at least 10 sec [9]. After the patients' conditions were stable, MMSE and MoCA scores were evaluated by the same physician in a quiet room. MMSE was used to evaluate cognitive function involving memory, orientation, attention and calculation, recall, and language abilities. There were 30 questions in total, with a total score of 30 points (1 point for each correct answer and 0 points for each wrong answer). The higher the score was, the better the cognitive function would be. MoCA can be used as a simple and sensitive neurocognitive assessment tool to detect cognitive impairment in patients with OSAHS, which should be completed within 10 min according to the evaluation criteria. It evaluates the functions of 7 cognitive regions of patients, including visual space and execution, naming, attention, language, abstraction, memory and delayed recall, and time and place orientation. A total score ≥ 26 points suggests normal cognition. It is a screening tool for mild cognitive impairment.

In the early morning, 3 mL of fasting blood was drawn from the median cubital vein of each patient in the three groups, loaded in an anticoagulant tube, and centrifuged at 3,000 rpm for 15 min. Then the supernatant was collected and stored in a refrigerator at -20 °C for later detection. According to the instructions of corresponding kits (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China), the activities of SOD and serum glutathione peroxidase (GSH-Px) were detected by colorimetry, and the level of MDA was measured by the thiobarbituric acid method.

SPSS 25.0 software (IBM Corp, Armonk, NY, USA) was used for statistical analysis. All data were tested for homogeneity of variance and normal distribution, and the normally distributed measurement data were expressed as mean ± standard deviation. An independent sample *t*-test was conducted for comparison between groups, and F-test was adopted for comparison among multiple groups. The measurement data that did not conform to normal distribution were represented as median (P_{25}, P_{75}), and the Kolmogorov-Smirnov test was used for comparison between groups. The qualitative data were expressed as frequency or percentage, and the χ^2 test was performed for comparison between groups. In combination with clinical factors, variables with significant differences in univariate analysis were taken as candidate variables and introduced into the multivariate logistic regression model of cognitive impairment to establish a prediction model aiming to predict the probability of cognitive impairment in patients with OSAHS. The predictive value of oxidative stress-related indices for prediction was analyzed by the receiver operating characteristic (ROC) curve. The area under the ROC curve (AURC) of each index was calculated, and the diagnostic efficiency was higher when AURC was closer to 1. AURC had low predictive accuracy when it was 0.5–0.7, it was relatively accurate when AURC was 0.7–0.9, and it was highly accurate when AURC exceeded 0.9. AURC of 0.5 indicated that the diagnostic method was completely ineffective and had no diagnostic value. Sensitivity = number of true positive cases/(number of true positive cases + number of false negative cases) × 100%. Specificity = number of true negative cases/(number of true negative cases + number of false positive cases) × 100%. The difference was considered statistically significant when $p < 0.05$.

Results

There were no significant differences in the general data such as gender, age, years of education, neck circumference, and ESS score among the snoring group, mild to moderate OSAHS group, and severe OSAHS group ($p > 0.05$), suggesting that the groups were comparable in the follow-up indices. Significant differences were observed in snoring loudness, BMI, AHI, ODI, MoCA, and MMSE scores, and levels of MDA, GSH-Px, and SOD among the three groups of patients. Compared with the snoring group, the mild to moderate OSAHS group had slightly higher snoring loudness, BMI, AHI, ODI, and level of MDA ($p > 0.05$), and the severe OSAHS group had significantly higher values ($p < 0.05$). Besides, compared with the snoring group, the SOD and GSH-Px levels and MoCA and MMSE scores of the mild to moderate OSAHS group were slightly lower ($p > 0.05$), and those of severe OSAHS group were significantly lower ($p < 0.05$) (Table 1).

There were no statistically significant differences in age and gender between the cognitive impairment group and the normal cognitive group ($p > 0.05$). GSH-Px, MDA, neuroglobin, hypoxia-inducible factor, and AHI were significantly higher, while BMI, SOD, and lowest nocturnal oxygen saturation were significantly lower in the cognitive impairment group than those in the normal cognitive group ($p < 0.05$ or $p < 0.01$).

Establishment of prediction model: Multivariate analysis was performed based on the factors influencing cognitive impairment in the 220 OSAHS patients in our hospital. With the presence of cognitive impairment as the dependent variable (normal cognitive group = 0, cognitive impairment group = 1), the variables with statistically significant differences shown in

univariate analysis were included in the analysis. The results showed that BMI, GSH-Px, MDA, SOD, neuroglobin, hypoxia-inducible factor, AHI, and lowest nocturnal oxygen saturation were independent risk factors for cognitive impairment in patients with OSAHS (Figure 1).

The results of Pearson's correlation analysis demonstrated that MoCA and MMSE scores of cognitive impairment in OSAHS patients had positive correlations with GSH-Px and SOD and negative associations with MDA ($p < 0.05$) (Table 2).

ROC curve analysis revealed that when the cut-off values of GSH-Px, MDA, and SOD were 251.07 U, 9.46 nmol/mL, and 98.32 mmol/L, respectively, the AURC were 0.670, 0.702, and 0.705, respectively, showing predictive values for cognitive impairment. Moreover, the AURC of the three parameters combined for prediction was 0.836 ($p < 0.001$), indicating that the diagnostic value of the combination of the three indices is higher (Table 3, Figure 2).

Table 1

Baseline clinical data

Parameter	Snoring group (n = 60)	Mild to moderate OSAHS group (n = 80)	Severe OSAHS group (n = 80)	*p*
Age (years)	46.3 ± 10.5	50.0 ± 10.3	51.5 ± 12.5	0.154
Male/female	41/19	53/27	56/24	0.878
Years of education (years)	10.7 ± 2.4	11.02 ± 2.56	10.94 ± 2.63	0.115
Snoring loudness (dB)	6.45 ± 1.48	6.98 ± 1.77	8.03 ± 1.74	0.002
Neck circumference (cm)	39.82 ± 3.68	40.03 ± 3.06	39.45 ± 4.13	0.097
ESS score	7.06 (3.15, 10.28)	7.25 (2.94, 10.33)	4.26 (3.15, 10.58)	0.329
BMI (kg/m^2)	25.51 ± 3.69	27.02 ± 4.34	28.25 ± 4.83	0.009
AHI (times/h)	1.81 ± 0.83	13.99 ± 3.06	53.68 ± 17.92	< 0.001
ODI (times/h)	7.25 (3.24, 11.25)	29.21 (14.54, 43.22)	64.53 (41.52, 82.58)	< 0.001
MoCA score	26.72 ± 0.53	24.86 ± 1.32	24.01 ± 1.00	0.014
MMSE score	29.58 ± 1.06	28.26 ± 1.84	27.32 ± 2.95	0.021
MDA (nmol/mL)	7.52 ± 1.80	10.64 ± 1.73	12.00 ± 2.01	0.003
GSH-Px (U)	283.49 ± 30.52	238.92 ± 31.67	229.67 ± 28.13	0.011
SOD (U/L)	117.16 ± 20.35	99.57 ± 18.62	86.21 ± 10.83	0.009

Results are given as number of patients or mean ± standard deviation or median (25th percentile, 75th percentile). OSAHS − obstructive sleep apnea-hypopnea syndrome; ESS − Epworth sleepiness scale; BMI – body mass index; AHI – apnea-hypopnea index; ODI – oxygen desaturation index; MoCA – Montreal cognitive assessment; MMSE – mini-mental state examination; GSH-Px – glutathione peroxidase; MDA − malondialdehyde; SOD − superoxide dismutase.

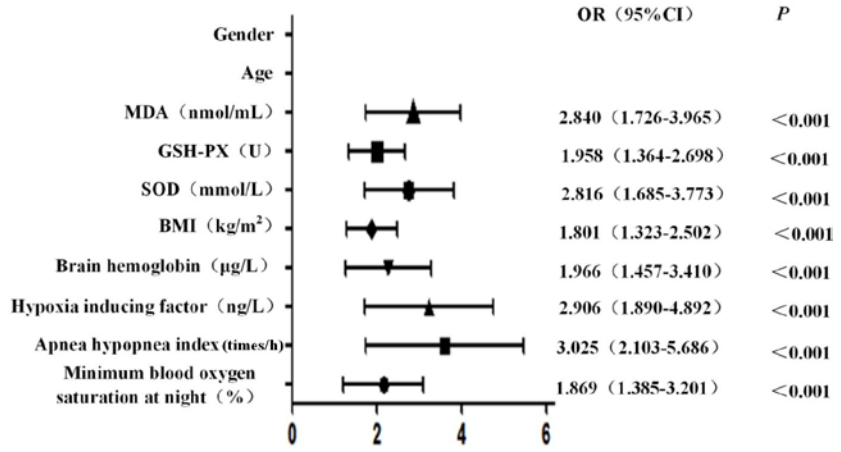

Fig. 1 – Forest plot of prediction model of cognitive impairment in patients with OSAHS. OSAHS − obstructive sleep apnea-hypopnea syndrome; MDA − malondialdehyde; GSH-PX – glutathione peroxidase; SOD − superoxide dismutase; BMI – body mass index; OR – odds ratio; CI – confidence interval.

Table 2

Correlation between cognitive impairment and oxidative stress

Score	GSH-Px		MDA		SOD	
	r	*p*	*r*	*p*	*r*	*p*
MoCA	0.421	0.013	-0.536	< 0.001	0.354	0.013
MMSE	0.625	0.004	-0.378	0.031	0.213	0.047

MoCA – Montreal cognitive assessment; MMSE – mini-mental state examination. GSH-PX – glutathione peroxidase; MDA − malondialdehyde; SOD − superoxide dismutase; *r* – Pearson's correlation coefficient.

Table 3

Predictive values of oxidative stress indices

Diagnostic index	AUC	95% CI	Cut-off	*p*	Sensitivity (%)	Specificity (%)
GSH-Px (U)	0.670	0.591–0.748	251.07	< 0.001	63.39	75.68
MDA (nmol/mL)	0.702	0.623–0.781	9.46	< 0.001	68.96	75.41
SOD (U/L)	0.705	0.629–0.782	98.32	< 0.001	67.53	76.27
Combination	0.836	0.781–0.889	–	< 0.001	70.16	83.24

MDA – malondialdehyde; GSH-Px – glutathione peroxidase; SOD − superoxide dismutase.; AUC – area under curve; CI – confidence interval.

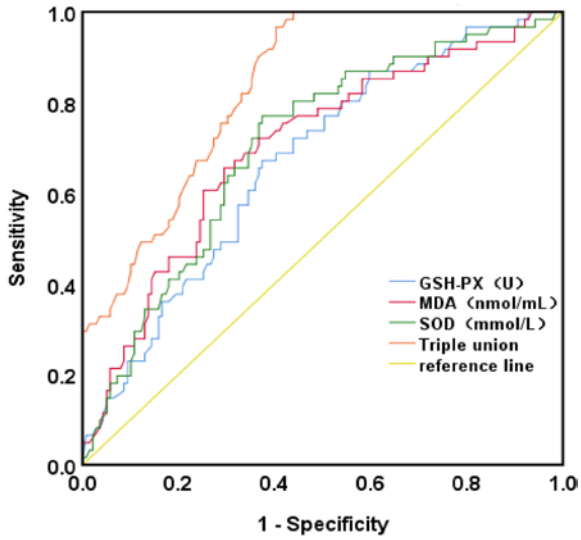

Fig. 2 – ROC curves for predictive values of oxidative stress indices for cognitive impairment in patients with obstructive sleep apnea-hypopnea syndrome.
GSH-PX – glutathione peroxidase; MDA − malondialdehyde; SOD − superoxide dismutase.

Discussion

OSAHS, a chronic disease characterized by recurrent partial or complete airflow obstruction during sleep, is increasingly becoming the cause of morbidity and death. During sleep, the muscle regulation function of the nervous system to the upper airway is decreased, and the upper airway becomes narrow, which can cause a partial or complete collapse of the airway, resulting in hypoventilation. If the airway is completely obstructed, it will lead to apnea [10]. OSAHS can induce damage to multiple systems of the whole body, and when the nervous system is injured, the major manifestation is cognitive impairment in the patients. Metabolic disorders and endocrine and neurological dysfunctions are the pathophysiological causes of OSAHS. The major inducing factor of OSAHS is concentric obesity with familial aggregation [11]. Besides daytime sleepiness and lack of cognitive ability, the clinical manifestations of OSAHS also include CV, respiratory, and nervous system injuries. Moreover, OSAHS is also an independent risk factor for morbidity and death from CVD, hypertension, and CEVD (such as atherosclerosis, arrhythmia, and ischemic heart disease), seriously affecting people's health and quality of life [12]. Some patients with severe cognitive impairment caused by OSAHS may develop Alzheimer's disease [13]. It is believed that the increased risk of CVD, CEVD, respiratory failure, and cognitive impairment is mediated by several mechanisms, such as sympathetic activation and oxidative stress [14]. It is speculated that the cause of oxidative stress in patients may be related to intermittent hypoxia, increased sympathetic excitability, and sleep disorder. There are few reports on the possible role of oxidative stress in the pathogenesis of cognitive impairment in patients with OSAHS [1]. Therefore, in this study, the indices of oxidative stress were measured, factors for cognitive impairment in patients with OSAHS were analyzed, and whether oxidative stress is connected with cognitive impairment in patients with OSAHS was explored.

In recent years, the influencing factors and pathogenesis of cognitive impairment in patients with OSAHS have been extensively studied [15]. The factors influencing cognitive impairment in patients with OSAHS include education, obesity, and intermittent hypoxia at night [16]. Young et al. [17] found that age, BMI, and lowest oxygen saturation are risk factors for cognitive impairment in patients with OSAHS. In this study, 220 patients were enrolled and divided into three groups, i.e., the snoring group, the mild to moderate OSAHS group, and the severe OSAHS group. These results suggest that patients with OSAHS suffer from cognitive impairment, which positively correlates with the severity of OSAHS. According to the analysis of risk factors for cognitive impairment, BMI, GSH-Px, MDA, SOD, neuroglobin,

hypoxia-inducing factor, AHI, and lowest nocturnal oxygen saturation were independent risk factors for cognitive impairment in patients with OSAHS, in line with the results of a previous study [17]. The identification of these high-risk factors is helpful for screening the high-risk factors of patients as soon as possible and reducing the incidence rate of cognitive impairment in patients with OSAHS.

OSAHS is a type of oxidative stress disease. The recurrent process of hypoxia and reoxygenation in patients results in the damage of various cellular structures of the human body, which is the cytological basis of various systemic diseases in patients with OSAHS [18]. The oxidative stress index of MDA content is able to directly reflect the degree of lipid peroxidation in the body and indirectly reflect the degree of cell injury. GSH-Px protects the integrity of cell membrane structure and function, which has a negative correlation with the severity of the disease. The function of SOD is to eliminate excessive oxygen free radicals and prevent tissues and organs from being damaged by free radicals [19]. Therefore, it was speculated that the abnormal oxidative stress level might be the molecular mechanism of cognitive impairment in patients with OSAHS. According to the study of Li et al. [6], MDA and SOD were representative indices of oxidative stress response of biological function for cognitive impairment in patients with OSAHS. OSAHS patients have a higher incidence rate of cognitive impairment, and oxidative stress may be one of the pathogeneses of OSAHS-related cognitive impairment [20]. In this study, there were significant differences in the levels of MDA, GSH-Px, and SOD among groups, which is consistent with previous literature [21] On this basis, the correlations between oxidative stress indices and cognitive impairment status were studied, and it was found that MoCA and MMSE scores of cognitive impairment were positively correlated with GSH-Px and SOD and negatively correlated with MDA. ROC curve analysis of cognitive impairment diagnosed by oxidative stress indices revealed that when the cut-off values of GSH-Px, MDA, and SOD were 251.07 U/mL, 9.46 nmol/mL, and 98.32 mmol/L, respectively, the AURCs were 0.670, 0.702, and 0.705, respectively, showing diagnostic values. When the three indices were adopted to evaluate the cognitive impairment of patients with OSAHS, it was found that each index had high sensitivity and specificity, indicating a certain predictive value. ROC curve analysis of the combination of the three indices showed that the maximum AURC was 0.836, suggesting that the combination of the oxidative stress parameters (GSH-Px, MDA, and SOD) can be used as a better tool to evaluate the severity of cognitive impairment in patients with OSAHS.

Conclusion

The incidence rate of cognitive impairment is high in patients with OSAHS, and there is oxidative stress in these patients. Oxidative stress is an early predictor and indicator of cognitive impairment in these patients, so it is of great significance to evaluate cognitive impairment and guide the treatment of OSAHS patients in clinical practice.

Conflict of interest

None of the authors report any conflict of interest.

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Root and canal-specific features of maxillary first molars with fused roots

Specifičnosti korenova i kanala prvih maksilarnih molara sa spojenim korenovima

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Abstract

Background/Aim. Maxillary first molars are one of the most challenging teeth for endodontic therapy. There are certain disparities in the number of roots and canals and canal interrelationships within the same root, particularly those with fused roots. The aim of the study was to assess *ex vivo* features of roots, root canals, and canal walls in maxillary first molars with fused roots. **Methods.** Out of the total of 366 maxillary first molars, 64 extracted maxillary first molars with fused roots were included in the study using cone-beam computed tomographic and microscopic examining. Tooth dimensions at the level of pulp chamber floor, number, location and distance between orifices, number and canal morphology, canal wall thickness, and features of apical *foramina* were examined and measured. **Results.** The incidence of maxillary first molars with root fusion was 17.5%, of which 60.0% was palatal fused to distobuccal root. At the level of the pulp chamber floor, the bucco-palatal dimension was significantly larger at 10.4 mm than M-D with 7.0 mm. Four canal orifices were detected in 65.6%, with the shortest distance of 1.95 mm between MB1 and MB2. In fused roots, two or three canals most frequently correlated strongly with the number of major apical *foramina*. No fusion of canals was found in fused roots. The thinnest canal wall in the mesiobuccal and distobuccal fused root was mesial with 1.25 mm, and distal with 1.31 mm, while for the palatal root, the thinnest was the palatal wall with 1.97 mm. Two or three large apex *foramina* were registered with a significant correlation with the number of canals in the fused root. **Conclusion.** The most frequent type of fusion was between the palatal and distobuccal roots. Bucco-palatal dimension at the level of the pulp chamber floor was significantly larger than the mesiodistal, with the shortest inter-orifice distance between the MB1 and MB2 orifice. The number of canals was either two or three, coinciding with the number of major apical *foramina*. There was no fusion of the canals in fused roots. The thinnest canal wall was either mesial or distal.

Key words:
maxilla; molar; multidetector computed tomography; tooth root.

Apstrakt

Uvod/Cilj. Prvi maksilarni molari su među najkompleksnijim zubima za endodontsko lečenje. Postoje određene razlike i odstupanja u morfologiji njihovih kanala, posebno kod maksilarnih prvih molara sa spojenim korenovima. Cilj rada bio je da se *ex-vivo* ispitaju morfološke specifičnosti korenova, korenskih kanala i njihovih zidova, kod prvih maksilarnih molara sa spojenim korenovima. **Metode.** Od ukupno 366 maksilarnih prvih molara, primenom kompjuterizovane tomografije konusnog snopa i stereo-mikroskopa, u studiju je uključeno i proučeno 64 zuba sa spojenima korenovima. Izmereni su i analizirani dimenzije zuba na nivou dna komore pulpe, broj, oblik, lokacija i rastojanje između ulaza u kanale, broj i morfologija kanala, debljina zidova kanala u spojenim korenovima i karakteristike apeksnih otvora (*foramina*). **Rezultati.** Učestalost spajanja korenova registrovana je kod 17,5% prvih maksilarnih molara, od čega je kod 60% zuba palatinalni koren bio spojen sa distobukalnim korenom. Bukopalatinalna dimenzija od 10,4 mm bila je značajno veća od meziodistalne, koja je iznosila 7,0 mm. Kod 65,6% zuba otkrivena su 4 ulaza u kanale, a najkraće rastojanje od 1,95 mm bilo je između MB1 i MB2 ulaza. Kod fuzionisanih korenova dva ili tri kanala su najčešće snažno korelirali sa brojem velikih apeksnih otvora. U spojenim korenovima nije registrovana fuzija kanala. Najtanji zid bio je ili mezijalni, sa prosečnom vrednošću od 1,25 mm ili distalni sa 1,31 mm, osim u palatinalnom spojenom korenu, sa palatinalnim zidom značajno veće debljine – 1,97 mm. Registrovana su dva ili tri velika apeksna otvora, uz značajnu korelaciju sa brojem kanala u spojenom korenu. **Zaključak.**

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Najčešće spajanje registrovano je između palatinalnog i distobukalnog korena. Bukopalatinalna dimenzija na nivou dna pulpne komore bila je značajno veća od mezio-distalne, a najmanje rastojanje izmereno je između MB1 i MB2 ulaza u kanale. Broj kanala bio je najčešće dva ili tri, uz direktnu korelaciju sa brojem velikih apeksnih otvora. Spajanje korenova nije pratilo spajanje kanala. Najtanji dentinski zid svih kanala bio je ili mezijalni ili distalni.

Ključne reči:
maksila; molari; tomografija, kompjuterizovana, multidetektorska; zub, korenski kanal.

Introduction

Besides adequate and thorough knowledge about usual external and internal root canal morphology and its possible variations [1], it is of utmost importance to evaluate each individual case for aberrant anatomy and to identify any morphological variation before and during the endodontic procedure of such teeth [2, 3]. Clinicians often have to treat teeth with unusual anatomy of their root canal system and atypical configurations, which is a constant challenge for diagnosing and managing such teeth [4]. Maxillary first molars are one of the most complex and challenging teeth in endodontology and endodontic practice, known as "possibly the most treated, least understood, posterior teeth" [5]. There are certain disparities and aberrations in their root morphology and configuration of the canal system, particularly in maxillary first molars with fused roots, mostly presented in various case reports and experimental studies but less in clinical evaluations or retrospective assessments [6–8]. Those variations have been attributed to differences in either ethnicity, i.e., national background, gender, or differences in study design, evaluation method, or sample size and structure [9–12].

The very beginning of the 21st century brought Cone-Beam Computed Tomography (CBCT), or Digital Volumetric Tomography (DVT), into endodontic practice, which provides three-dimensional images in a noninvasive and nondestructive way [13]. Importantly, it has been proved as a more accurate method for precise and detailed detection of root canal morphology in clinical conditions, especially in the maxillary region [9, 14, 15]. Literature that deals with the use of CBCT for revealing root canal anatomy presents and describes wide variations in morphological features of maxillary first molars, but reports are mostly focused on the number and configuration of mesiobuccal (MB) root canals, supernumerary roots, or root canals [16–19].

There are quite a few articles targeting maxillary first molars with fused roots, with or without C-shaped canals, often describing their endodontic treatment, and they are generally confined to case reports [20–22]. However, incidence, type of root fusion, root and root canal relation to other anatomical parameters that may influence and interfere with the endodontic treatment of maxillary first molars with fused roots have been presented in a few studies and literature reviews [6, 7, 23–25].

The aim of the study was to evaluate *ex vivo* anatomomorphological characteristics of the roots, root canals, and dentin canal walls in maxillary first molars with fused roots with the aid of CBCT and light microscopy.

Methods

The materials used for this study were human maxillary first molars collected from individuals of both genders, 25–60 years of age, and from both sides of the jaw. According to the Approval of the Ethics Board of the Faculty of Dental Medicine, University of Belgrade, Serbia (No 36/30 from December 21, 2011), after signing the written consent, patients' teeth were extracted due to advanced periodontal disease, prosthetic or orthodontic demands, or extremely poor prognosis for endodontic treatment. Teeth with cracked or fractured roots, apical root resorption, massive coronal destruction or restorations, as well as those undergoing endodontic treatment, were excluded from the further procedure. Tooth samples were then stored in a 3% NaOCl solution (Parcan, Septodont, Saint-Maur-des-Fossés, France) for one hour to dissolve periodontal ligaments. After cleaning the root surface, all teeth were stored in a saline solution with 0.2% thymol at 4 °C temperature until examining procedure.

From the total number of 366 collected maxillary first molars, only those teeth with two or all roots entirely fused from the cement-dentinal junction (CDJ) to the very apical portion were included in this study. Coronal preparation, trepanation, and removal of the entire pulp chamber roof were done using high-speed round diamond bur with a water spray as a coolant. Occlusal walls were flattened using diamond cylindrical bur (F011 series; Dentsply/Maillefer, Ballaigues, Switzerland), and lateral walls were refined using conical carbide bur with passive tip (EndoZF.G; Dentsply/Maillefer). Ultrasonic tips Start-X1 and Start-X2 (Dentsply/Maillefer) were used to remove dentin deposits interfering with canal orifices, which were then identified and marked using × 3.5 loupes and Micro Opener tip #1 (Dentsply/Maillefer) with neither widening nor reshaping from the original. Respecting the original root canal diameter, K-Reamers size 0.6, 0.8, and 1.0 (C-Pilot, VDW GmbH, Munich, Germany) were used to establish patency of each canal until the tip of the instrument was visible at the anatomical *foramen* under × 3.5 magnification.

After completing this procedure, four teeth were placed with their roots in a round block of impression paste Zeta Plus (Zhermak, Rovigo, Italy) with the pulp chamber floor parallel to the horizontal plane and mounted at the CBCT device with the aid of a laser positioner. CBCT examination was performed using Scanora® 3DX (Soredex, Tuusula, Finland) with a small field of view 50 x 50 mm, with a voxel size of 100 μm, 90 kVp, 10 mA. All data were analyzed in the OnDemand 3D Application computer program (CyberMed, Seoul, South Korea). Images were processed and analyzed from axial, sagittal, and coronal planes. All measurements for

each tooth sample and at each predetermined point along the roots from the coronal to the apical portion of each canal were conducted and recorded by two independent examiners, both endodontic specialists trained in CBCT techniques.

At the pulp chamber floor level, a quadrangle was drawn around each cross-section of the scan tangenting the most prominent spot on the mesial (M), buccal (B), distal (D), and palatal (P) borderline. Dimensions were measured in four directions: a) mesiodistal (M-D); b) bucco-palatal (B-P); c) mesiopalatal-distobuccal (MP-DB); d) distopalatal-mesiobuccal (DP-MB) (Figure 1a). Centers of each consecutive orifice were connected by straight lines, which formed a multi-angle, presenting a specific "dentin map" at the pulp chamber floor. The number, shape, and distance between the centers of the orifices and the "dentin map" for each tooth were recorded on CBCT scans. Distance between two neighboring centers was measured with a precision of 0.01 mm (Figure 1b). The angle between two lines connecting three consecutive orifices was expressed in degrees (Figure 1c). That enabled to determine precise orientation and localization of the canal orifices.

The statistical analysis contained a correlation between the following parameters: a) number of orifices to the number of anatomical *foramina*; b) number of orifices to the number of canals; c) number of canals to the number of anatomical *foramina*; d) distance between MB1 and MB2 canal orifices in MB fused root.

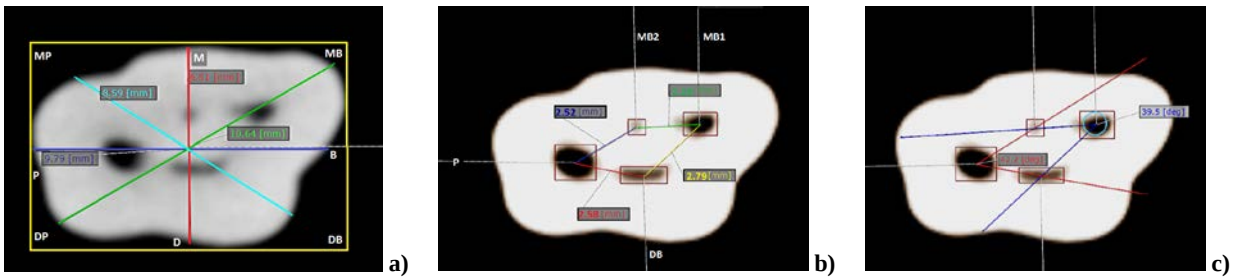
Canal morphology within the same root was categorized according to Vertucci classification [5]. To determine the thickness of the canal dentin, wall measurements were conducted on axial CBCT sections at each consecutive 100 μm of each fused root. Values were grouped as averages for the coronal (c), middle (m), and apical (a) levels for each root canal. Measurements were done from four directions: a) (M), b) (B), c) (D), and d) (P).

Following canal irrigation with 2 mL of 3% NaOCl solution tooth was impressed and centered with its coronal portion in a cube of red wax to accomplish the best position to visualize each individual *foramen*. The location and number of apical *foramina* were registered and photographed under a microscope with × 24 magnification.

Statistical methods contained descriptive analysis and the Spearma's correlation analysis that reflects the level of agreement. Inter-rater reliability was analyzed with Cohen's kappa-test for two examiners. All data were computed using the software package SPSS 20 (IBM Corporation).

Results

Of the total 366 maxillary first molars, 294 (80.3%) had three distinctive roots, 64 (17.5%) were with fused roots, and eight (2.2%) teeth were with four separate roots. Types of fusion are presented in Table 1 and Figure 2. The most



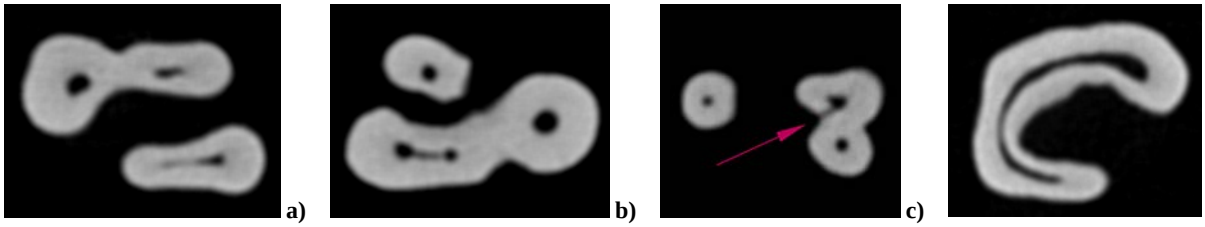
a) b) c)

Fig 1 – Tooth, distances, and angles between canal orifices at the level of pulp chamber floor:
a) measuring lines and tooth dimensions at the pulp chamber floor from various directions (M – mesial, B – buccal, P – palatal, D – distal, MB – mesiobuccal, DB – distobuccal, DP – disto-palatal, MP – mesio-palatal);
b) measuring lines and distances between canal orifices (MB1 – MB first canal, MB2 – MB second canal);
c) lines connecting three consecutive orifices with angles between the two.

Table 1
Type of fusion of maxillary first molar roots

Type of fusion	n (%)
All in one	2 (3.1)
DB - P	38 (59.4)
MB - P	16 (25.0)
MB - DB	8 (12.5)

DB – distobuccal; P – palatal; MB – mesiobuccal.



a) b) c) d)

Fig. 2 – Types of root fusion in maxillary first molars: a) all roots and canals fused into a single one (C-shape configuration); b) palatal fused to distobuccal (DB) root; c) palatal fused to mesiobuccal (MB) root; d) MB fused to DB root.

frequent root was P fused to DB (Figure 2a), followed by P to MB (Figure 2b), and MB to DB root (Figure 2c) with significant differences among all those types (Table 1; $p < 0.001$). Consecutively, P showed the strongest tendency towards fusion and MB root the least. Of 64 fused-rooted maxillary first molars, only two had all roots fused into one (Figure 2 d).

At the pulp chamber floor level, the dimension was significantly larger than MD (Table 2; $p < 0.05$). The diagonal dimension MB-DP was larger compared to MP-DB with a significant difference (Table 2; $p < 0.05$). Analysis of the orifice shape showed that circular or oval shape was found to be absolutely dominant, with a few crevice-like orifices. Four canal orifices were found in two-thirds of fused rooted maxillary first molars with a high statistical difference from other variations (Table 3; $p < 0.001$). The sides and angles of the quadrangle formed by connecting those four orifices were measured. The longest distance was between the P-DB and P-MB2 orifice, while the shortest distance was between the MB1-MB2 orifice (Table 4; $p < 0.005$). The largest angle was between neighboring lines connecting centers of MB1-MB2 with MB2-P canal orifices, and the smallest was between MB2-P and P-DB sides (Table 4; $p < 0.001$).

Either two or three canals were found in the same percent with dominant prevalence over four or five, with a high statistical difference (Table 5; $p < 0.001$). No canal fusion was detected in any of the fused roots. Canal configuration, according to Vertucci classification [5], could not be accomplished in 26 of 64 fused roots (Figures 3a and 3b).

Table 5 presents the distribution of configuration types with significant differences between types IV, VI, and VIII ($p < 0.01$). In 40% of fused roots, pulpo-periodontal

Table 2

Tooth dimensions (mm) at the level of the pulp chamber floor

Dimension	Mean ± SD	Median	Min–Max
BP	10.42 ± 0.78	10.36	9.03–11.67
MD	7.06 ± 0.41	7.04	6.27–7.97
MB-DP	11.52 ± 0.52	11.42	10.61–12.72
MP-DB	8.68 ± 1.09	8.64	6.66–10.51

BP – bucco-palatal; MD – mesiodistal; MB – mesiobuccal; DP – distopalatal; DB – distobuccal; SD – standard deviation; Min – minimum; Max – maximum.

Table 3

Distribution of the number of orifices at the pulp chamber floor

Number of orifices	n (%)
1	2 (3.1)
2	4 (6.2)
3	9 (14.1)
4	42 (65.7)
5	5 (7.8)
6	4 (3.1)

Table 4

Distance between orifice centers (mm) and angles formed by sides of a quadrangle (°)

Distance/Sides	Mean ± SD	Median	Min–Max
Distance			
MB1-MB2	1.95 ± 0.45	1.95	1.29–3.04
MB2-P	3.62 ± 0.85	3.82	2.27–4.68
P-DB	3.63 ± 0.78	3.46	2.60–5.19
DB-MB1	2.69 ± 0.51	2.92	1.60–3.36
Angle			
MB1-MB2→MB2-P	143 ± 13.68	141.00	121–170
MB2-P → P-DB	37 ± 7.05	36.50	25–51
P-DB→ DB-MB1	117 ± 15.07	117.00	85–137
DB-MB1→MB1-MB2	62 ± 10.95	60.00	44–90

MB – mesiobuccal; P – palatal; DB – distobuccal; SD – standard deviation; Min – minimum; Max – maximum.

Table 5

Distribution of the number of canals in fused roots and Vertucci-type classification [5]

Parameter	n (%)
Number of canals in a fused root	
2	30 (46.9)
3	30 (46.9)
4	2 (3.1)
5	2 (3.1)
Vertucci-type	
IV	18 (47.4)
VI	10 (26.3)
VIII	10 (26.3)

communications (PPCs) were detected at different levels of root canals (Figures 4 a–d).

Table 6 shows that the thinnest canal wall of the P fused root was mesial, followed by distal and palatal, but with no significant difference ($p > 0.1$). The buccal wall had the greatest thickness with a highly significant difference from the other three ($p < 0.001$).

The thinnest canal walls of the DB fused root were mesial and distal, with no mutual differences (Table 7; $p > 0.1$). The buccal wall was slightly thicker than the latter two (Table 7; $p < 0.05$), while the palatal wall was the thickest at all three levels, with a highly significant difference from the other three ($p < 0.001$).

The smallest values of dentin wall thickness for the MB1 canal in the MB fused root at all three levels were found for the distal one (Table 8). There was no statistical difference between values for distal, mesial, and buccal walls (Table 6; $p > 0.1$). The thickest wall was the palatal at all three levels, with significant differences from the other three (Table 8; $p < 0.005$).

Mesial and distal walls of the MB2 canal were significantly thinner than the other two at all three levels of the MB fused root (Table 9). The thickest wall was the buccal one, with a significant difference from the latter two (Table 9; $p < 0.001$). Values for the palatal wall of the MB2 canal were significantly different from the distal and mesial ($p < 0.05$), as well as from the buccal one ($p < 0 .01$).

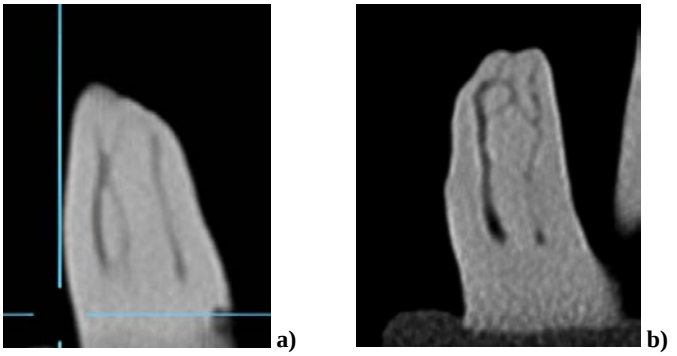


Fig. 3 – Feature of fused roots with unclassified canals upon Vertucci types [5]: a) sagittal section of fused roots with specific morphology which could not be included in Vertucci [5] classification; b) sagittal section of fused roots with specific unclassified canals, particularly in the apical third, with extreme variability of canal pathways.

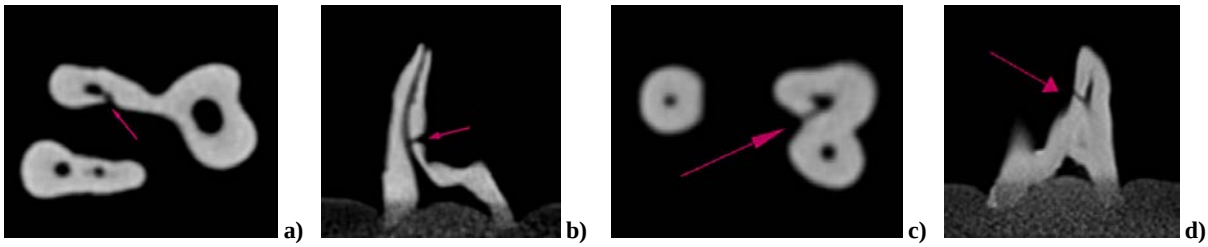


Fig. 4 – Cone-beam computed tomography (CBCT) sections presenting pulpo-periodontal communications (PPCs) specific for maxillary first molars with fused roots: a) axial section showing palatal (P) to distobuccal (DB) type of root fusion with PPCs in DB root canal with mesio-palatal (MP) orientation (arrow); b) sagittal section of the same PPCs at the coronal to mid-root location (arrow); c) axial scan of mesiobuccal (MB) to DB type of root fusion with PPCs in MB root canal (arrow); d) sagittal scan of the same MB root showing mid-root located PPCs (arrow).

Table 6

Values of the canal wall thickness (mm) in palatal (P) fused root

Localization	Mean ± SD	Median	Min–Max
C m	2.04 ± 0.22	2.03	1.72–2.58
C d	2.10 ± 0.13	2.03	2.00–2.42
C b	6.56 ± 0.40	6.60	6.03–7.14
C p	2.24 ± 0.27	2.20	1.58–2.63
M m	1.69 ± 0.38	1.73	1.15–2.44
M d	1.79 ± 0.19	1.87	1.21–2.01
M b	6.47 ± 0.65	6.60	5.60–7.76
M p	1.74 ± 0.17	1.75	1.44–2.15
A m	1.31 ± 0.25	1.29	0.88–1.87
A d	1.32 ± 0.22	1.35	0.72–1.60
A b	5.57 ± 1.10	5.32	3.50–7.67
A p	1.47 ± 0.34	1.52	0.77–2.31

C – coronal; M – middle; A – apical; m – mesial; d – distal; b – buccal.
SD – standard deviation; Min – minimum; Max – maximum.

Table 7

Values of the canal wall thickness (mm) in distobuccal (DB) fused root

Localization	Mean ± SD	Median	Min–Max
C m	1.18 ± 0.28	1.24	0.72–1.70
C d	1.43 ± 0.18	1.40	1.13–1.72
C b	2.25 ± 0.14	2.29	2.02–2.58
C p	6.34 ± 0.47	6.36	5.56–7.44
M m	1.05 ± 0.20	1.15	0.57–1.29
M d	1.18 ± 0.13	1.20	0.86–1.39
M b	2.02 ± 0.15	2.01	1.72–2.27
M p	6.31 ± 0.60	6.31	5.46–7.25
A m	1.01 ± 0.18	0.95	0.80–1.41
A d	0.97 ± 0.21	0.95	0.72–1.43
A b	1.50 ± 0.28	1.58	1.10–1.87
A p	5.75 ± 0.82	5.72	3.47–7.52

C – coronal; M – middle; A – apical; m – mesial; d – distal; b – buccal; p – palatal; SD – standard deviation; Min – minimum; Max – maximum.

Table 8

Values of the wall thickness for MB1 canal (mm) in mesiobuccal (MB) fused root

Localization	Mean ± SD	Median	Min–Max
C m	1.42 ± 0.26	1.38	1.15–2.03
C d	1.42 ± 0.26	1.45	1.00–1.87
C b	1.93 ± 0.18	1.89	1.60–2.44
C p	4.46 ± 0.34	4.50	3.44–4.86
M m	1.23 ± 0.13	1.23	0.86–1.44
M d	1.20 ± 0.14	1.17	1.00–1.43
M b	1.66 ± 0.26	1.72	1.29–2.09
M p	3.53 ± 0.70	3.31	2.30–4.39
A m	1.18 ± 0.21	1.12	0.87–1.63
A d	1.05 ± 0.20	1.09	0.71–1.41
A b	1.26 ± 0.22	1.27	1.00–1.67
A p	2.66 ± 0.841	2.73	1.15–3.68

C – coronal; M – middle; A – apical; m – mesial; d – distal; b – buccal; p – palatal; SD – standard deviation; Min – minimum; Max – maximum.

Table 9

Values of the wall thickness for MB2 canal (mm) in mesiobuccal (MB) fused root

Localization	Mean ± SD	Median	Min–Max
C m	1.15 ± 0.23	1.15	0.72–1.60
C d	1.19 ± 0.18	1.27	0.86–1.43
C b	4.26 ± 0.69	4.24	2.30–5.36
C p	2.15 ± 0.59	2.04	1.28–4.02
M m	1.04 ± 0.16	1.00	0.72–1.32
M d	0.98 ± 0.19	0.99	0.57–1.30
M b	3.51 ± 0.49	3.31	2.87–4.30
M p	2.01 ± 0.28	1.96	1.65–2.58
Am	0.99 ± 0.18	1.00	0.57–1.32
A d	0.95 ± 0.18	0.97	0.72–1.36
A b	2.55 ± 0.85	2.78	1.15–3.52
A p	1.74 ± 0.46	1.72	1.15–2.60

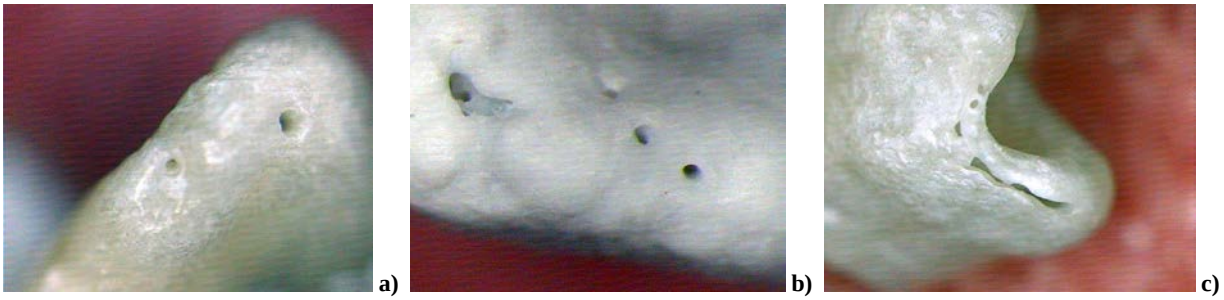
C – coronal; M – middle; A – apical; m – mesial; d – distal; b – buccal; p – palatal; SD – standard deviation; Min – minimum; Max – maximum.

Half of the fused roots had two, and approximately one-third had three major apical *foramina* (Table 10) (Figures 5a and 5b). There was a strong direct correlation between the number of major *foramina* and the number of canals in fused roots (Tables 10 and 5; Rho = 0.509; $p < 0.003$). Table 10 also presents that more than half of fused roots had no minor, i.e., accessory *foramina*, and one-quarter had one accessory *foramen* (Figures 5a and 5c).

Table 10

Distribution of the number of *foramina* on apices of fused roots with respect to the diameter

Number of *foramina*	n (%)
major	
1	6 (9.4)
2	34 (53.1)
3	22 (34.4)
4	2 (3.1)
minor	
0	36 (56.2)
1	16 (25.0)
2	6 (9.4)
3	2 (3.1)
4	4 (6.2)



a) b) c)

Fig. 5 – Stereo-microscopic images of specific anatomical *foramina*: a) minor, i.e., accessory *foramen* (left) and major anatomical *foramen* (right) on the apex of mesiobuccal (MB) fused to distobuccal (DB) root; b) huge anatomical *foramen* (left) with two anatomical *foramina* (right) on the apex of palatal (P) fused to MB root; c) long and curved crevice-like anatomical *foramen* (right) and three minor *foramina* at the apex of C-shaped fused roots.

Cohen's kappa-test showed high inter-examiner reliability of 94% (kappa-test > 0.90).

Discussion

Material for this *ex vivo* study was primarily chosen with respect to the previous study in the same population [26, 27], aiming to reveal wide scope of different morphological features and variables that characterize first maxillary molars with fused roots. There are quite a few research articles dealing with this topic, as well as case presentations, mostly accompanied by endodontic treatment or retreatment of those teeth [8, 21, 28, 29].

CBCT and scanning technique used in this study enabled to detect and register not only anatomical details of the main canal but also the presence of accessory canals, intercanal communications, their dividing and deviations, to follow their entire paths along different roots and measure the thickness of the canal walls from different aspects at various levels of the root. All those anatomical features are more complex and specific in teeth with fused than in teeth with three distinctive, i.e., separate roots. Micro-CT, even though proven as the most precise method in presenting morphological details of the root canal, is limited only to extracted teeth [30, 31]. Results of several retrospective studies of the morphology of maxillary first molars and quite a few case reports showed high precision of CBCT in revealing tiny details of their root canal anatomy both *ex vivo* and, more importantly, *in vivo*, in clinical conditions [32-34]. Bauman et al. [35] showed that the voxel size has a great impact on the accuracy in detecting multiple canals of first maxillary molars, stressing that only 60.3% of canals were detected when voxel size was 400 μm, and 93.3% with a voxel size of 125 μm for the same group of teeth. Therefore, the CBCT technique with a voxel size of 100 μm was used in this study to detect and describe important and specific morphological details in fused roots of maxillary first molars with root fusion.

The term fused root is defined as two or more roots that are united either through the deposition of cementum from the cement-enamel junction to the root apex [9], formed in the course of an individual's life, or with more histological and anatomical support, as the result of an alteration in the development of the Hertwig epithelial root sheath in the furcation area of multirooted tooth [34]. Moreover, the presence of extra canals in maxillary first molars is more frequent than the presence of fused roots, which is supported by the statement of Vertucci [20] that root fusion in three-rooted teeth is an exception from the usual anatomy.

This study showed that of the total number of examined maxillary first molars, 17.5% were found to be with fused roots. Studies that have been conducted using different methods as well as review articles and case reports, showed a wide range in the incidence of this anatomical entity, rising from none [15, 36] to 23.9% [9]. The incidence of fused roots was found to be significantly lower in the first than in the second maxillary molars, from an extreme difference of 0.7% vs.

10.7% [23] or 1.4% vs. 23.9% in Chinese patients [9]. The same incidence with almost the same values but with less reciprocal differences of 7% vs. 21% and 7.1% vs. 25.2% was found in Saudi Arabians [24] and Portuguese individuals [34], respectively. Marcano-Caldera et al. [37] found in Columbian patients an extremely high incidence of root fusion in maxillary molars of 23.3% in the first vs. 57.7% in the second, with a lower difference ratio between the two. High percentages and variations, even within the same population, could be attributed to the fact that different authors presumably applied different criteria for defining three-rooted first maxillary molars. Silva et al. [38] stated that differences could also be found due to an erroneous assessment method of morphological details. All authors pointed out the impact of ethnicity, i.e., race origin, on the prevalence and anatomical characteristics of fused roots in maxillary first molars, which was one of the main reasons to conduct this study, specifically on the Serbian population.

Age factor may affect the detection of the root canals and their morphology; therefore, patients between 25–50 years old were included in this study, as in the previous survey [26], revealing no influence of age on examined characteristics of maxillary first molars with fused roots. Mohara et al. [39] used individuals 18–45 years old, similar to this study, while Naseri et al. [40] included patients with a very wide age range from 10–70 years old, and both found no statistical difference between patients' age. The late result may be attributed to the sample size and higher concentration of individuals in particular age groups. However, most studies generally showed that as age progresses, the number of detected MB2 canals decreases [41].

Considering the influence of gender on the incidence of fused roots, this study showed no difference in the results concerning patients' sex, which coincides with results by Naseri et al. [40] and Lee et al. [41]. Conversely, Ross and Evanchik [42] reported a 13% higher incidence of root fusion in females than in males in the multinational group, which was supported by findings of Martins et al. [34] for Portuguese individuals. Marcano-Caldera et al. [37] found in that Latin Americans, 64.1% of all fused roots belonged to women, similar to results by Al-Shehri et al. [43], who found 71.4% of root fusion in females in the Saudi population.

No significant difference between left- and right-sided teeth was found in this study, confirmed by Zheng et al. [44] and previously cited authors. Zhang et al. [15] found that 84% of maxillary molars had perfect symmetry in the root and canal morphology of homonym teeth on the opposite side, similar to Felsypremila et al. [32], with 77.5% of bilateral symmetry of root fusion. In Saudi Arabians, Mashyajkhy et al. [24] found no statistical difference between patients' sex and left- and right-sided teeth in fused rooted maxillary molars. On the contrary, in the same subpopulation, Al-Shehri [43] reported a significantly higher prevalence of fused roots in the right-sided teeth. Those findings support the statements that anatomical variations between different and within the same morphological group of teeth could be affected besides ethnic factors by the sample characteristics or the varieties in methodology.

Most articles that have studied root fusion paid either no attention to the type of fusion [25, 40, 42, 45] or presented only rare cases [8, 22, 46]. Since the palatal root dominates on periapical radiography, it is clear why fusion between the massive palatal and one of the buccal roots is very hard to detect. Thus, the CBCT technique with a voxel size of 100 μm was used as it revealed the entire anatomy from all three scanning planes enabling the detection of many tiny details. The most frequent fusion was found between the P and DB roots (Table 1). Marcano-Caldera et al. [37] confirmed this result with a frequency of 58.9%, while Mashyajkhy et al. [24] in Saudi Arabians and Martins et al. [34] in Portuguese found an even higher incidence of P-DB fusion with 66.7% and 85.3%, respectively. On the contrary, among Malaysians, Al-Kadhim et al. [47] reported only the MB-DB type of fusion, which supports the impact of the ethnic foundation of root morphology, and further justifies the use of a specific national population in this study.

Of 64 maxillary first molars with root fusion, only two had all roots fused into one conical shape (Table 1). Single-rooted maxillary first molars are considered an extreme anatomical feature or certain root anomaly and have been presented as rare cases [29, 48–50] or with no incidence of such entity [24, 51]. Conversely, Marcano-Caldera et al. [37] found 16.1% of maxillary first molars with all three roots joined into a single cone-shaped, and when the authors added teeth with all three fused roots associated with one or more lateral grooves, the percentage rose to 21%. There is an enormous discrepancy between those results and the result from the present study, as well as the findings by other authors [23, 52], confirming diversity in criteria when defining root fusion.

The results revealed that the first maxillary molars with fused roots have irregular shapes and contours at their cross-section at the pulp chamber floor level and different levels of their roots. Additionally, there is a geometric inability to define measuring spots and lines that hinder or interfere with obtaining the most precise and reproducible measurements. In order to overcome those problems, a quadrangle was drawn around the axial section of each CBCT scan at the pulp chamber floor level with lines tangenting the four most prominent spots on the contour borderline, accompanied by two diagonal lines. Measurements showed that the B-P dimension was significantly larger than M-D ($p < 0.005$). Diagonally oriented diameter MB-DP was the largest one, significantly larger than the MP-DB diagonal line ($p < 0.05$), determining the cross section in a trapezoid-like shape. Quite a few authors reported on the external and internal anatomy of maxillary molars [39, 44, 45] with no information on tooth dimensions at the pulp chamber floor level, particularly not in maxillary first molars with fused roots, as presented in this study.

Results showed that a regular oval shape of the canal orifice was found in the absolute majority of cases, and the rest were crevice-like or a combination of those two, with no information in the available literature on these characteristics of maxillary first molars with fused roots. Information on the number of orifices in maxillary first molars with fused roots may be found in fewer case reports, mostly associated with their endodontic management [8, 28, 46, 50].

Considering inter-orifice distances, the most intriguing and clinically important is the one between MB1 and MB2. There are a few reports for maxillary first molars with three separate roots, and values varied from 1.20 mm detected by Spagnuolo et al. [53] to 2.90 mm presented by Magat and Hakbilen [54]. Keçeci et al. [55] measured an MB1-MB2 distance of 1.97 mm, which strongly coincides with the 1.95 mm found in this study (Table 4). Differences in those results have been attributed to variations in race, sample and voxel size, and/or experimental methods. The review of the current literature revealed no study on special geometry formed by canal orifices in maxillary first molars with fused roots. Presented results have clinical relevance when a dentist tends to negotiate canal orifices in maxillary first molars with fused roots, stressing the great importance of having proper insight into the "dentin map", particularly on MB1 and MB2 relation, which is the first instance a practitioner meets when approaching root canal instrumentation.

Roots formed by the fusion of two or more roots showed specific morphological features different from a single root, and thus they were considered a separate anatomical unit. Complex morphology complicates and hinders canal instrumentation and thus decreases the success rate of endodontic therapy, as proved by many case reports [17, 40, 41, 51]. The same incidence of either two or three canals was detected in fused roots, and no case was found with one single canal (Table 5), indicating that the fusion of the roots is not associated with the fusion of the canals. That was confirmed by Tian et al. [56] and Mashyajkhy et al. [24], with only 4.5% and 8.3% of merged canals in DB-P type of root fusion, respectively. On the contrary, Martins et al. [34] found multiple merging canals in 25%, where the confluence position was usually between the DB root and the palatal canal. Several case reports presented two rooted maxillary first molars with two canals, where the buccal orifice was the large one, most likely C-shaped, and another was a regular single palatal canal [8, 55, 57]. All those authors estimated that root fusion is not always accompanied by the merging of the canals, confirming the results from this study.

Of all multiple canals detected in fused roots, 40% could not be classified according to Vertucci types, which emphasizes their complexity (Figure 3a, b). Interestingly, PPCs were revealed in the significant incidence of 40% of all fused root canals, irrespective of the type of fusion. PPCs have always been detected on the furcation aspect of the fused root, meaning that any ingress of noxious stimuli through PPCs will inevitably cause either inter-radicular bone lesion or, vice-versa, pulp pathology. Depending on location and diameter, PPCs could complicate and cause the failure of endodontic and periradicular treatment, particularly due to the lack of their precise revealing and detection on the periapical radiographs. Therefore, CBCT should be applied whenever there is a hint of PPCs' presence. Those findings couldn't be discussed since there is no information in the available literature.

At the same apex of the fused root, there were *foramina* of various diameters, and the numerical threshold for the major (large) *foramen* was defined to be 0.3 mm and over [58], while below that value, they were classified as small, i.e., accessory *foramina*. On the apices of fused roots, more than half were with two, and one-third with three large anatomical *foramina*. The degree of correlation showed a direct and strong correlation between the number of canals and the number of major apical *foramina* in fused roots (Rho = 0.509; $p < 0.003$), indicating that the larger the number of major *foramina*, the larger the number of canals was in the "curtain-shaped" fused root. Importantly, no *foramen* coincides with the anatomic apex. As for accessory *foramina*, more than half of the apices were without any, a quarter was with one, and the rest were with two, three, or four small *foramina*. No analysis of this kind was found in the available literature. The Spearman's correlation coefficient showed no statistically significant difference between the number of canal orifices and the total number of apical *foramina* in maxillary first molars with fused roots (Rho = 0.285; $p = 0.114$). There was a tendency that a higher number of orifices was associated with a higher number of *foramina*, but with low correlation and with no significant differences between those two anatomical entities. Therefore, in clinical situations, a practitioner might predict the number of apical *foramina* upon the clear insight into the number of canal orifices when treating maxillary first molars with fused roots.

During root canal preparation with manual or engine-driven instruments, a certain amount of paracanal dentin is removed, which may often lead to either extreme thinning of walls or to worse complications in the form of strip perforation at any level of the root canal, often followed by microcracks or vertical fractures [59, 60]. The main intention of measuring the canal wall thickness in this study was to reveal critical zones, i.e., critical instrumentation areas for the specific root canal in a fused root, which would help to prevent excessive instrumentation and consequences of such endodontic preparation [6, 59, 60].

In the fused P root, the buccal wall of the canal was three to four times thicker at all three levels than the other three walls since the palatal root was always fused with one of the buccal roots with a huge inter-canal dentine layer. Mesial and distal walls were the thinnest along its entire length; therefore, it is important to bear in mind that this area is potentially a risk zone for extreme thinning, despite the massiveness of the palatal root.

A slightly different situation was with canal walls in a fused DB root, as it was fused most frequently with a P root, with three to five greater dentine thicknesses for the palatal wall. The thinnest wall was mesial at all three levels, with no statistical difference compared to the distal but significantly thinner than the buccal one. Oval canal shape in DB fused root with smaller M-D dimension is exposed to stress on mesial and distal walls as potentially prone to weakening and strip perforation during mechanical instrumentation. No data of such measuring on the palatal and DB root canals in maxillary first molars with fused roots were found in the available literature.

Considering the canal complexity in MB fused root, the dentin wall thickness of the MB1 and MB2 canals has been

put in focus. Regardless of the fact whether the MB root was fused to P or the DB root, the thinnest walls around MB1 and MB2 canals were distal and mesial, thus they could be considered a dangerous zone or "critical instrumentation areas" and most prone to procedural errors during their mechanical instrumentation. In contrast to those two, the palatal wall of the MB1 and the buccal wall of the MB2 canal were several times thicker, with very similar values at all three levels. No article particularly dealing with measuring and assessing canal wall thickness in maxillary first molars with fused roots was found. There are a few studies presenting dentin thickness from different aspects around the MB1 and MB2 canal, but only in maxillary first molars with three separate roots. Matus et al. [61] found that mesial and distal walls for MB1 and MB2 canals were the thinnest, with mean values ranging from 0.81 mm to 1.28 mm, which correlates to the values for a fused MB root in this study. Furthermore, the same authors showed the palatal and buccal walls of similar thickness to the values presented in this study. Degerness and Bowles [62] measured mesial and distal walls as the thinnest towards the coronal portion of MB root and emphasized that the average canal wall thickness decreases for one-third on the distal aspect, suggesting this area for a "danger zone" for maxillary molars at the level where MB root joins the crown of the tooth. This statement corroborates the findings by Yoo et al. [63], pointing to the distal wall as the thinnest one for both MB canals and that dentin walls around MB1 are generally thicker than around the MB2 canal, which corresponds to the results from this study. Previous authors also found that the palatal wall of MB1 and the buccal wall of the MB2 canal were approximately three times thicker, which also coincides with the results from this study. Respecting results from this and other articles, weakening of distal and mesial walls in the MB root of maxillary first molars should be avoided, particularly in those with fused roots. Thus, there is little room for procedural errors with an increasing possibility for strip perforation, which might lead to vertical root cracks and fractures. Knowledge and awareness of the presented discrepancy in the wall thickness between distal and mesial on one vs. buccal and palatal canal walls on the other side would help clinicians keep in mind that real thickness is always less than what appears in intra-oral radiographs.

Generally, mesial and distal walls of all fused roots are more sensitive to thinning at mid-root and coronal third due to the greater tapered design of endodontic instruments and specific "brushing motion" during canal preparation with rotary files. That is particularly important for moderately curved canals and in situations where the canal orifice has to be dislocated away from furcation. Therefore, the combination of variously designed canal instruments during preparation sequences could significantly decrease the production of the "dangerous zone" and thus increase the final success of the entire endodontic treatment.

For judging inter-rater reliability regarding all conducted measurements and calculated data, both CBCT scans and micro-photographs, Cohen's kappa test was used [64]. Results showed 94% agreement between the two examiners (κ-test > 0.90). This high inter-rater reliability is to be expected due to standardized and reproducible levels and locations for the detection of each anatomical entity and each of the measurements. The calculating program was calibrated to the precision of 1/10,000 of unit (four decimals), and the final score was shown with two decimals (1/100) in order to present data in a less complicated and confusing manner, with no effect on the accuracy and significance of each value.

It should be emphasized that there were neither studies nor reviews or reports that have been focused on the anatomical details in such variety and on such morphological specificities of maxillary first molars with fused roots as was presented in this study. The collected number of extracted maxillary first molars from patients of Serbian origin was representative when correlated with various studies on other ethnic groups. Due to those facts, only a few comparisons with the findings of other authors have been discussed. However, results from this study may be of great help for endodontic practice and should facilitate clinical diagnosis when one aims to predict which of those canal variations exist in the specific case. Recognizing and revealing major anatomical aberrations using all available recourses, such as CBCT and the operating microscope, is the first step towards more predictable root canal preparation and higher long-term success of endodontic therapy.

Conclusion

Of the total number of maxillary first molars collected from patients of Serbian origin, 17.5% were with fused roots. The most frequent type was P fused to DB root and significantly less P to MB and MB to DB root. At the pulp chamber floor level, the B-P dimension was significantly larger than M-D. The number of canal orifices was four in two-thirds of teeth, with the shortest MB1-MB2 orifice distance and the longest between P-DB orifices. There were either three or two canals in fused roots with a strong correlation to the number of major apical *foramina*. In the vast majority, there was no inter-canal communication. In MB and DB fused roots, the thinnest canal wall was either mesial or distal, while in the P fused root, the thinnest wall was palatal. Those walls are considered critical areas during mechanical instrumentation. CBCT scanning technique with 100 μm voxel size enabled the detection of tiny details and precise measurements. Comparing data from available literature with the results from this study, certain specificities of the anatomical characteristics were shown in maxillary first molars with fused roots within the Serbian population.

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Relationship between heat storage and parameters of thermotolerance and fatigue in exertional heat stress

Povezanost između stepena akumulacije toplote u organizmu i pokazatelja termotolerancije i zamora kod toplotnog stresa usled fizičkog napora u uslovima povišene temperature spoljne sredine

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Abstract

Background/Aim. The risk assessment of heat illness and fatigue development is essential in military service. The aim of our study was to investigate the relationship between heat storage and various psychophysiological parameters of heat stress, as well as potential peripheral markers of fatigue in soldiers performing exertional heat stress tests. **Methods.** Fifteen young, healthy, and unacclimatized men underwent an exertional heat stress test (EHST) with the submaximal workload in warm conditions (WBGT 29 °C) in a climatic chamber. Every 10 min, the following parameters of thermotolerance were measured or calculated: core temperature (Tc), mean skin (Tsk) and body temperature (Tb), heart rate (HR), heat storage (HS), physiological strain index (PSI), as well as peripheral markers of fatigue [blood concentrations of ammonia, urea nitrogen (BUN), lactate dehydrogenase (LDH), cortisol and prolactin] and subjective parameters: thermal sensation (TS) and rate of perceived exertion (RPE). **Results**. Tolerance time varied from 45 to 75 min (mean 63 ± 7.7 min). Average values of Tc, Tb, and HR constantly increased during EHST, while Tsk reached the plateau after 10 min. Concentrations of all investigated peripheral markers of fatigue were significantly higher after EHST compared to baseline levels (31.47 ± 7.29 vs. 11.8 ± 1.11 μmol/L for ammonia; 5.92 ± 0.73 vs. 4.69 ± 0.74 mmol/L for BUN, 187.27 ±2 8.49 vs.152.7 3± 23.39 U/L for LDH, 743.43 ± 206.19 vs. 558.79 ± 113.34 mmol/L for cortisol, and 418.08 ± 157.14 vs. 138.79 ± 92.83 μIU/mL for prolactin). **Conclusions**. This study demonstrates the relationship between heat storage and Tc, HR, TS, and RPE, but also with PSI. Concentrations of cortisol and especially prolactin showed a significant correlation with parameters of thermotolerance.

Key words:
body temperature; heat stress disorders; hormones; military personnel; physical exertion.

Apstrakt

Uvod/Cilj. Procena rizika od nastanka zamora i nekog oblika toplotne bolesti je od velikog značaja za vojnu službu. Cilj ovog istraživanja bio je da se utvrdi povezanost između stepena akumulacije toplote i različitih psihofizioloških parametara toplotnog stresa, kao i mogućih perifernih markera zamora u populaciji vojnika izloženih toplotnom stresu kombinovanim sa fizičkim naporom. **Metode**. Petnaest mladih, zdravih, utreniranih i neaklimatizovanih muškaraca podvrgnuto je testu toplotnog stresa (TTS) tokom fizičke aktivnosti submaksimalnog opterećenja u uslovima povišene temperature spoljne sredine (29 °C) u klimatskoj komori. Na svakih 10 min registrovane su ili izračunavane vrednosti sledećih parametara termotolerancije: unutrašnje (timpanične) temperature (Tu), srednje temperature kože (Tsk), temperature tela (Tt), frekvence srčanog rada (FSR), akumulacije toplote (AT), indeksa fiziološkog napora (IFN), kao i perifernih markera zamora [koncentracije amonijaka, uree u krvi (BUN), laktat—dehidrogenaze (LDH) kortizola i prolaktina] i subjektivnih parametara – osećaja toplote (OT) i stepena napora (SN). **Rezultati**. Vreme tolerancije variralo je između 45 i 75 min (srednja vrednost 63 ± 7,7 min). Prosečne vrednosti Tu, Tt i FSR konstantno su rasle tokom TTS, dok je Tsk dostigla plato nakon prvih 10 min. Vrednosti svih ispitivanih perifernih markera zamora bile su značajno veće nakon TTS u odnosu na vrednosti pre testa (amonijak 31,47 ± 7,29 vs. 11,8 ± 1,11 μmol/L, BUN 5,92 ± 0,73 vs. 4,69 ± 0,74

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mmol/L, LDH 187,27 ± 28,49 vs. 152,73 ± 23,39 U/L, kortizol 743,43 ± 206,19 vs. 558,79 ± 113,34 mmol/L i prolaktin 418,08 ± 157,14 vs. 138,79 ± 92,83 μIU/mL). **Zaključak**. Rezultati su ukazali na povezanost između stepena akumulacije toplote i Tu, FSR, OT i SN, ali takođe i IFN. Koncentracije kortizola i, naročito, prolaktina pokazale su značajnu povezanost sa parametrima termotolerancije.

Ključne reči:
telesna temperatura; stres uzrokovan toplotom, poremećaji; hormoni; kadar, vojni; napor, fizički.

Introduction

Fatigue is generally a useful mechanism in preventing harmful exertion and damage to the organism. On the other hand, the development of fatigue/exhaustion during strenuous physical work is of major importance for performance in the context of sports activities as well as in military service. There are several physiological and psychological factors related to the onset of fatigue: environmental conditions (especially high temperature and/or high humidity), duration and intensity of physical activity, supplementation, hydration status, motivation, and level of physical fitness. The feeling of fatigue is triggered by complex processes resulting from peripheral and central factors. Peripheral fatigue occurs within the muscle and is related to impairment in neuromuscular and muscular structures and functions [1], while central fatigue considers alterations in efferent neurons and impaired neurochemistry in the brain, such as the interplay between dopamine and serotonin, which affects mood and motivation [2]. Hence, some peripheral parameters in the blood may serve as markers of fatigue, such as lactate, ammonia, stress hormones, and pro-inflammatory interleukins [3].

The risk assessment of heat illness development is also essential in military service. There are numerous indices used in the prediction of excess heat strain during physical activity in hot conditions. The most commonly used are environmental parameters or their combination, such as Wet Bulb Globe Thermometer (WBGT) [4]. Furthermore, physiological parameters of thermotolerance are also used, such as core (tympanic) temperatures (Tc) and skin temperatures (Tsk) and heart rate (HR), with models developed to predict heat stress, such as physiological strain index (PSI). Estimating heat storage in the body is also used to evaluate both fatigue and the potential risk of overheating. Finally, some subjective parameters such as thermal sensation (TS) and rate of perceived exertion (RPE) may also serve in the prediction of the development of heat strain and fatigue [5].

Considering the importance of heat strain and fatigue in military personnel, the aim of our study was to investigate the relationship between heat storage and various psychophysiological parameters as well as potential peripheral markers of fatigue in soldiers performing an exertional heat stress test (EHST).

Methods

The study population consisted of 15 male soldiers aged 19–21, healthy, fit, and unacclimatized. The investigation was conducted at the Military Medical Academy (MMA) in Belgrade, Serbia designed as an experimental study. The study was conducted according to ethical principles for investigations in biomedical science, and signed informed consent was obtained from each participant. The subjects performed an EHST by walking on a treadmill with a submaximal workload in warm conditions [40 °C, WBGT 29 °C] in a climatic chamber (Weiss Technik, Germany). Tc and mean Tsk, as well as HR, were continuously measured every 10 min using a system for data acquisition MP 150 SKT100C (BIO PAC Systems Inc., USA) and Q4500 Exercise Test Monitor (Quinton Instruments, USA), respectively. Detailed descriptions of methods of temperature measurements were presented in our previous study [6]. The protocol of EHST and criteria for termination were also previously presented [7]. Before and immediately after EHST, venous blood samples were collected for analysis of peripheral markers of fatigue: concentrations of ammonia, blood urea nitrogen (BUN), lactate dehydrogenase (LDH), cortisol, and prolactin, and analyzed at the Institute of Medical Biochemistry, MMA. At the beginning of the EHST and every 10 min during the test, as well as at the moment when the subjects finished their tests, they assessed their subjective TS and RPE using a modified Gagge 8-point scale [8] with verbal descriptions between “cool” (ranking 5) and “unbearably hot” (ranking 13), and Borg 15-point scale of RPE [9] with verbal descriptions of physical workload between “very, very light” (ranking 6) and “very, very hard” (ranking 20), respectively.

Calculations

Body temperature (Tb) was calculated as:

$$K * Tc + (1 - K) * Tsk$$

In warm conditions, K has a constant value of 0.9 [10].

We used Tikuisis’ modification of Moran’s calculation for:

$$\mathrm{PSI} = 5 * \frac{\mathrm{Tc} - \mathrm{Tc(0)}}{39.5 - \mathrm{Tc(0)}} + 5 * \frac{\mathrm{HR} - \mathrm{HR(0)}}{180 - \mathrm{HR\ (0)}}$$

where Tc and HR represent current values of tympanic temperature and heart rate, while Tc (0) and HR (0) represent values of the same parameters at rest [5].

Heat storage (HS) was determined using Havenith’s calculation as follows:

$$\mathrm{HS} = ((0.8 * (\mathrm{Tc} - \mathrm{Tc(0)}) + (0.2 * (\mathrm{Tsk} - \mathrm{Tsk(0)})) * 3.49\ \mathrm{J/g}$$

where Tsk represents the current value of mean skin temperature, Tsk(0) represents the initial mean skin temperature, and 3.49 J/g is the specific heat of body tissues [11].

Since some subjects had shorter exposures than others, we introduced the rate of change (ROC) in investigated parameters with calculation as follows [12]:

$$\mathrm{ROC\ (x)} = \frac{\mathrm{x\ (end)} - \mathrm{x\ (0)}}{\mathrm{T}}$$

where x (end) is the investigated parameter (Tc, HR, PSI, HS, TS, RPE) at the end-point of EHST, x (0) is the value of the same parameter before the start, and T is the total exercise time.

Statistical analysis

The normality of data was tested by the Kolmogorov-Smirnov test. Data were presented as mean ± standard deviation (SD). The significance of differences between time points was tested using a *t*-test and Tukey's test for pairwise comparisons. The significance of relations was tested using the Pearson's correlation test. The statistical significance was accepted at $p < 0.05$. All statistical analyses were performed using SPSS 18 package (Chicago, USA).

Results

The baseline anthropometric and ergometric characteristics of the participants are presented in Table 1.

Tolerance time before termination of EHST (due to reaching the ethical barrier for Tc of 39.5 °C or unbearable subjective discomfort) varied between 45 and 75 min (the average time was 63 ± 7.70 min). Average values of core and body temperatures were very close and constantly increased during EHST, while mean skin temperature reached the plateau after the first 10 min, i.e., when sweating occurred (Figure 1). The average HR increased in the same manner (Figure 2).

Table 1

Anthropometric and ergometric characteristics of subjects

Characteristic	Mean ± SD	Range
Body weight (kg)	76.14 ± 7.12	65.86–88.32
Body mass index (kg/m^2)	22.9 ± 1.82	20.3–25.8
Body surface area (m^2)	1.97 ± 0.09	1.84–2.14
Body fat (%)	17.53 ± 3.33	13.8–23.1
LBM (kg)	62.77 ± 5.15	55.65–72.89
VO_{2max} (mL/kg LBM)	68.22 ± 12.16	52.13–89.98

LBM – lean body mass; VO_{2max} – maximal oxygen consumption; **SD – standard deviation.**

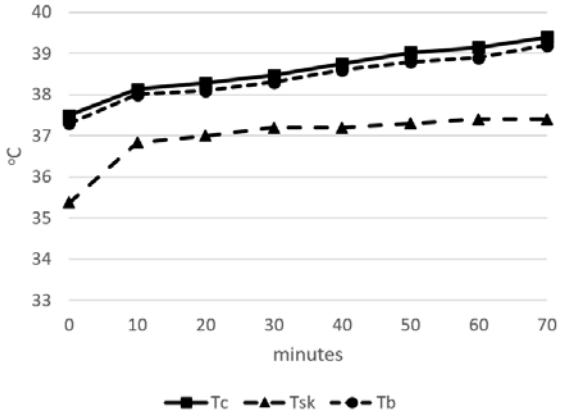

Fig. 1 – Average values of core, skin, and body temperatures during EHST.
EHST – exertional heat stress test; Tc – core (tympanic) temperature;
Tsk – mean skin temperature; Tb – body temperature.

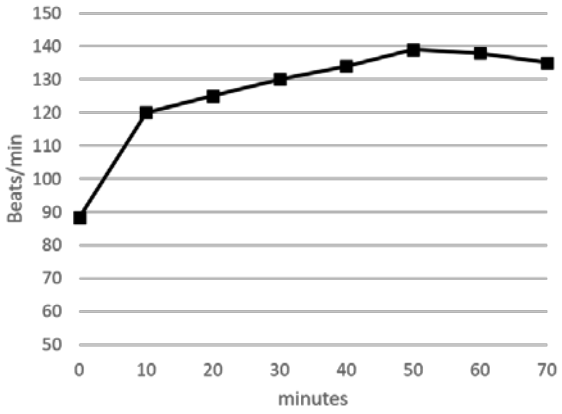

Fig. 2 – Average values of heart rate during EHST.
EHST – exertional heat stress test.

Values of all investigated peripheral parameters of fatigue were significantly higher after EHST compared to basal values (Table 2).

Average values of HS and levels of PSI constantly increased during EHST, following an almost identical pattern (Figure 3). Subjective measures of TS increased in the first 40 min, and after that, we recorded the plateau close to maximal values for the given scale, while RPE values continued to rise to the end of the test, approaching the maximal value (Figure 4).

Heat storage strongly correlated with average levels of Tc in time points between 10 and 60 min: r values varied between 0.6061 ($p < 0.05$) and 0.7894 ($p < 0.01$). HS also correlated with average HR from 20 to 60 min (r values varied between 0.54484 and 0.8498), and the strongest correlation was recorded with PSI in time points between 10 and 60 min (r values varied between 0.6778 and 0.8451). On the other hand, RPE showed a significant correlation to parameters of thermotolerance (Tc, HR, and PSI) only in the second half of the test, i.e., between 40 and 60 min (values of r coefficient varied between 0.5266 and 0.8498).

When analyzing the end-point values (the last measured values at the moment of exhaustion) of all the parameters of thermotolerance, TS, and peripheral markers of fatigue, only values of RPE and prolactin significantly correlated with HS [r = 0.59221 ($p < 0.05$) and r = 0.5516 ($p < 0.05$), respectively). There was also a significant correlation between end-point HS and VO_{2max} (r = 0.564983; $p < 0.05$), but not with

Table 2

Average concentrations of peripheral markers of fatigue before and after EHST

Marker	Before EHST	After EHST	*p*
Ammonia (μmol/L)	11.8 ± 1.11	31.47 ± 7.29	< 0.001
BUN (mmol/L)	4.69 ± 0.74	5.92 ± 0.73	< 0.001
LDH (U/L)	152.73 ± 23.39	187.27 ± 28.49	< 0.001
Cortisol (mmol/L)	558.78 ± 113.34	743.43 ± 206.19	= 0.001
Prolactin (μIU/mL)	138.79 ± 92.83	418.08 ± 157.14	< 0.001

EHST – exertional heat stress test; BUN – blood urea nitrogen; LDH – lactate dehydrogenase.

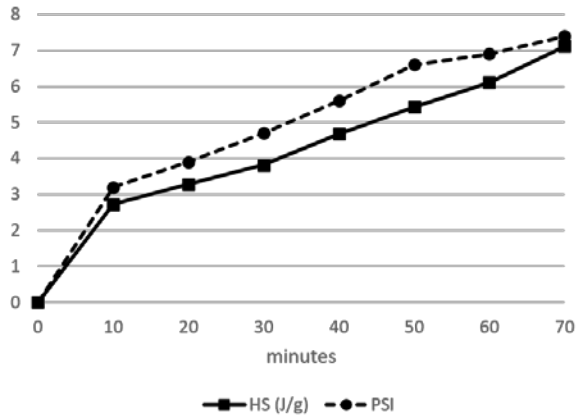

Fig. 3 – Average values of heat storage (HS) and physiological strain index (PSI) during EHST. EHST – exertional heat stress test.

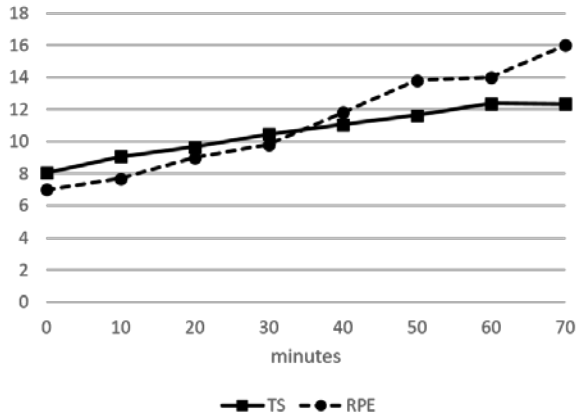

Fig. 4 – Average values of TS and RPE during EHST. TS – thermal sensation (modified Gagge's scale); RPE – rate of perceived exertion (Borg's scale); EHST – exertional heat stress test.

any anthropometric parameter whatsoever. The concentration of prolactin at the end of EHST significantly correlated with end-point values of thermotolerance: Tc, HR, and PSI (r = 0.5306; r = 0.5758; r = 0.6126, respectively; $p < 0.05$). The correlation was also highly significant between prolactin concentrations and RPE (r = 0.750084; $p < 0.001$) but not with TS.

In order to incorporate the tolerance time, we calculated rates of change in parameters of thermotolerance and fatigue between values at the very end of the EHST and the start values (Table 3).

Table 3

Rate of change (ROC) in parameters of thermotolerance and fatigue

ROC	Values
HS (J/g/min)	0.112 ± 0.016
Tc (°C/min)	0.032 ± 0.005
Tb (°C/min)	0.032 ± 0.004
HR (beat/min)	1.03 ± 0.30
PSI	0.135 ± 0.028
TS	0.064 ± 0.009
RPE	0.152 ± 0.040
Ammonia (μmol/L/min)	0.315 ± 0.142
BUN (mmol/L/min)	0.021 ± 0.008
LDH (U/L/min)	0.522 ± 0.203
Cortisol (mmol/L/min)	3.080 ± 2.255
Prolactin (μIU/mL)	4.44 ± 1.76

HS – heat storage; Tc – core temperature; Tb – body temperature; HR – heart rate; PSI – physiological strain index; TS – thermal sensation; RPE – rate of perceived exertion; **BUN – blood urea nitrogen; LDH – lactate dehydrogenase.**

When we introduced the ROC values, we found an even stronger correlation between HS and ROC RPE (r = 0.68311; $p < 0.01$), but the correlation between HS and ROC HR was also significant (r = 0.5915; $p = 0.05$).

We also analyzed the relationship between the rate of change in heat storage, i.e., the speed of increase in body heat, and end-point values of other investigated parameters. The ROC HS showed a statistically highly significant correlation with end-point values of HR and PSI (r = 0.636531; $p < 0.01$ and r = 0.570339; $p < 0.05$, respectively), as well as with concentration of prolactin after EHST (r = 0.51278; $p < 0.05$). The significance was borderline in the relation between ROC HS and values after EHST of two other peripheral markers of fatigue: concentrations of LDH and cortisol (r = 0.41812; $p = 0.054$ and r = 0.45442; $p = 0.051$, respectively).

Discussion

The high ambient temperature combined with physical activity plays an important role in physically demanding occupations such as military service [13]. Acclimatization is the most helpful method of alleviating physiological strain in hot conditions [14, 15]. Unacclimatized persons are prone to operational mean error rates when engaged in high-temperature surrounding conditions [16]. In this study, we investigated the physiological parameters of heat strain in a relatively homogenous population of young, fit male soldiers to establish a relationship between heat storage during heat stress tests and various markers of fatigue.

Finding suitable models of heat exchange between the human body and the environment has been an important issue for more than 70 years. The problem is especially pronounced when physical activity is involved. Besides classical parameters of thermotolerance such as Tc, Tsk, and HR, over 100 different heat stress indices have been explored [4]. The study conducted by Cuddy et al. [17] with 56 male participants performed EHST in conditions similar to our study and revealed several parameters which showed significant accuracy in assessing the risk of heat illness. The authors concluded that HR and Tsk, as well as PSI, may serve as predictors of heat risk. According to PSI values, subjects were divided into groups "at risk" (PSI > 7.5) and "not at risk" (PSI ≤ 7.5). Subjects in the "not at risk" group also showed significantly lower RPE, especially between 60 and 90 min of the test, which coincided with lower values of Tc and HR. In our study end-point, PSI was 8.35 ± 0.70. In the first 30 min, all subjects showed PSI under 7.5 ("no risk"). After 40 min, two subjects had PSI over 7.5, and at the end of the test, 12 of 15 had PSI over 7.5.

In a previously mentioned study [17], authors reported a significant relationship between subjective perception of heat strain and total exercise time, which is in disagreement with our results. We found no significant correlation between RPE and time before exhaustion. Our results rather support the theory of "critical Tc", proposed by Gonzales-Alonso et al. [18]. They suggested that the absolute value of "critical Tc" triggers fatigue, regardless of the total exercise time. In accordance with their results, we found that exhaustion occurred at a similar Tc when all participants rated their RPE at the almost same level, close to the upper limit of the scale.

Several studies reported that the rate of heat storage is well correlated with acute fatigue during physical work in hot conditions. That was confirmed in an experiment conducted on animal model [19], but the results of the given study are in disagreement with the hypothesis of "critical Tc". The rate of body heat storage is also related to body composition, i.e., the content of body fat [20], which is expected due to different specific heat of tissues. The cumulative value of heat storage in our investigation showed a constant increase, with a significant correlation with Tc, HR, and PSI from 10–60 min. That confirms the findings of other authors [19, 21].

Temperature sensation and thermal comfort may contribute to the self-regulation of exercise intensity. In addition, acceptability and comfort were found to be closely correlated. Zhang and Zhao [22] investigated local TS of different body parts, as well as overall TS in 30 subjects, and reported the positive correlation between these factors and thermal comfort. Other authors reported the linear correlation between the TS and ambient temperature and suggested using physiological parameters such as Tc, Tsk, and HR as predictors of thermal comfort [16]. However, the evaluation of TS is still a challenging issue [12]. Assessment of individual perception of the thermal state is commonly obtained using several stand-

ard scales, with a various number of points [23], which contributes to the difficulties in comparing the results. In our investigation, we used a modified Gagge 8-point scale [8] with verbal descriptions between "cool" (ranking 5) and "unbearably hot" (ranking 13). Gagge et al. [8] indicate that lower ambient temperature affects the subjective sensation of discomfort more than higher temperatures, and one may expect a rapid increase in discomfort with lowering Tsk. Nevertheless, values of subjective thermal comfort in our investigation showed a constant linear increase from the beginning to the end of EHST, with an average end-point value of 12.2 ± 0.6. At the end of the EHST, 12 out of 15 subjects showed values of 12, and the rest ranked their TS as "unbearably hot" (rank 13). This relatively high ranking of thermal discomfort is in agreement with the results of the previously mentioned study by Davey et al. [12], where the subjects reaching the thermal tolerance limit ranked their subjective TS with an average of 18.8 ± 1.3 using a 20-point scale. Considering the similar ambient temperature (WBGT 29 °C in our investigation and 28.79–31.85 in the given study), similar results of TS are expected.

RPE increase was faster between the 40th and 70th min compared to the first 30 min. After 30 min, it correlates with TC, HR, and PSI. Some authors suggest that participants who were allowed to self-select their exercise work by maintaining the RPE level, mobilize an anticipatory mechanism by adjusting the work rate regulating the degree of motor-unit recruitment in order to prevent a harmful increase in Tc and thus the onset of premature fatigue [21], which is confirmed by findings that RPE correlates with changes in electroencephalogram.

Finally, in our study, we wanted to investigate the potential importance of peripheral markers of fatigue and their relation to psychophysical parameters of thermotolerance. Concentrations of prolactin showed the most prominent role out of all the investigated markers. Prolactin is a stress hormone that may indicate the rate of central fatigue since its secretion is stimulated and inhibited by serotoninergic and dopaminergic neurons in the brainstem [2]. Fatigue may be considered an impairment of balance between brain secretion of serotonin and dopamine, which is reflected in prolactin concentration in blood. The increase in prolactin concentrations is expected in high-intensity and/or long-duration exercise in both cool and warm environments and passive thermal stress. Manfredelli et al. [24] found a correlation between the increase in prolactin and lactate levels during high-intensity exercise. However, prolactin response has been more pronounced during exercise in heat compared to cool conditions [25]. The investigation conducted on 21 young males exercising in the heat showed that concentrations of prolactin were more sensitive in indicating heat stress than cortisol, and the most important stimulus to prolactin secretion was an increase in Tc [26]. Nevertheless, other authors did not find any significant increase in cortisol and prolactin levels during exercise-heat stress. In their investigation, ten young and ten older men performed short-time (30 min) bouts of physical activity [3]. The acclimatization tends to alleviate the increase in prolactin during exercise in the heat [27].

Our results show the association between prolactin concentrations at the end of EHST and end-point values of Tc, HR, and PSI. Other authors also found a correlation between prolactin and parameters of thermotolerance. In their study, Wright et al. [2] investigated peripheral markers of central fatigue in a group of 23 healthy men, of which 12 were well-trained, with an average VO_{2max} of 70 ± 2 mL/kg of lean body mass (similar to our participants). They performed EHST under similar ambient conditions and similar workloads. Values of Tc, HR, and change in Tc at the moment of exhaustion were in agreement with our results. The same authors also reported a sudden decrease in circulating free tryptophan levels at Tc over 39.5 °C consistent with levels of thermal strain, which may be the consequence of increased permeability of the blood-brain barrier. However, the results of this study, as well as our findings, indicate that the increase in prolactin concentrations may serve as a peripheral marker of central fatigue, reflecting an increase in serotonin and a decrease in dopamine secretion in the brain.

Conclusion

Prevention of heat illness is one of the most important issues regarding physical activity in hot conditions. As expected, this study demonstrates the relationship between heat storage and physiological and psychological parameters of thermotolerance (Tc and HR, as well as TS and RPE). It also demonstrates the suitability of using PSI as a reliable index of thermal strain. The perception of heat strain agreed with physiological strain parameters. Concentrations of prolactin, and to some extent cortisol, showed the strongest correlation with these parameters and may thus be considered peripheral markers of fatigue.

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Burnout and coping strategies among future healthcare professionals: a structural equation modelling approach

Sindrom sagorevanja i strategije za suočavanje sa stresom kod budućih zdravstvenih radnika: pristup zasnovan na modeliranju strukturnih jednačina

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Abstract

Background/Aim. To reduce the risk of burnout development in medical professionals, it is important to identify the contributing factors as early as in their schooling years. The aim of this study was to propose a model for determining the relationship between the coping strategies used by medical high school and medical faculty students and burnout. **Methods.** The cross-sectional study included 164 students of Medical High School (80.5% female and 19.5% male) and 344 students of the Faculty of Medicine University of Belgrade, Serbia (76.9% female and 23.1% male). The model exploring the relationship between coping strategies (measured by the Brief COPE scale) and burnout [measured by the Copenhagen Burnout Inventory-student version (CBI-S) scale] was tested using structural equation modelling (SEM) analysis. **Results.** When copied with stress, Medical High School students used Acceptance, Venting, Behavioural Disengagement, and Planning, which increased their burnout, but they did not use any strategies to help them reduce burnout. When copied with stress, Faculty of Medicine students used Planning, Acceptance, Humour, Venting, Behavioural Disengagement, Self-Blame, and Substance Use, which increased their burnout, and Positive Reframing, which helped them reduce burnout. **Conclusion.** The results of this research showed an evident lack of using adaptive coping strategies with both groups of respondents. Proper education could help them replace these dysfunctional coping strategies with constructive ones.

Key words:
adaptation, psychological; burnout, psychological; health personnel; models, psychological; schools; stress, psychological; students, medical; risk factors.

Apstrakt

Uvod/Cilj. U cilju smanjenja rizika od pojave sindroma sagorevanja (SS) kod zdravstvenih radnika, važno je identifikovati faktore koji doprinose njegovom razvoju još tokom njihovog školovanja. Cilj rada bio je da se predloži model za utvrđivanje povezanosti između različitih strategija za suočavanje sa stresom, koje koriste učenici srednje medicinske škole i studenti medicine, sa SS. **Metode.** Studijom je obuhvaćeno ukupno 164 učenika Srednje medicinske škole (80,5% ženskog i 19,5% muškog pola) i 344 studenta Medicinskog fakulteta Univerziteta u Beogradu, Srbija (76,9% ženskog i 23,1% muškog pola). Model za ispitivanje veze između strategija za suočavanje sa stresom (merene skalom „Brief COPE“) i SS merenog skalom *The Copenhagen Burnout Inventory-student versio*n (CBI-S) testiran je upotrebom modeliranja strukturnih jednačina. **Rezultati.** Pri suočavanju sa stresom, učenici Srednje medicinske škole koristili su Prihvatanje, Ventiliranje, Bihevioralno neangažovanje i Planiranje, koje su im povećavali SS, a nisu koristili nijednu strategiju za pomaganje u smanjenju SS. Kod suočavanja sa stresom, studenti Medicinskog fakulteta koristili su Planiranje, Prihvatanje, Humor, Ventiliranje, Bihevioralno neangažovanje, Samookrivljavanje i Konzumiranje psihoaktivnih supstanci, što im je povećavalo SS, a Pozitivnu reinterpretaciju (redefinisanje) koristili su kako bi smanjili SS. **Zaključak.** Rezultati istraživanja pokazali su jasan nedostatak upotrebe adaptivnih strategija suočavanja sa stresom kod obe grupe ispitanika. Pravilno obrazovanje bi pomoglo da se nefunkcionalni način reagovanja na stres zameni konstruktivnim.

Ključne reči:
adaptacija, psihološka; sagorevanje na radu, sindrom; zdravstveno osoblje; modeli, psihološki; škole; stres, psihološki; studenti medicine; faktori rizika.

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Introduction

Medical professions involve high risks of burnout development [1–4]. The figures show that 25% to 80% of all healthcare professionals experience mild to severe burnout [5], while 30–50% of nurses demand clinical intervention regarding burnout [6]. The presence of burnout levels in physicians and nurses is indicated as early as in medical schools [7], with increasing rates over time predicting lower vocational preparedness and actual clinical achievement [8]. More specifically, burnout prevalence among medical faculty students reaches up to 75.2% worldwide [9], with 19% suffering from high or very high overall burnout [10]. As for nursing students, the research showed that around 25% experience overall burnout [11], with 6% experiencing high burnout [12] and almost 90% reporting low levels of professional efficacy [11].

Student burnout development is proven to be related to the ways individuals cope with stressful situations [3, 13]. Moreover, poor coping can influence students' success, satisfaction, and career development before leading to burnout [3], while effective and adequate coping can significantly reduce the risk of burnout development [14]. Exploring the relationship between coping strategies and burnout occurrence among medical high school students and medical faculty students has, so far, received insufficient attention [15], both in Serbia and globally, and has been recommended for research [16].

Therefore, the present study aims to develop a conceptual model that will unveil how specific coping strategies impact the levels of different types of burnout among medical high school and medical faculty students. The model we propose is based on the literature review of what could be identified as a distinctive field of study, proposing several analogous models so far. For instance, in 2020, by using the structural equation modelling (SEM) analysis, de la Fuente et al. [17] verified a model that explores a mediating role of coping strategies with respect to achievement emotions and engagement-burnout variables. A methodological advance was thus made since such predictive relationships could not be identified using classic analyses. Similarly, in 2019, Vizoso et al. [18] used the SEM analysis to propose a model which examines the effects of optimism and adaptive and maladaptive coping on exhaustion, cynicism, and efficacy (as burnout dimensions) as well as on the performance of undergraduate students. Following these examples, we designed a model that would test the impact of 14 distinct coping strategies [19] on medical students' overall burnout as well as on their personal, colleagues-related, studies-related, and teachers-related burnout [20].

The aims of the presented model could be multifold. Firstly, the verification of the proposed conceptual model would be done on two different populations – medical high school students and medical faculty students. Secondly, the findings would contribute to the existing body of literature as the influence of the 14 examined coping strategies on specific medical students' burnout types has not been examined yet. Finally, as the effectiveness of coping strategies is shown to be occupation-specific [4, 21], the identification of the beneficial as well as maladaptive coping strategies related to medical students' burnout should provide a basis for the appropriate training that would enable them to deal with stress more effectively [22] both at school and university and later once they become doctors and nurses [23].

Methods

This study was designed as cross-sectional and included the students of the High School of Medicine, Belgrade, and the students of the University of Belgrade, Faculty of Medicine (Serbia), who were willing to participate and gave their written informed consent. The survey was anonymous and was conducted by filling in provided online questionnaires in January and February 2020. The study protocol was approved by the Ethics Committee of the Faculty of Organizational Sciences, University of Belgrade (from June 10, 2021) and carried out in accordance with the principles of the Declaration of Helsinki.

Instruments

Sociodemographic characteristics were investigated by the 5-item sociodemographic self-reported questionnaire designed for this study. Five items were gender, age, academic year, tuition payment source (budget of the Republic of Serbia, self-funding, or other), and hometown schooling (yes or no). Student burnout syndrome was assessed using the student version of the Copenhagen Burnout Inventory (CBI-S) [20]. CBI-S is composed of four scales - Personal burnout, Studies-related burnout, Colleagues-related burnout, and Teachers-related burnout. Each scale consists of 6, 7, 6, and 6 items, respectively. Items' responses were measured on a five-point Likert-type scale (1 = Never or 0% of times and 5 = Always or 100% of times). Coping strategies were measured with Brief COPE [19]. Brief COPE consists of 28 items measuring 14 distinguishable coping reactions (2 items per scale): Active Coping, Planning, Positive Reframing, Acceptance, Humour, Religion, Using Emotional Support, Using Instrumental Support, Self-Distraction, Denial, Venting, Substance Use, Behavioural Disengagement, and Self-blame [19]. Responses were assessed on a four-point scale ranging from 0 (I have not been doing this at all) to 3 (I have been doing this a lot).

To identify the relationship between coping strategies and specific and overall burnout, we used the SEM analysis. The initial step in the SEM analysis is to inspect the internal consistency of the proposed constructs using Cronbach's alpha [24]. The relationships we aimed to measure are presented in Figure 1. After the survey had been conducted, the statistical analysis was done using SPSS 25 (IBM Corporation, Armonk, NY, USA), whereas the SEM analysis was performed using AMOS 22 (IBM Corporation, Armonk, NY, USA).

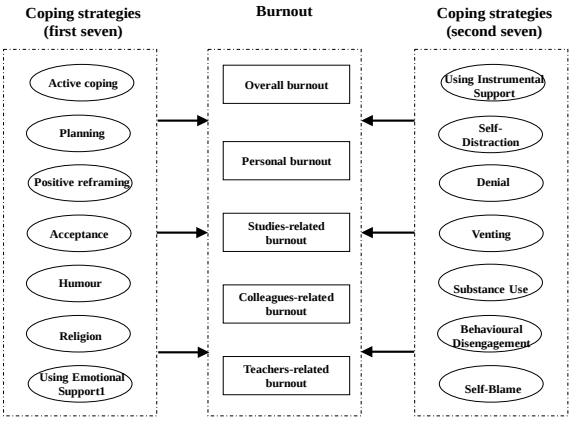

Fig. 1– Proposed conceptual model.

Results

Sample characteristics

The sociodemographic characteristics of the participants are presented in Table 1. In the sample of the High School of Medicine, 80.5% were female students. The age range was from 15 to 18. Most (53.0%) respondents were second-year students studying in their hometowns (79.3%). The sample of the Faculty of Medicine included 76.9% of female students, with an age range from 18 to 24. Most respondents were second-year students, almost 80% of the respondents had their tuitions covered by the state budget of the Republic of Serbia, and more than half of them studied in their hometown (59.3%).

Comparison of burnout levels between Medical High School and Medical Faculty students

The comparison of the levels of burnout between Medical High School and Medical Faculty students was presented in Table 2. Medical High School students were significantly more prone to studies-related, teachers-related, and overall burnout.

Table 1

Sociodemographic characteristics of students

Parameter	Medical High School in Belgrade	Faculty of Medicine, University of Belgrade
Gender		
male	32 (19.5)	77 (23.1)
female	132 (80.5)	257 (76.9)
Year of study		
first	10 (6.1)	8 (2.4)
second	87 (53.0)	102 (30.5)
third	67 (40.9)	96 (28.7)
fourth	0 (0.0)	71 (21.3)
higher years of study	/	57 (17.1)
Study in their hometown		
yes	130 (79.3)	198 (59.3)
no	34 (20.7)	136 (40.7)
Tuition payment source		
self-funding	113(68.9)	70 (21.0)
RS budget	35 (21.3)	264 (79.0)
other	16 (9.8)	0 (0.0)

All values are expressed as numbers (percentages).
RS – Republic of Serbia.

Table 2

Comparison of burnout levels between Medical High School students and Medical Faculty students

Respondents	Personal burnout	Studies-related burnout	Colleagues-related burnout	Teachers-related burnout	Overall burnout
Medical High School students	3.331 ± 1.016	3.240 ± 0.903	2.295 ± 1.029	2.678 ± 1.150	2.886 ± 0.790
Medical Faculty students	3.150 ± 0.942	2.853 ± 0.894	2.201 ± 0.989	2.261 ± 1.018	2.616 ± 0.779
Mann-Whitney test statistics	-1.691	-4.299**	-0.862	-3.864**	-3.399**

All values are expressed as mean ± standard deviation.
****p* < 0.01.**

Validation of the proposed conceptual models

Concerning Medical High School students, Cronbach's alpha ranged from 0.573 (Venting) to 0.942 (Substance Use). For Medical Faculty students, the internal consistency ranged from 0.421 (Venting) to 0.950 (Substance Use). Interestingly, the same constructs had the highest and lowest consistency for both groups of respondents. These results showed that the data is suitable for the SEM analysis.

Herein we assessed four SEM models: models of the impact of coping strategies on overall burnout and specific burnout dimensions for Medical High School students and Medical Faculty students.

The first model we assessed was the model of the impact of coping strategies on overall burnout for Medical High School students. The initial model had a low fit to the data (χ^2 = 1345.552, p < 0.000, SRMR = 0.129). In the following steps, we modified the model by removing the paths which were not statistically significant. To assess the significance of the paths, we employed bootstrapping with 1,000 samples. We first removed the paths with the highest p-value. The final model had a better fit to the data (χ^2 = 150.090, p < 0.000, SRMR = 0.089). In the final model, three coping strategies proved to have a statistically significant impact: Acceptance, Venting, and Behavioural Disengagement, with standardized coefficients of 0.169, 0.191, and 0.274. All coefficients were positive, indicating that the increase in employing these coping strategies increases burnout. The adjusted R-squared value was 0.188, indicating that 18.8% of the variability of overall burnout can be explained by the three predictors (Table 3).

The second observed model was the model of the impact of coping strategies on specific burnout dimensions for High School students. The initial model had a low fit to the data (χ^2 = 1302.753, p < 0.000, SRMR = 0.078). In the following steps, we modified the model in the same manner as the previous model. The final model had a better fit to the data (χ^2 = 231.376, p < 0.000, SRMR = 0.070). The obtained equations are given in Table 3.

Personal burnout proved to have three predictors, Planning, Venting, and Behavioural Disengagement. All coping strategies had a positive statistically significant impact on this particular burnout, meaning that these coping strategies slightly increased burnout. The adjusted R-squared value was 0.209, showing that these three coping strategies are responsible for 20.9% of the variability of personal burnout. When it comes to teachers-related burnout, the obtained model was of low quality as one significant predictor, coping strategy Behavioural Disengagement, explains only 5.6% of this specific burnout. Two coping strategies had a statistically significant impact on colleagues-related burnout: Acceptance and Behavioural Disengagement. The coefficients were stable and explained 13.1% of the variability. Finally, the only strategy that had an impact on studies-related burnout was Venting. Nevertheless, the sole predictor explained 12.2% of the variability.

Now we proceed to the SEM analysis on the Medical Faculty students. The third model we assessed was the model of the impact of coping strategies on overall burnout. The initial model had a low fit to the data (χ^2 = 1941.671, p < 0.000, SRMR = 0.066). In the following steps, we modified the model. The final model had a better fit to the data (χ^2 = 887.480, p < 0.000, SRMR = 0.083), and its assessment is given in Table 4.

In the case of the Faculty students, the overall burnout proved to have seven predictors: Planning, Positive Reframing, Acceptance, Humour, Venting, Behavioural Disengagement, and Self-Blame. All coefficients were positive, except for Positive Reframing. Therefore, the remaining six coping strategies lead to increased burnout, while the coping strategy of Positive Reframing decreased burnout. Some of the coefficients were quite low and close to zero, but they were not removed from the model as we aimed to create a high-quality model and find as many predictors as possible [25]. The coefficients on the original sample and the mean coefficients did not differ, indicating that the coefficients were stable.

The fourth observed model was the model of the impact of coping strategies on specific burnout dimensions for Faculty students. The initial model had a low fit to the data

Table 3

[1]Coping strategies' impact on different burnout levels of Medical High School students

Construct/predictors	Std. coeff.	Std. coeff. (mean)	SD	*t*	R^2
Overall burnout					
Acceptance	0.[1]69	0.176	0.083	2.032*	0.188
Venting	0.191	0.197	0.075	2.559**	
Behavioural Disengagement	0.274	0.279	0.080	3.440**	
Personal burnout					
Planning	0.198	0.207	0.085	2.336*	0.209
Behavioural Disengagement	0.212	0.215	0.084	2.505*	
Venting	0.248	0.246	0.082	3.009**	
Teachers-related burnout					
Behavioural Disengagement	0.235	0.234	0.087	2.714**	0.056
Colleagues-related burnout					
Acceptance	0.172	0.178	0.079	2.170*	0.131
Behavioural Disengagement	0.310	0.311	0.083	3.726**	
Studies-related burnout					
Venting	0.324	0.328	0.066	4.899**	0.122

[1]Assessment of the model on the impact of coping strategies on the level of different types of burnout among Medical High School students: construct, predictors, obtained standardized coefficients (std. coeff.), mean std. coeff., standard deviation (SD), *t* statistics, and the R-squared.
*** p < 0.05; ** p < 0.01.**

(χ^2 = 2024.310, p < 0.000, SRMR = 0.059). In the following steps, we modified the model in the same manner as the previous models. The final model had a better fit to the data (χ^2 = 874.919, p < 0.000, SRMR = 0.059). The obtained equations are given in Table 5. All indicators were positive, stable, and statistically significant.

Personal burnout of the Faculty students was impacted by coping strategies Humour, Venting, Substance Use, and Self-Blame. All coefficients were positive, meaning that they increased this burnout. The four predictors explained 30.8% of the variability, thus creating a model of solid quality. Similarly, teachers-related burnout can be predicted with the use of coping strategies Humour, Venting, Behavioural Disengagement, and Self-Blame. Again, the obtained model was of solid quality as the adjusted R-squared value was 0.215. Burnout related to colleagues had just two predictors, Humour and Behavioural Disengagement, which explain more than 10% of the variability (13.8%). Burnout related to studies had the most predictors, five, which create a model of the highest quality that explains 31.7% of the variability.

Discussion

There is a visible disproportion in the number of male and female students in both samples. That could have been expected as more female students enrol in the High School of Medicine [26] and the University of Belgrade, Faculty of Medicine [27].

The first conspicuous insight provided by the proposed conceptual model is that both the Medical High School and Medical Faculty students do not use coping strategies that could help them reduce burnout occurrence risk (namely faculty students use only one of such strategies while high school students use none). The strategies they use to combat stress are dysfunctional and serve only to increase their risk of experiencing burnout. Both groups of students use Acceptance, Venting, and Behavioural Disengagement, which are herein shown to be positively related to their overall burnout.

As for Acceptance, the finding is in line with the results of Shin's et al. [4] meta-analysis showing that Acceptance is

Table 4

[2]Coping strategies' impact on the overall burnout of Medical Faculty students

Construct/predictors	Std. coeff	Std. coeff (mean)	SD	*t*	R^2
Overall burnout					
Planning	0.124	0.119	0.055	2.272*	0.203
Positive Reframing	-0.134	-0.125	0.056	-2.374*	
Acceptance	0.096	0.097	0.047	2.032*	
Humour	0.213	0.209	0.045	4.753**	
Venting	0.161	0.165	0.048	3.358**	
Behavioural Disengagement	0.191	0.193	0.049	3.857**	
Self-blame	0.242	0.243	0.049	4.946**	

[2]Assessment of the model on the impact of coping strategies on the level of overall burnout among medical students: construct, predictors, obtained standardized coefficients (std. coeff.), mean std. coeff., standard deviation (SD), *t* statistics, and the R-squared.
*** p < 0.05; ** p < 0.01.**

Table 5

[3]Coping strategies' impact on different burnout levels of Medical Faculty students

Construct/predictors	Std. coeff.	Std. coeff. (mean)	SD	*t*	R^2
Personal burnout					
Humour	0.101	0.101	0.047	2.131*	0.308
Venting	0.170	0.172	0.047	3.638**	
Substance Use	0.132	0.132	0.048	2.764**	
Self-blame	0.387	0.387	0.051	7.661**	
Teachers-related burnout					
Humour	0.228	0.228	0.051	4.474**	0.215
Venting	0.158	0.161	0.058	2.733**	
Behavioural Disengagement	0.197	0.196	0.059	3.337**	
Self-blame	0.124	0.125	0.055	2.247*	
Colleagues-related burnout					
Humour	0.235	0.236	0.053	4.464**	0.138
Behavioural Disengagement	0.175	0.176	0.052	3.372**	
Studies-related burnout					
Planning	0.151	0.155	0.049	3.062**	0.317
Humour	0.134	0.134	0.048	2.767**	
Venting	0.139	0.142	0.051	2.697**	
Behavioural Disengagement	0.187	0.186	0.054	3.446**	
Self-blame	0.288	0.286	0.053	5.425**	

[3]Assessment of the model on the impact of coping strategies on the level of different types of burnout among medical students: construct, predictors, obtained standardized coefficients (std. coeff.), mean std. coeff., standard deviation (SD), *t* statistics, and the R-squared.
*** p < 0.05; ** p < 0.01.**

positively related to burnout. This finding might seem counter-intuitive as accepting the reality of a situation might imply an individual's attempt to consequently deal with the stressor [19], which makes it a positive coping strategy. Indeed, a few studies prove its negative relation to burnout [28]. However, as Lazarus [29] observed, Acceptance can be an adaptive strategy only in such circumstances when there is nothing an individual can do to change them; the relation was, for instance, later confirmed for medical professionals [30] facing dying patients. Since Acceptance leads to burnout in our examined groups, it leads us to the conclusion that the stressors could be changed, and Acceptance herein is rather a passive strategy that should be replaced and/or coupled with an active strategy. With regard to Venting and Behavioural Disengagement, both of them are confirmed to be positively related to students' overall burnout, which is in congruence with a number of other studies testing their relations to burnout and distress [31, 32]. The ineffectiveness of both strategies is referred to in Carver's [19] study that provided this very instrument (Brief COPE). Chao [33] states that using the Venting of emotions dominantly suggests that emotions are focused on distress with no adaptive behavioural strategies. On the other hand, when people tend to use Behavioural Disengagement, they stop attempting to deal with stressors, while the stress remains [34]. Therefore, both strategies are unfruitful and should be replaced with effective ones.

Apart from these three dysfunctional strategies, Medical Faculty students also use Planning, Humour, and Self-Blame, which increase their burnout, and only one strategy that is negatively related to their burnout – Positive Reframing.

Planning as a strategy occurs during secondary appraisal and is different from active coping, and mainly involves thinking about the potential active strategies and the solutions for dealing with the problem [34]. Previous studies found that planning itself is perceived as stressful by some medical students and trainees, and not following the plan is seen as even more stressful [35]. Herein, not only has planning been shown to be positively related to faculty students' overall and studies-related burnout but also to high school students' personal burnout. It may also indicate that 'a delay' in action-based coping has a deteriorating effect on the younger generations. Previous results of using Humour as a coping strategy are ambivalent, suggesting both its negative correlation with various burnout dimensions [36] and its ineffectiveness in burnout reduction [37]. Other studies suggest that different types of humour can either decrease or increase job burnout [38] and that, for instance, self-enhancing humour is negatively correlated to burnout, while self-defeating humour is positively correlated [39]. In addition, using humour to make fun of the situation can be a marker of cynicism which can be a sign of burnout. Humour was also linked with increased levels of emotional exhaustion [40]. Since it is linked with all dimensions of faculty students' burnout herein, it should be substituted with a more effective strategy. Self-blame has been shown to be the most or one of the most maladaptive coping strategies in the medical profession [32]. Overuse of Self-blame can be indicative of the lack of self-confidence and the increased levels of insecurities in students who can inaccurately perceive errors as self-made, which can, in return, paralyze them from moving further and learning how to behave in less-than-ideal situations with patients. Self-blame, along with Venting and Disengagement, has been found to be connected to elevated depersonalization as well as emotional exhaustion scores [31]. On the other hand, counseling interventions aiming to reduce self-blame have been proven to reduce emotional exhaustion as a burnout component [41]. This approach should be applied to Serbian medical students as well. Positive Reframing or, as Lazarus and Folkman [42] named it, Positive Reappraisal, is, according to them, an emotional coping strategy that manages stress-induced emotions and does not focus on the stressor *per se*. In addition, as Carver et al. [34] state, dealing with the stressful reaction positively leads an individual 'to continue' or subsequently use an active coping strategy. Its negative correlation with burnout has been confirmed by previous studies [4]. Shin et al. [4] add that it facilitates the expansion of positive emotions, which, in turn, increase one's ability for positive reframing in situations of intense and chronic stress. Herein, it has been shown as a positive and functional coping strategy and should be promoted as such and included in student stress management training.

The last strategy that appeared to be related to burnout herein is Substance Use. It is positively related to faculty students' personal burnout. van der Merwe et al. [43] confirm that students who use maladaptive coping strategies, like Substance Use, have decreased resilience to stress. Since high rates of substance abuse have frequently been reported among both medical students and health care professionals [44], this result confirms that the deteriorating effect of this dysfunctional mechanism should be communicated more seriously at the university level and students taught to use more functional strategies instead.

If the models of overall burnout are compared, the three predictors in the model of high school students (Acceptance, Venting, and Behavioural Disengagement) are among the seven predictors in the model of faculty students. This result indicates that faculty students use more coping strategies that can increase burnout than high school students and that they use one coping strategy that decreases it. When it comes to comparing the models for each specific burnout, there are some similarities. For personal and studies-related burnout, both of the groups use Venting. For teachers-related and colleagues-related burnout, both of the groups use Behavioural Disengagement. As seen, Behavioural Disengagement is herein used for people-related burnout and Venting for the dimensions of burnout which are more under the control of the self, but both strategies have already been associated with burnout and lower personal accomplishment of future physicians [40] and are, therefore, very dysfunctional.

Finally, our results show that Medical High School students are statistically significantly more prone to personal, studies-related, teachers-related, and overall burnout than Medical Faculty students, while both groups have the same level of colleagues-related burnout. That is an interesting insight emphasizing the increased need to work with medical

high school students on developing functional coping strategies and thus prevent future nurses' burnout.

Limitations of the study

This study has some limitations. It should be followed by a qualitative one to obtain more thorough insights into why certain strategies, such as humour or planning, are positively linked to burnout. Moreover, a larger sample of students, including those from other medical schools and universities in Serbia, should be included in the research to get more representative results for the entire future Serbian healthcare workforce.

Conclusion

The results of this study show an evident lack of using adaptive coping strategies, such as active coping or instrumental or emotional support, which would help students deal effectively with stress, thus decreasing the risk of developing burnout. On the contrary, the proven positive relationship between a large number of strategies the students use and burnout is alarming and should be a red flag to educators and the medical community. An adequate training should be created to help students substitute these dysfunctional ways of reacting to stress with more functional ones, which they would be able to use once they become a part of the professional workforce.

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Health professions education in Serbia: evaluation and measures for quality improvement through experiential education, interprofessional education, and teaching competencies development

Obrazovanje zdravstvenih radnika u Srbiji: procena i mere za unapređenje kvaliteta kroz praksu, interprofesionalno obrazovanje i razvoj nastavničkih kompetencija

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Abstract

Background/Aim. Health professions education is facing emerging issues. A comprehensive situation analysis was performed among academic staff, healthcare practitioners, and healthcare science students to address and respond to new trends. The aim of the study was to investigate the attitude, perception, and the recognized needs towards experiential education (EE), interprofessional education (IPE), and teaching competencies development (TCD). The critical evaluation of the existing quality standards for further quality improvement in health professions education in Serbia was provided. **Methods.** The survey on EE, IPE, and TCD was conducted within the Reinforcement of the Framework for Experiential Education in Serbia (ReFEEHS) project, co-funded by the Erasmus+ program of the European Comission at four Serbian universities (the University of Belgrade, the University of Kragujevac, the University of Niš, and the University of Novi Sad). Four task groups were appointed to perform a desk review of the existing curricula, recommendations, and practices within each of the four health professions education (Medicine, Pharmacy, Dentistry, and Nursing) in Serbia and assess the level of compliance with relevant educational policies and practices in the European Union . **Results.** A total of 1,507 respondents completed the survey. A highly expressed positive attitude was found towards EE, IPE, and TCD among all the respondents. The majority of the respondents (> 70%) shared that EE should be organized in real-life practice and involve students' work under the supervision of a qualified supervisor, as well as interactions with patients and healthcare professionals. About 90% of the respondents supported the inclusion of IPE teaching activities into EE, with 77% of students expressing high motivation to attend those classes, whereas 93% of academic staff was eager to deliver and teach joint IPE subjects. Only 20% of academic staff has already attended some TCD program, while 75% recognized the need for its organization. Moreover, 90% of healthcare practitioners have recognized that mentors/clinical supervisors also need additional skills for effective mentoring work within health science education. Based on the survey results, recommendations for improvement were given within three educational fields, healthcare science curricula, professional practice (traineeship), teaching staff, and regulations. **Conclusion.** The results derived from the survey served as a starting but also a vital point for higher education improvement in Serbia. All interested parties – academia, students, healthcare professionals, and regulatory bodies should collaborate on achieving improved, contemporary, and transformative health professions education.

Key words:
delivery of health care; education; health personnel; professional competence; serbia; students; surveys and questionnaires; teaching.

Apstrakt

Uvod/Cilj. Obrazovanje u oblasti zdravstva suočava se sa novim pitanjima. Da bi se odgovorilo na savremene zahteve obrazovanja zdravstvenih radnika, sprovedena je sveobuhvatna situaciona analiza. Cilj istraživanja bio je da se istraže stavovi, percepcije i prepoznaju potrebe akademske zajednice, zdravstvenih radnika i studenata zdravstvenih

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profesija, u odnosu prema učenju u realnom radnom okruženju, tj. nastavi u praksi (*experiential education – EE*), interprofesionalnom obrazovanju (*interprofessional education – IPE*) i unapređenju nastavničkih kompetencija (*teaching competencies development – TCD*). Radi daljeg unapređenja u oblasti obrazovanja, za zdravstvene radnike u Srbiji obezbeđeno je kritičko vrednovanje postojećih standarda kvaliteta. **Metode.** Istraživanje je sprovedeno u okviru projekta *Reinforcement of the Framework for Experiential Education in Serbia* (ReFEEHS), ko-finansiranog od strane Erasmus+ programa Evropske komisije na četiri univerziteta u Republici Srbiji (Univerzitet u Beogradu, Univerzitet u Kragujevcu, Univerzitet u Nišu i Univerzitet u Novom Sadu). Određene su četiri radne grupe, sa zadatkom razmatranja trenutnih kurikuluma, preporuka i prakse u okviru svake zdravstvene profesije (medicina, farmacija, stomatologija, sestrinstvo), kao i procene njihove usklađenosti sa relevantnim preporukama i praksom u obrazovanju u Evropskoj uniji. **Rezultati.** U istraživanju je učestvovalo 1 507 ispitanika. Pozitivan stav prema EE, IPE i TCD zabeležen je među svim ispitanicima. Više od 70% ispitanika iskazalo je stav o tome da je potrebno organizovati EE, tj. nastavu u praksi u realnom radnom okruženju, što bi podrazumevalo stručni rad studenata pod nadzorom kompetentnog mentora, ali i interakciju sa bolesnicima, kao i interakciju sa zdravstvenim radnicima. Oko 90% ispitanika podržalo je uključivanje IPE nastavnih aktivnosti u kurikulume; 77% studenata izrazilo je motivisanost da prisustvuju zajedničkim predmetima, dok je čak 93% nastavnika i saradnika iskazalo volju da učestvuju u kreiranju i podučavanju nastavnih jedinica u okviru IPE. Prethodno je samo 20% nastavnika/saradnika pohađalo neki TCD program, dok je čak 75% prepoznalo potrebu za organizacijom tih programa; 90% zdravstvenih radnika smatralo je da su za efikasan mentorski rad u obrazovanju u oblasti zdravstvene nauke potrebne dodatne veštine mentora/kliničkih supervizora. Na osnovu sagledanih rezultata upitnika, date su preporuke za unapređenje visokog obrazovanja u okviru tri obrazovne oblasti budućih zdravstvenih radnika, koje se odnose na kurikulum, studentsku stručnu praksu/klinički staž, nastavno osoblje i regulatorne aspekte. **Zaključak.** Dobijeni rezultati korišćeni su kao polazna, ali veoma značajna tačka za unapređenje visokog obrazovanja u Srbiji. Sve zainteresovane strane – akademska zajednica, studenti, zdravstveni radnici i regulatorna tela, treba da sarađuju u cilju postizanja unapređenog i savremenog obrazovanja zdravstvenih radnika.

Ključne reči:
zdravstvena zaštita; obrazovanje; zdravstveno osoblje; kompetencija, profesionalna; srbija; studenti; ankete i upitnici; učenje.

Introduction

The quality of healthcare delivery has been acknowledged as one of the global imperatives of contemporary society. It assumes the provision of safe, effective, and patient-centered health services based on collaborative interprofessional practice [1, 2]. Quality health professions education is one of the prerequisites to achieving the goals stated, including improved patient health outcomes and strengthened national health systems. However, it has been noted that "health professions education has not complied with the new global challenges, largely because of fragmented, outdated, and static curricula that produce ill-equipped graduates" [3].

As regulated professions with the anticipated mutual recognition of qualifications between the European Union (EU) member states, health professions (medical doctors, pharmacists, dental practitioners, and nurses) need to be compliant with specific and rigorous professional standards on the national and international levels. General standards and harmonized minimum training requirements are specified in the EU Directives 2005/36 and 2013/55 on the recognition of professional qualifications [4, 5], as well as the number of documents issued by the relevant national and international authorities and professional bodies [6–10].

Higher education institutions (HEI) are expected to provide evidence of continuous quality improvement, which is regularly evaluated externally through study program accreditation. There is general agreement that the accreditation process can encourage institutional improvement [11–13]. However, in order to be meaningful, this process should be based on a set of clearly defined, specific standards and procedures that reflect the societal needs and roles of each health profession. Complementary to general Standards and Guidelines for Quality Assurance in the European Higher Education Area [14], leading international and national professional bodies and associations have issued guidelines and quality standards for study programs accreditation in Medicine, Pharmacy, Dentistry, and Nursing. Contemporary trends in health professions education emphasize the importance of quality experiential education (EE)/clinical practice in the real workplace environment, opportunities for interprofessional education (IPE) of all health science students, and continuing teaching competencies development (TCD) of academic staff and teacher practitioners/clinical supervisors engaged in health professions education.

In the Republic of Serbia (RS), National Entity for Accreditation and Quality Assurance in Higher Education is responsible for HEIs and study programs accreditation. Relevant standards and procedures are defined through general rules and regulations that include certain aspects of the various fields of study [15–19]. Although particularities of the individual fields of arts and science have been recognized to a certain extent, there is a need to further develop and implement profession-specific standards in line with the provisions of the EU Directive on recognizing professional qualifications and contemporary requirements and expectations, including EE of health science students in clinical practice, IPE and TCD of academic staff and teacher practitioners.

It has been recognized that, in order to introduce these advanced teaching and learning practices and improve the quality of health professions education in RS, explicit capacity building was necessary at both the institutional and regulatory levels. In order to address the emerging issues in health professions education, representatives of the four RS state universities (the University of Belgrade, the University of Kragujevac, the University of Niš, and the University of Novi Sad) in collaboration with four EU universities (the University of Dublin, the University of Lisbon, the University of Pecs, and the University of Medicine in Sofia) took the initiative for joint action which was elaborated and shaped into the project proposal titled "Reinforcement of the Framework for Experiential Education in Healthcare in Serbia" (ReFEEHS). The Project has been approved for co-funding by the Erasmus+ program KA2-Cooperation for innovation and the exchange of good practice−Capacity Building in the field of Higher Education in the selection year 2015.

In the present paper, outcomes of the comprehensive situation analysis performed based on the survey on the attitude, perception, and recognized needs of academic staff, healthcare practitioners, and healthcare science students towards EE, IPE, and TCD are presented, including the critical evaluation of the existing quality standards for further quality improvement in health professions education in the RS.

Methods

Survey on experiential education, interprofessional education, and teaching competencies development

ReFEEHS Survey on EE, IPE, and TCD was distributed online via Google Forms in February 2016. The survey was conducted at the four RS universities (the University of Belgrade: Faculty of Medicine, Faculty of Pharmacy, Faculty of Dentistry; the University of Kragujevac: Faculty of Medical Sciences; the University of Niš: Faculty of Medicine; the University of Novi Sad: Faculty of Medicine) with the support of the professional chambers, the professional associations, and the healthcare institutions. All responses were received anonymously.

Four sets of questions were designed to assess the following: the demographic profile of the respondents (age, gender, educational background, professional title/degree, postgraduate education, professional experience); perceptions and attitudes on EE; perceptions and attitudes on IPE; perceptions and attitudes on the need for TCD. Questions were designed as close-ended, with the possibility of choosing one or more offered answers, followed by a list of twelve statements describing medical teacher roles as defined by Harden and Crosby [20]. The five-point Likert scale has been employed to rate each of the potential roles as very important (5), important (4), moderately important (3), slightly important (2), and unimportant (1). The data obtained were extracted into an Excel worksheet (Microsoft Office Excel). Descriptive and inferential statistics were performed using SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Qualitative data were presented as the number of respondents with percentage, whereas quantitative data were presented by median values with interquartile range (IQR) and total range (minimum-maximum). Mann-Whitney or Kruskal-Wallis test was used to test the difference in quantitative variables between two or among three groups of respondents, respectively, whereas the difference in proportions was tested using χ^2 or Fisher's exact test, where appropriate. A p-value < 0.05 was considered statistically significant.

The sample size was calculated based on the estimated population of 5,000 academics in higher health education, 1,000 healthcare practitioners involved in students' learning in professional practice placements, and 15,000 health science students. The required number of respondents was estimated to be 357 academic staff, 218 healthcare practitioners, and 375 students (confidence level 95%; margins of error 5%).

Survey and measures for quality improvement

Four task groups were appointed to perform a desk review of the existing curricula and practices in each of the four health professions education (Medicine, Pharmacy, Dentistry, and Nursing) in the RS and assess the level of compliance with the relevant educational policies and practices in the EU.

Professional task groups (medicine, dental medicine, pharmacy, and nursing) were composed of the representatives of the academic staff members from different EU and RS participating universities. Each task group collected and desk-reviewed available national and international documents (law, directives, guidelines, and national education policy), reports, and necessary information and performed comparative situation analysis for the eight participating universities.

Based on the outcomes of the desk review performed and insight gained, quality improvement recommendations have been drafted for each health profession, followed by the joint ReFEEHS Need for Change report [21].

Results

A total of 1,507 respondents completed the survey, with the highest proportion of students (57.9%), followed by academic staff (26.8%), and healthcare practitioners (15.3%). More than half of the academic staff (53.7%) was involved in medical students' education, whereas the majority of the students (43.5%) were attending pharmacy studies. About 80% of students were enrolled in the final year of undergraduate studies (fifth or sixth). More details on the descriptive characteristics of the study sample are presented in Table 1.

Table 1

Study sample characteristics (n = 1,507)

Parameter	Academic staff (n = 404)	Healthcare practitioners (n = 231)	Students (n = 872)	*p*-value
Age (years), median [IQR] (total range)	47 [40–54] (24–69)	42 [35–50] (26–64)	24 [24–25] (19–54)	< 0.001
Gender, n (%)				
female	273 (67.6)	191 (82.7)	665 (76.3)	0.002
Study program, n (%)				
medicine	217 (53.7)	-	293 (33.6)	< 0.001
dental medicine	77 (19.1)	-	173 (19.8)	0.737
pharmacy	78 (19.3)	-	379 (43.5)	< 0.001
nursing	13 (3.2)	-	16 (1.8)	0.124
other	19 (4.7)[a]	-	10 (1.1)[b]	< 0.001
University, n (%)				
Belgrade	135 (33.4)	-	393 (45.1)	< 0.001
Kragujevac	44 (10.9)	-	183 (21.0)	< 0.001
Niš	106 (26.2)	-	146 (16.7)	< 0.001
Novi Sad	119 (29.5)	-	149 (17.1)	< 0.001
Academic title, n (%)				
full professor	89 (22.0)	-	-	
associate professor	96 (23.8)	-	-	
assistant professor	133 (32.9)	-	-	
teaching assistant/associate	80 (19.8)	-	-	
Undergraduate degree, n (%)				
medicine	237 (58.7)	47 (20.3)	-	< 0.001
dental medicine	70 (17.3)	24 (10.4)	-	0.018
pharmacy	49 (12.1)	138 (59.7)	-	< 0.001
nursing	3 (0.7)	1 (0.4)	-	0.635
other	45 (11.1)[c]	20 (8.7)[d]	-	0.322
Postgraduate degree, n (%)				
PhD	370 (91.6)	62 (26.8)	-	< 0.001
MSc	186 (46.0)	40 (17.3)	-	< 0.001
postgraduate specialization	226 (55.9)	92 (39.8)	-	< 0.001
postgraduate diploma	28 (6.9)	35 (15.2)	-	0.001
Duration of professional practice (years), median [IQR] (total range)	20 [11–27] (0–40)	15 [8–23] (0–41)	-	< 0.001
Year of the undergraduate studies, n (%)				
1	-	-	3 (0.3)	
2	-	-	3 (0.3)	
3	-	-	10 (1.1)	
4	-	-	171 (19.6)	
5	-	-	397 (45.5)	
6	-	-	287 (32.9)	

IQR – interquartile range.

a – study program special education and rehabilitation or all four study programs (medicine, dental medicine, pharmacy, nursing); b – study program pharmacy-medicinal biochemistry; c – undergraduate studies in psychology, special education and rehabilitation, biology, chemistry, mathematics, technical science (pharmaceutics), philology (English language); d – undergraduate studies in psychology, special education and rehabilitation, biology, chemistry, mathematics, technical science (pharmaceutics).

Experiential education

The majority of respondents (> 70%) shared the perception that EE should be organized in real-life practice and involve students' work under the supervision of a qualified supervisor and interactions with patients and other healthcare professionals (Figure 1A). However, disagreement was observed regarding EE curricular aspects. More than half of the academic staff (57.7%) perceived the necessity of a strict definition of learning and teaching activities, including learning outcomes, whereas only 33% of students support such an approach to the EE ($p < 0.001$) (Figure 1A).

Educator and role model were recognized as the most important roles of EE supervisor by all three subgroups of participants, 92.6–94.6% and 64.4–77.2%, respectively (Figure 1B). Interestingly, students expected significantly more from supervisors as facilitators, friends, and advisers in comparison to academic staff and healthcare practitioners ($p < 0.05$). Concurrently, academic staff saw the EE supervisor role in the evaluation as more significant compared to

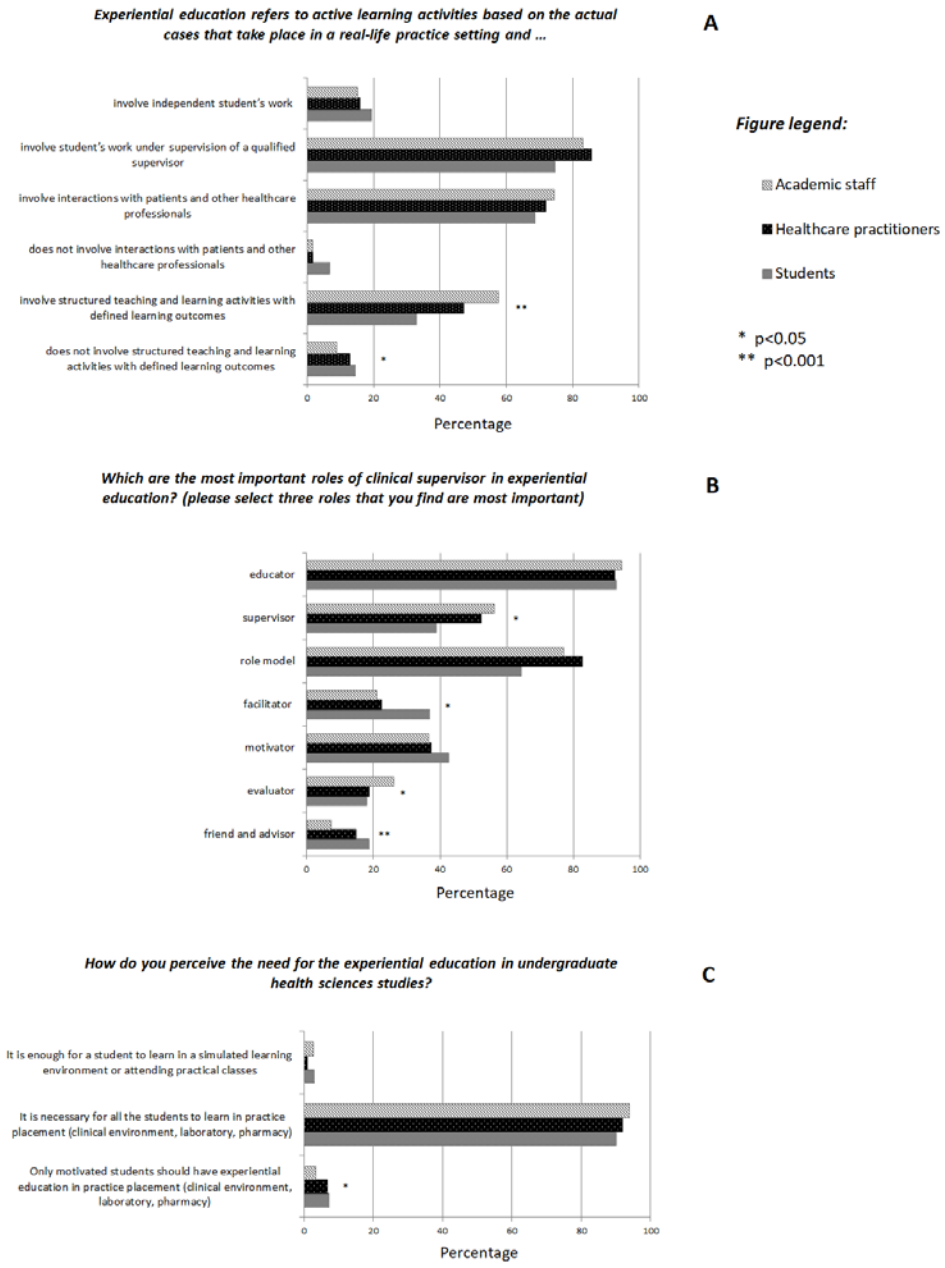

Fig. 1 – Attitudes towards experiential education.

students and healthcare practitioners ($p < 0.05$) (Figure 1B). The need for EE as an integral part of undergraduate health science studies is acknowledged by more than 90% of participants (Figure 1C).

The vast majority of healthcare practitioners (73.6%) reported the difference in professional competence between students who attended EE in practical placements and those who did not have such experience during their undergraduate studies. The following specific differences were recognized by the following proportion of healthcare practitioners: professional knowledge (55.4%), responsibility at the working place (51.1%), independence in professional practice (44.6%), self-esteem (37.7%), communication with patients (24.7%), and relations with colleagues (19.0%). However, despite acknowledged positive features of EE, healthcare practitioners stated the following available time for students: plenty of time, 0.9%; enough time, 46.8%; not having enough time, 49.4%; not having time at all, 2.6%.

Interprofessional education

The main aspects of IPE were recognized well by the majority of respondents. A higher proportion (77.4%) of correct answers regarding IPE definition was recorded among students, in comparison to healthcare professionals (74%) and academics (68.1%), $p < 0.05$. A conclusive agreement was reached with 88.5% of students and 92% of healthcare practitioners and academics related to joining IPE activities

Table 2

Attitudes towards interprofessional education (IPE), experiential education (EE) and teaching competencies development (TCD)

Attitude	Academic staff (n = 404)	Healthcare practitioners (n = 231)	Students (n = 872)	*p*-value
IPE				
Which of the following statements better describes the IPE of health science students?				
IPE occurs when two or more professions learn with, from, and about each other to improve collaboration and the quality of care.	275 (68.1%)	171 (74.0%)	675 (77.4%)	0.002
IPE occurs when students, within their study program, learn about other professions to improve collaboration and the quality of care.	129 (31.9%)	59 (25.5%)	194 (22.2%)	0.001
Do you think that EE should include some IPE teaching activities?				
yes	372 (92.1%)	214 (92.6%)	772 (88.5%)	0.061
Are you interested in jointly attending theoretical or practical lessons with other health science students?				
yes	-	-	673 (77.2%)	
Are you interested in planning and delivering teaching for joint subjects on two or more study programs in health science?				
yes	376 (93.1%)	-	-	
TCD				
Have you attended any TCD program, course, or education?				
yes	81 (20.0%)	-	-	
Is there a TCD program available at your faculty/university?				
yes	105 (26.0%)	-	-	
Is there a need for a TCD program?				
yes	304 (75.2%)	-	-	
Do you think you need additional skills for mentoring work with students/interns (e.g., assessment of their professional competencies/knowledge during practice, skills needed for successful communication between mentor and student, etc.)?				
yes, numerous skills are definitely needed	-	56 (24.2%)	-	
yes, some skills are needed	-	149 (64.5%)	-	
no, a mentor with active professional practice has all skills needed for EE	-	25 (10.8%)	-	
Would you attend a program/education devoted to TCD?				
yes, I am very interested	-	166 (71.9%)	-	
yes, but only if the employer requires	-	44 (19.0%)	-	
no, I am not interested in those activities	-	20 (8.7%)	-	

within EE. Furthermore, about 77% of students reported their interest in attending IPE, while 93% of the academic staff was eager to prepare and deliver IPE units (Table 2).

Teachers' roles and teaching competencies development

High grades were attained in the assessment of teacher roles. Over 50% of respondents evaluated eleven out of twelve roles as important or very important (Figure 2). However, academic staff stated significantly higher importance of the majority of roles compared with the rest of the contributors, particularly the students.

The following three roles were identified as the most valued by all three subsamples: "Transferring knowledge through mentoring in a real work environment", "Role model for professional and competent behavior in a real work environment", and "Directs students and facilitates learning by solving case studies, working in small groups within hands-on teaching at a faculty or in a real work environment". The first role, "Transferring knowledge through lectures in the amphitheater", was the least valued by all three groups of respondents – seen as important or very important by 65.1% of academic staff, 58.3% of healthcare practitioners, and 42.6% of students.

Only 20% of academic staff has already attended some TCD program, course, or education, whereas 26% reported that a TCD program was available at their faculty or university. Three-fourths of academics (75.2%) recognized the need for organizing a TCD program, and 71.9% expressed high interest in attending such a program (Table 2). Moreover, even 90% of healthcare practitioners have recognized that mentors/clinical supervisors also need additional skills for effective mentoring work within health science education (Table 2).

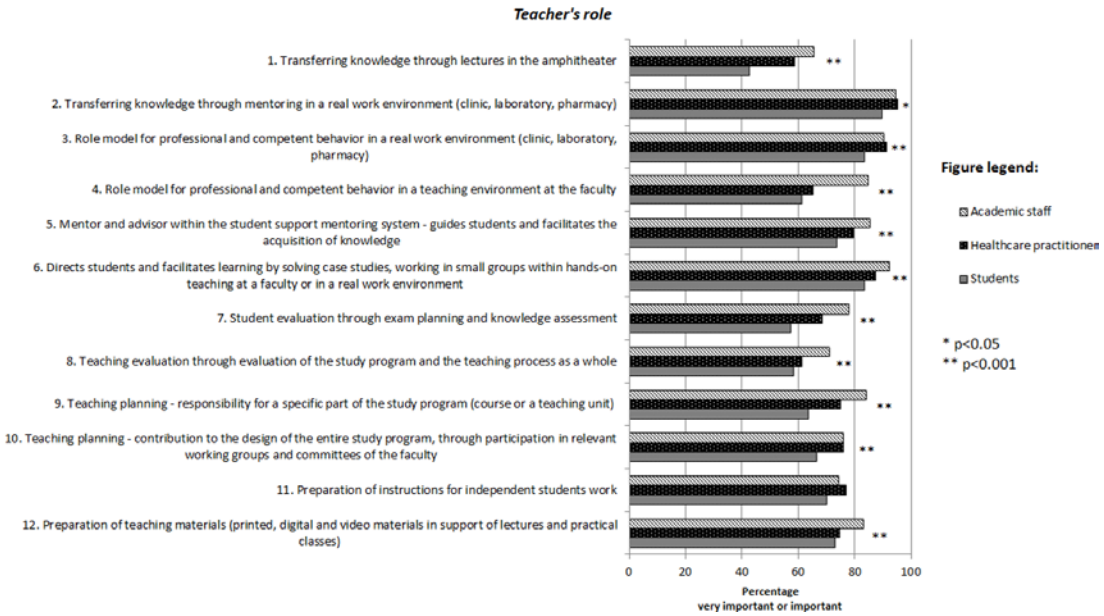

Fig. 2 − Perceptions of teacher's role in health professions education.

Follow-up of the ReFEEHS

The need for changing the recommendations for improvement was given within three educational fields, healthcare science curricula, professional practice (traineeship), teaching staff, and regulations.

In the second year of the project outputs follow-up, all six recommendations for curricular improvement were adopted completely or at least partially. Introducing an IPE course established at all participating HEIs in RS is among the main curricular innovations. Three generations have successfully completed the course until now, including almost six hundred students, with the highest number, 287, being from the University of Belgrade.

However, follow-up of the curricular outputs pointed out several critical issues which should be additionally reinforced. Complete adoption of EU directives 2005/36 and 2013/55 requirements, dominantly in study programs and practical training duration, are particularly important for pharmacy, dentistry, and nursing curricula. There is only one pharmacy study program and one dentistry program in the RS completely harmonized with the requirements of EU directives in the abovementioned fields. Moreover, the use of students' feedback in the development of the educational process should be improved through the monitoring of the received feedback implementation level. Finally, although active student participation in curricula evaluation and development is highly recommended, this is only partially attained through their membership of the curricula committee at several HEIs.

The level of implementing the ReFEEHS recommendations for professional practice (traineeship), teaching staff, and regulations improvement was also analyzed. Development and adaptation of common standards and requirements for professional practice are one of the major project outputs reached for all study programs. However, there are some differences in implementing defined standards between different HEIs since the learning experience within students' professional practice is still not completely comparable at the national level. Innovative elements within the field of professional practice include the implementation of Objective Structured Clinical Examination at the pharmacy study program (the University of Belgrade) and the foundation of two simulated labs (the University of Belgrade and the University of Novi Sad) equipped with manikins acquired through the project.

One of the advanced project outcomes is the development of the formal program for TCD and its implementation at the University of Belgrade and the University of Niš. Since the establishment of the program, two generations involving 49 academic staff and 19 teacher practitioners at the University of Belgrade and one generation of 16 academic staff at the University of Niš have completed the program.

Discussion

To the best of our knowledge, this is the first large-scale study conducted to explore the attitudes of academic staff, healthcare professionals, and students related to three pivotal aspects of contemporary healthcare professions education – EE, IPE, and TCD. In order to address the fast-changing landscape of healthcare, which will be even more prominent in the coming years, it was necessary to strongly encompass education, practice, and science [22].

Experiential education

EE is defined by the Association for Experiential Education as "a philosophy that informs many methodologies in which educators purposefully engage with learners in direct experience and focused reflection in order to increase knowledge, develop skills, clarify values, and develop peo-

ple's capacity to contribute to their communities" [23]. The current research pointed out that the attitudes of the study participants are compliant with the abovementioned definition and other publications [24–29]. However, quite a low number of academic staff (57.7%), healthcare professionals (47.2%), and students (33%) recognized it as important to define structured teaching and learning activities, including learning outcomes. A lack of structured approach to delivering the EE activities might lead to inequality in EE teaching activities delivery among academic staff and supervisors, who are relied on their personal experience, attitude, and daily professional responsibilities. In this situation, reaching expected learning outcomes could be seriously jeopardized. These concerns gathered the ReFEEHS project team members into four EE working subgroups (medicine, dentistry, pharmacy, and nursing), with the main tasks to identify EE competency-based outcomes, develop EE educational contents and resources, develop/reinforce EE curricula, and design and adopt EE Quality Assurance (QA) documents. All tasks are completed and published within the QA Standards for Student Professional Practice Placement in Health Professions Education [30].

Along with EE teacher practitioners' roles assessed as the most important by all study participants (educator, role model, and motivator), some contrary attitudes of academics compared to students were recorded. The role of supervisor and evaluator was the most valued by the academics, and the lowest by the students, whereas facilitator, friend, and adviser was the role of the highest importance for students and the lowest for academics. Converging different attitudes and expectations from both sides is essential in reaching constructive communication, a positive learning atmosphere, and defined EE learning outcomes.

Interprofessional education

Assessing the knowledge and attitudes towards IPE, a forward-looking and driving approach was found among all the participants. As a matter of fact, IPE attracted high interest among the interviewees. Further, both academics and students expressed a very high level of interest in preparing, teaching, and attending the IPE course. Consequently, an IPE working group was established to develop and introduce IPE courses at all participating HEIs in the RS. EU Consortium partners contributed through presentations of their IPE curricula and discussions of various models for course development and implementation. In order to ensure a consistent quality of IPE teaching activities, the Interprofessional Education Handbook was published in October 2018 [31].

Teaching competencies development

The research results pointed out the necessity for TCD within healthcare education in Serbia. Accordingly, the TCD working group has been appointed with the main task of developing and implementing the TCD program. At the beginning of this process, 12 members of the RS academic staff were enrolled in the external, international postgraduate courses in health professions education provided by the University of Dundee and the International Association of Medical Education. They serve as a driving force for the Teaching Certificate in Health Professions Education development. Additionally, EU partner institutions (dominantly Trinity College Dublin) hosted academic staff from RS within the Teaching & Learning Center to discuss TCD program development. The program was designed and approved by the Senate of the University of Belgrade (February 2018) as continuous professional education for academic staff and teacher practitioners. Moreover, the TCD working group developed recommendations and guidelines for TCD and evaluation in the RS, compiled in the document entitled "Teaching competencies development and evaluation: Guidelines for quality health professions education" [32].

ReFEEHS project outputs follow-up

Results of the ReFEEHS project outputs follow-up, two years after the project ended, pointed out some important issues for further improvement and identified the main challenges for healthcare professions education in the RS. Particularly important items are those related to the harmonization with EU directives 2005/36 and 2013/55 on the recognition of professional qualifications. It is very clear that the overall study program duration has to be extended up to the required number of hours for dental (5,000 hrs) and nursing (4,600 hrs) education at three HEIs [4]. Additionally, pharmacy students' traineeship has to be extended up to six months at three HEIs [4]. Without the implementation of these requirements, harmonization with EU directives will not be completed, while recognition of the professional qualifications of graduates from Serbian HEIs will be further aggravated.

Recommendations related to reinforcement and QA of healthcare students' professional practice are, in most cases, only partially or even not at all fulfilled. That presents a threat to reaching common and comparable learning outcomes for healthcare graduates and, accordingly, the potential cause of gaps in acquiring an equable level of healthcare at the national level. Therefore, harmonizing professional practice issues (e.g., the adoption of the national framework for professional practice experience, ensuring comparable learning experiences for all students, etc.) for each study program is essential to be attained in the future.

Finally, the main challenges for improvement in the upcoming years will be establishing relevant quality standards for professional practice, which should be recognized by the National Rules and regulations on accreditation standards, as well as remodeling the state exams in the RS in order to be focused on practical skills and knowledge. Although recommendations related to the abovementioned fields were given four years ago, there has been no evident progress until now. QA Standards for Student Professional Practice Placement in Health Professions Education defined and adopted within the ReFEEHS project could be particularly useful in developing national standards for professional practice. This guideline involves well-developed recommendations organized within six standards, including curriculum, competency-based

learning outcomes, teaching methods, learning outcomes assessment, practical placement sites, and students' obligations and responsibilities [30].

ReFEEHS project follow-up results have also shown some important progress and innovations in healthcare curricula achieved during the project and maintained after the project completion. Among these outputs, there is the establishment of the IPE courses, which present a base for underpinning interprofessional and collaborative practice defined as "the foundation for high quality, safe, and compassionate care, which is truly integrated and person-centered" [33]. Additionally, the introduction of the formal program for TCD designed explicitly for academic staff and teacher practitioners involved in healthcare professions education presents advanced output. It brings the universities that implemented such courses to the list of a limited number of EU universities that established such programs.

Strengths and limitations of the study

A broad questionnaire was applied in data collection, providing an in-depth understanding of three pivotal aspects of contemporary healthcare professions education, EE, IPE, and TCD, expressed by main partners in the higher education process, academic staff, healthcare professionals, and students. Moreover, four healthcare professions (medical doctors, dentists, pharmacists, and nurses) were involved, thus increasing the validity and generalizability of our results.

Among the 1,507 respondents, students were more frequent, with a share of 58%, which may reflect their motivation to influence and foster the changes in higher education curricula. Moreover, about 80% of student respondents were enrolled in the final years of undergraduate studies, which provides more relevant information on students' needs and concerns. Further, almost equal distribution among academic staff was found for different academic ranks (teaching assistant/associate, assistant professor, associate professor, full professor), which may reduce the study bias in expressing their attitude. Healthcare professionals were, on average, highly experienced, with a median duration of professional practice of 15 years (IQR 8-23); they are thus also considered a good representative sample.

Applied methodology with anonymized data collection enabled us to assess and contrast beliefs between the academic staff, healthcare professionals, and students (pairwise). Not only were attitudes explored within one focus group in the higher education process, but also was the insight into others' needs and concerns provided. Covering different points of view, a summary of current practice, challenges, as well as recommendations for improvement were retrieved from the responses.

It could be supposed that participants in the current study are those particularly enthusiastic and motivated to improve healthcare education in the RS. Accordingly, one should be careful with the generalization of the results, despite recognizing a high number of participants as interested parties. However, no differences could be explored between health professions since nursing was represented by a small number of students and practitioners. Nursing is not well-established in higher education in Serbia, having a relatively small number of students enrolled per year. Additionally, representatives of the regulatory bodies, such as important stakeholders in the researched fields and their attitudes, are missing in the current study, which could significantly affect the overall study results.

The results of the current study should be interpreted in line with the abovementioned strengths and limitations.

Conclusion

The results derived from the ReFEEHS survey on EE, IPE, and TCD served as a starting but also a crucial point for higher education improvement in the RS. All interested parties – academy, students, healthcare professionals, and regulatory bodies should collaborate on achieving improved, contemporary, and transformative health professions education. Implementing the reinforced EE and IPE will contribute to the long-term continuous improvement of the professional competencies of health science students, graduates, and experienced practitioners. It is anticipated that through the reinforced EE, students will develop professional and personal competencies needed for continuing professional development as self-centered lifelong learners. The introduction of IPE activities will form a foundation for future interprofessional collaborative practice, which is perceived as the preferable model for healthcare delivery. Attainments within EE and IPE will be braced by the improvement of teaching competencies. The Teaching Certificate in Health Professions Education may serve as a teacher/mentor/preceptor portfolio in applying contemporary principles and standards of quality assurance in academic teaching and delivering professional/clinical practice for health professionals – medicine, dental medicine, pharmacy, and nursing. The explored aspects of EE, IPE, and TCD represent the major interconnected trends in contemporary health professions education. The obtained results should serve as guiding principles to continuously work on and monitor the improvement of health professions education and healthcare delivery in our system. Barriers between education, policy, and clinical practice should be actively investigated, recognized, and appropriately addressed to improve the health of the community as the ultimate goal.

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The effect of cross-linking procedure on corneal wavefront aberrations in patients with keratoconus

Uticaj *cross-linking* procedure na kornealne optičke aberacije kod bolesnika sa keratokonusom

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Abstract

Background/Aim. Corneal cross-linking (CXL) treatment shows the best results in stabilizing the cornea and stopping the progress of the ectatic process. The aim of the study was to assess the impact of CXL on the keratoconus regarding higher-order aberrations (HOAs) and potential improvement of visual function. **Methods**. In 19 patients, a standard epithelium-off CXL was performed with an energy density of three mW/cm^2 for half an hour. The cornea was examined by Pentacam topography before CXL and one and six months after CXL. Best-corrected visual acuity (BCVA), topographic data, and aberrations were collected. **Results**. A significant reduction in vertical coma was observed from preoperative -1.03 ± 1.89 to -0.74 ± 1.73 (p = 0.004) six months after CXL, and in spherical aberration from preoperative -0.22 ± 1.05 to -0.08 ± 1.13 (p = 0.002) six months after CXL. Root mean square (RMS) HOAs six months after CXL also significantly reduced from 2.31 ± 1.82 to 2.26 ± 1.84 six months after CXL (p = 0.001). BCVA improved from preoperative 0.43 ± 0.15 to 0.71 ± 0.19 six months after surgery (p < 0.001). **Conclusion**. CXL is a very potent treatment method for keratoconus, which significantly reduces certain corneal wavefront aberrations, especially vertical coma, spherical aberration, and RMS, and leads to a significant improvement in visual acuity.

Key words:
cornea; corneal topography; keratoconus; ophthalmologic surgical procedures; visual acuity.

Apstrakt

Uvod/Cilj. Procedura *corneal cross-linking* (CXL) pokazuje najbolje efekte u stabilizovanju rožnjače i zaustavljanju napretka ektatičnog procesa. Cilj rada bio je da se ispita uticaj CXL procedure na optičke aberacije višeg reda – *higher-order aberrations* (HOAs) i poboljšanje funkcije vida u lečenju keratokonusa. **Metode**. Standardna CXL procedura sa uklanjanjem epitela rožnjače izvedena je kod 19 bolesnika korišćenjem energije jačine 3 mW/cm^2 u trajanju od 30 min. Kod svakog bolesnika rožnjača je snimljena na Pentacam aparatu pre procedure i jedan i 6 meseci posle CXL procedure. Ispitivani su najbolje korigovana oštrina vida, topografski podaci i vrednosti optičkih aberacija. **Rezultati**. Utvrđeno je značajno smanjenje vrednosti vertikalne kome sa -1,03 ± 1,89 preoperativno na -0,74 ± 1,73 šest meseci postoperativno (p = 0,004) i sferne aberacije sa -0,22 ± 1,05 preoperativno na -0,08 ± 1,13 postoperativno (p = 0,002). Vrednosti *root mean square* (RMS) HOAs takođe su se značajno smanjile sa 2,31 ± 1,82 preoperativno na 2,26 ± 1,84 šest meseci postoperativno (p = 0,001). Najbolje korigovana oštrina vida poboljšana je sa 0,43 ± 0,15 preoperativno na 0,71 ± 0,19 šest meseci postoperativno (p < 0,001). **Zaključak**. Procedura CXL je uspešna metoda lečenja keratokonusa koja značajno smanjuje određene aberacije talasnog fronta rožnjače, posebno vertikalnu komu, sferne aberacije i RMS, i dovodi do značajnog poboljšanja oštrine vida.

Ključne reči:
rožnjača; kornealna topografija; keratokonus; hirurgija, oftalmološka, procedure; vid, oštrina.

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Introduction

Keratoconus is an ectatic dystrophic disease of the cornea that occurs in working-age people. Etiology is unknown for this generally bilateral disorder, and only 10% of patients are proven to have inherited the disease with a 1 : 2,000 prevalence. The pathohistological base of the disease shows the weakness of corneal stromal tissue caused by structural abnormalities of stromal collagen. Therefore, there is a disorganized architectonics of collagenous lamellae, which is why the cornea loses its biomechanical stability. Due to such changes cornea is progressively getting thinner and deformed in the sense that it loses its anatomical shape and assumes a cone-like form [1].

In a perfect human eye (or perfect optical system), all incoming light waves from the observed object would interfere constructively at the fovea (or focal point). The term wavefront denotes the surface obtained by joining simultaneously all the points of the propagating light wave, which have an equal phase. The wavefront shape can vary depending on the geometry of the source and can be plane (flat), spherical, cylindrical, concave, or convex. In the perfect eye, the wavefront of reflected light should still be flat, but in reality, this is not the case. Due to an imperfect crystalline lens, an irregular cornea (as in keratoconus), and a variable refractive index of ocular media, the wavefront becomes irregular. These imperfections correspond to what is known as higher-order aberrations (HOAs) in wavefront optics. Since 1999, the Optical Society of America has recommended describing wave aberrations with Zernike decomposition, and Zernike polynomials became useful for the interpretation of aberrations and the description of the wavefront error of the human eye. The slopes of the wavefront across the pupil use the least square fit with Zernike polynomials [2, 3]. Each Zernike polynomial called a mod describes a certain type of shape, a certain type of three-dimensional surface. The second-order Zernike terms represent the conventional aberrations (lower-order aberrations – LOA): myopia, hyperopia, and astigmatism. The third-order of Zernike terms are coma and trefoil (trifolio), the fourth-order includes spherical aberration, and so on [4].

The wavefront aberration function summarises all information about the monochromatic optical system. However, it is desirable to use appropriate metrics for wavefront analysis to quantitatively analyze aberrations. The most commonly used metric is the metric in the pupil plane, the so-called root mean square (RMS) wavefront error. It indicates how strongly the measured wavefront deviates from the reference wavefront. RMS gives us information about the amount of aberration between the actual and ideal wavefront for every order. The higher-order RMS error represents the vector sum of all the Zernike terms from the 3rd order and above. Aberrations are defined by either negative or positive signs as well as magnitude. A positive sign means that the aberrated wavefront is in front of the perfect wavefront, whereas a negative sign means that the aberrated wavefront is behind the perfect wavefront.

HOAs are more complex than lower-order aberrations and can be detected with an aberrometer. These aberrations can result in vision disturbances such as night vision disturbance, glare, halos, blurring, or double vision [5].

Aberration measurement was used at an early stage as a sensitive measuring method in the analysis of keratoconus. At that time, it was found that the average RMS value (total coma RMS) in a 6 mm corneal zone in healthy eyes was 0.28 ± 015 μm compared to keratoconus eyes with a value of 3.10 ± 2.28 μm [6]. The fact that an eye with keratoconus with significantly higher values of third-order RMS errors could be measured compared to a healthy eye was confirmed in other studies [7]. These results are supported by other studies, where coma-aberration in a keratoconus eye was 3.74 times higher than in a healthy control group [8]. Very similar results were reported in another paper, where higher vertical coma and RMS values were found in an early stage of keratoconus [9]. The keratoconus leads optically to increased corneal HOAs and, consequently, to increased ocular HOAs, which leads to a decrease in visual acuity. In keratoconus, the most frequently affected are the increases in corneal spherical aberration and coma [10].

To stabilize the cornea and stop the progress of the ectatic process, corneal cross-linking (CXL) treatment shows the best results. This CXL procedure saves most patients from undergoing keratoplasty (deep anterior lamellar or penetrating keratoplasty), which is the only surgical solution for the most difficult stages of the disease.

CXL procedure implies corneal treatment with riboflavin and ultraviolet-A (UVA) radiation, which creates in stroma new covalent cross-links between collagenous fibers and thus improves the firmness and biomechanical stability of the diseased cornea. Apart from this effect, the excessive corneal curvature weakened by keratoconus becomes flat a few months after the procedure, which decreases the level of astigmatism [11, 12].

The aim of the study was to determine the importance of the CXL procedure along with the effect of reducing keratometry in a decrease of levels of corneal wavefront aberrations, especially HOAs, and also in the improvement of visual function.

Methods

Study design

This retrospective, single-center study systematically collected outcome data of keratoconus patients enrolled between January 2015 and December 2018. Patients had progressive, topographically confirmed keratoconus stages 2–4 according to the Amsler-Krumeich classification

The inclusion criterion for the study was a detectable progression of keratoconus. Exclusion criteria were ocular pathology other than keratoconus and previous ocular surgery.

The study has been approved by the Ethics Committee of the Military Medical Academy, Belgrade, Serbia, with de-

cision number 18/2020. Informed consent was obtained from all subjects involved in the study.

Clinical examination

Best-corrected visual acuity (BCVA) was assessed with Snellen charts at preoperative consultation and at one- and six-month follow-up visits. Corneal topography and tomography, as well as wavefront aberrometry assessing total RMS, HOA RMS, and lower-order aberrations (LOA) RMS, were also measured on scotopic conditions at the same time points by corneal Scheimpflug topography (Pentacam, Oculus Instruments, Wetzlar, Germany). Slit-lamp microscopic and fundoscopic examinations, as well as the measurement of intraocular pressure by applanation tonometry, were performed.

Surgical technique

The CXL procedure was performed using a modified Dresden protocol [13] under topical anesthesia (oxybuprocaine 4 mg/mL solution) in a surgery room. Using a hockey knife, the epithelial cells were removed on a diameter of 9 mm, followed by the application of iso-osmolar riboflavin solution 0.1% (10 mg riboflavin-5-phosphate in 10 mL dextran-T-500 20% solution) onto the cornea every 2 min for 30 min. Using a manual ultrasonic pachymeter (UP-100, Nidek), a minimum central pachymetry of more than 400 μm was measured immediately before the cross-linking treatment. If the corneal thickness was measured < 400 μm, additional hypoosmolar riboflavin solution 0.1% (10 mg riboflavin-5-phosphate in 10 mL physiological salt solution-sodium chloride 0.9%) was instilled every 20 sec for 2 min until the cornea had swollen to a thickness of at least 400 μm. The cornea was then irradiated for 30 min with 370 nm-UVA light from the corneal CXL system (UV-X 1000, IROC Innocross AG, Zurich, Switzerland) with a power density of 3 mW/cm^2. The riboflavin solution was further instilled every 2 min during the UVA irradiation to keep the corneal saturation in balance. After the procedure, a topical steroid combined with antibiotic (0.1% dexamethasone solution + 0.3% tobramycin solution) and soft therapeutic silicon contact lens (PureVision, Bausch&Lomb) were applied. The contact lens was usually removed on the third day after surgery if complete epithelial healing was achieved. After epithelial healing, patients used drops of a topical steroid combined with antibiotic (0.1% dexamethasone solution + 0.3% tobramycin solution) three times daily and artificial tears (0.1% sodium hyaluronate solution) eight times per day for one month.

Statistical analysis

IBM SPSS Statistics Version 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to analyze data sets, whether or not they are subject to normal distribution. A data set was considered normally distributed if $p > 0.05$. The parametric data sets are further analyzed using the ANOVA test, and the non-parametric data sets are further analyzed using the Friedman test. Significance exists if $p < 0.05$.

Results

Patients' characteristics

We performed CXL in 19 eyes of 19 progressive keratoconus patients consecutively. Their mean age was 41.7 ± 14.3 years, and 59.9% (n = 11) of patients were male. No eyes were lost to follow-up after one and six months. No intra- or postoperative complications were observed.

Overall effects of CXL

CXL improved BCVA significantly from preoperative 0.43 ± 0.15 to 0.56 ± 0.18 and 0.71 ± 0.19 after one and six months, respectively ($p < 0.001$). Mean spherical refraction changed after CXL from preoperative -2.14 ± 2.78 D to -1.79 ± 3.02 D, 6 months postoperatively ($p = 0.002$). The cylindric refraction was 2.43 ± 1.40 D preoperatively, 2.54 ± 1.58 D one month, and 2.48 ± 1.26 D six months postoperatively, which was not statistically significant ($p = 0.34$). The postoperative visual and refractive results are shown in Table 1.

After one month, 42.1% of eyes gained one line, and 15.8% of eyes even increased BCVA to two or more lines. In 26.2% of eyes, BCVA remained unchanged after one month. After one month, 10.5% of eyes lost two or more lines, and 5.2% of eyes lost one-line BCVA. After 6 months, there was a significant overall improvement in BCVA, i.e., 42.1% gained 2 or more lines, 31.6% gained one line, while 15.8% remained unchanged, and 10.5% lost one line of BCVA (Figure 1).

Table 1

Visual acuity, refractive and corneal parameter values before and one and six months after corneal cross-linking

Characteristics	Preoperative	One month	Six months	*p*
BCVA	0.43 ± 0.15	0.56 ± 0.18	0.71 ± 0.19	< 0.001
Spherical value D	-2.14 ± 2.78	-2.54 ± 3.38	-1.79 ± 3.02	= 0.002
Cylindrical value D	2.82 ± 2.16	2.61 ± 2.21	2.33 ± 1.76	= 0.34
Ksteep	47.04 ± 3.30	47.41 ± 3.79	46.61 ± 4.14	= 0.008
Kflat	44.70 ± 2.93	44.87 ± 3.76	44.10 ± 3.57	= 0.018
Kaverage	45.74 ± 3.02	46.09 ± 3.68	45.33 ± 3.78	= 0.002
Central corneal thickness (μm)	469.1 ± 51.52	434.6 ± 61.34	444.2 ± 58.34	< 0.001

Results are presented as mean ± standard deviation.
BCVA – best corrected visual acuity.

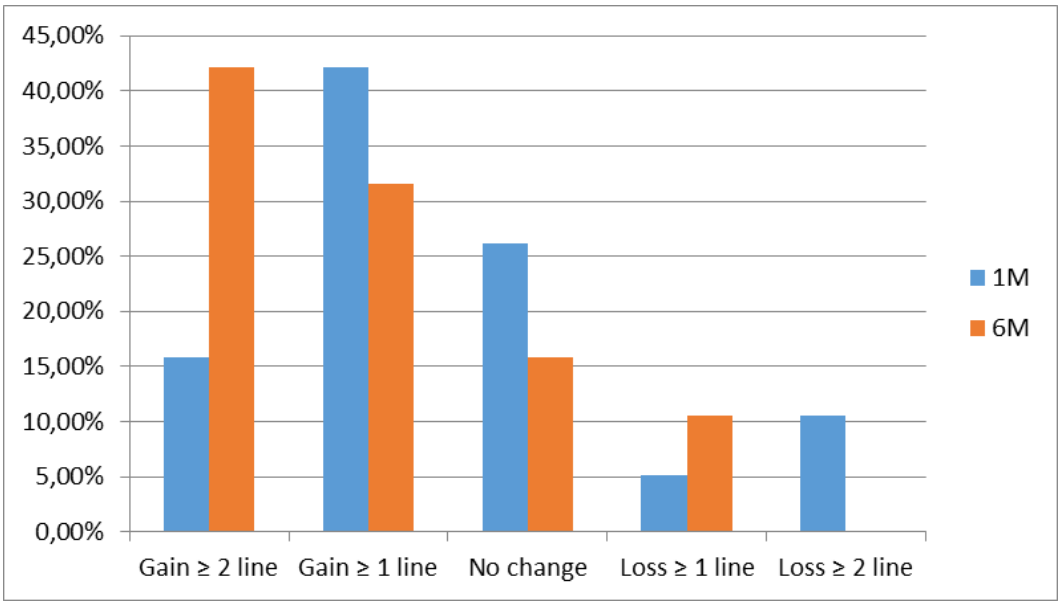

Fig. 1 – Best corrected visual acuity (BCVA) changes 1 and 6 months (M) after corneal cross-linking (CXL).

Topographic changes

Topographically, the mean value of Ksteep was 47.04 ± 3.30 D preoperatively, 47.41 ± 3.79 D one month postoperatively, and 46.61 ± 4.14 D six months postoperatively (p = 0.008). The mean value of Kflat was 44.70 ± 2.93 D preoperatively, 44.87 ± 3.76 D one month postoperatively, and 44.10 ± 3.57 D six months postoperatively (p = 0.018). Finally, the mean value of Kaverage preoperatively was 45.74 ± 3.02 D, whereas one month postoperatively the value was 46.09 ± 3.68 D, and six months postoperatively, 45.33 ± 3.78 D (p = 0.002). All three topographic values were statistically significant. The mean central pachymetry reduced from preoperative values of 469.1 ± 51.52 µm to 434.6 ± 61.34 µm one month postoperative and after 6 months to 444.2 ± 58.34 µm (p = 0.001) (Table 1). *K*average values were significantly changed in the 6-month controls (p = 0.002). One month after CXL, the value remained unchanged at 73.7%. In 21%, even a steepness of more than one diopter of the cornea was found. Only in 5.3%, a flattening of the cornea by 1-2 diopters was observed. Six months after CXL, the picture looked different: 42.1% of the corneas showed a flattening of the cornea by 1–2 diopters and 10.5% by more than 2 diopters; 36.8% remained unchanged, and only 10.5% of corneas with more than one diopter had a higher steepness (Figure 2).

Pachymetry showed a corneal thinning from preoperative 469.1 ± 51.52 µm to 434.6 ± 61.34 µm one month after CXL and 444.2 ± 58.34 µm six months after CXL, which was statistically highly significant (p < 0.001).

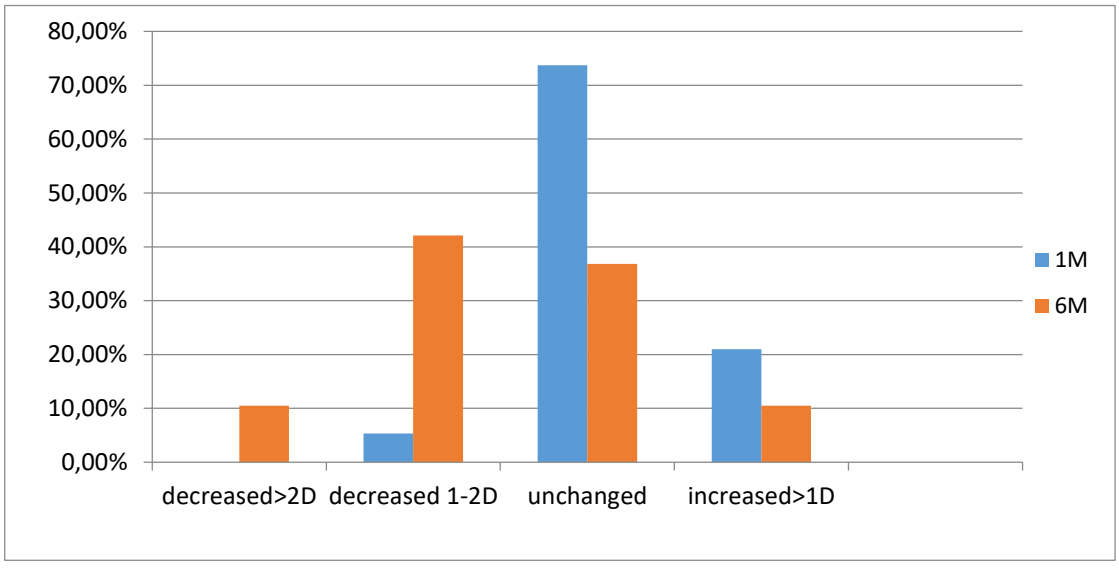

Fig. 2 – Changes of keratometry (Kaverage) 1 and 6 months (M) after corneal cross-linking (CXL).

Wavefront analysis

The wavefront analysis shows a significant reduction of vertical coma from -1.03 ± 1.89 preoperatively to -0.74 ± 1.73 six months postoperatively (p = 0.004) and spherical aberration from -0.22 ± 1.05 preoperatively to -0.08 ± 1.13 six months postoperatively (p = 0.002). The RMS values, total (p = 0.001), LOA (p = 0.002), and HOA (p = 0.001), decreased and improved during the 6-month postoperative course (Table 2).

All other wavefront parameters were not significant, especially those of the 5th and 6th order (p > 0.05). The aberration reduction per eye of the 3rd and 4th order is shown in Figure 3.

Table 2

Corneal wavefront aberrations before and one and six months after corneal cross-linking

Characteristics	Preoperative	One month	Six months	*p*
Vertical coma	-1.03 ± 1.89	-1.13 ± 1.65	-0.74 ± 1.73	0.004
Horizontal coma	0.10 ± 1.62	0.18 ± 1.72	0.12 ± 1.64	0.58
Trifolio 30°	0.10 ± 0.32	0.24 ± 0.50	0.23 ± 0.54	0.45
Trifolio 0°	0.21 ± 0.52	0.18 ± 0.86	0.11 ± 0.59	0.91
Spherical aberration	-0.22 ± 1.05	-0.38 ± 1.18	-0.08 ± 1.13	0.002
RMS total	8.63 ± 6.26	10.09 ± 6.61	8.65 ± 6.27	0.001
RMS LOA	8.31 ± 6.00	9.69 ± 6.39	8.31 ± 6.01	0.002
RMS HOA	2.31 ± 1.82	2.74 ± 1.82	2.26 ± 1.84	0.001

Results are presented as mean ± standard deviation.
RMS – root mean square; LOA – lower-order aberrations; HOA – higher-order aberrations.

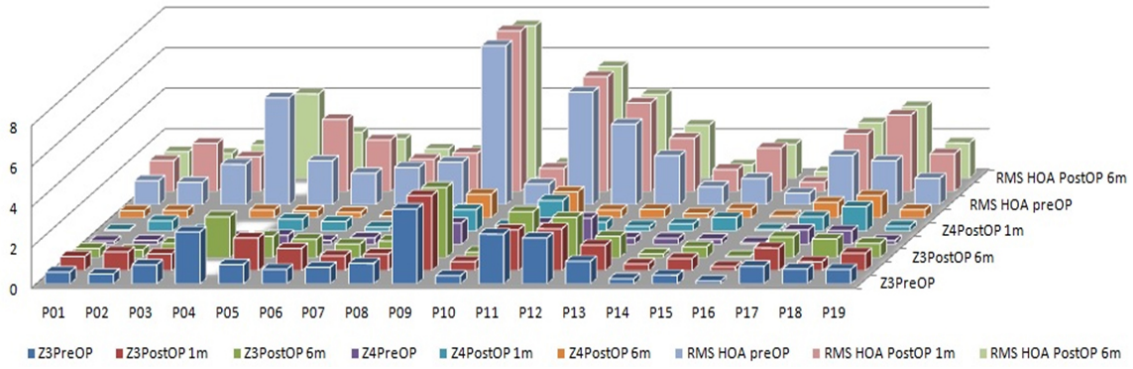

Fig. 3 – The values of root mean square (RMS) for 3rd order (Z3), RMS for 4th order (Z4) corneal wavefront aberrations, and RMS for higher-order aberrations (HOA), preoperatively (preOP), one month (1m) postoperatively (PostOP), and six months (6m) PostOP, with each patient's examined eye (P).

Discussion

Main findings

The CXL procedure not only improved BCVA and corneal topography parameters after one and six months of follow-up but also reduced corneal wavefront aberrations indicating a significant improvement of the distorted corneal shape, leading to a better visual function. The focus is on a significant improvement in spherical aberration and vertical coma.

Results in the context of the existing literature

The main therapeutic effect of the CXL procedure is increasing firmness of stroma in the diseased cornea, thus preventing or slowing further progress of the ectatic process in the diseased cornea. Besides this effect, the CXL procedure also leads to a decrease in the curvature of the diseased cornea, which was shown in our study through the results of Ksteep, Kflat, and Kaverage. According to the values of Kaverage, after 6 months, 42.1% of our patients had a flattening of 1–2 diopters, and in 10.5%, more than 2 diopters occurred. Our results are similar to the results of some other studies [14–16]. CXL procedure influences multiple corneal parameters, which has a direct positive influence on visual acuity. Two main processes lead to an improvement of the optical condition, i.e., a significant flattening of the cornea and a reduction of spherical aberration and vertical coma. The cornea thus becomes more regular and allows for a better optical system [16]. HOAs of the cornea, especially coma, are important optical parameters that say something about the image quality. The continuous analysis of these parameters can provide information about the effectiveness of CXL treatment. In analogy to our study, studies have shown that in keratoconus eyes, coma-like aberrations are dominant [7, 17]. Further studies show that CXL significantly reduces HOAs even in progressive keratoconus [18–20].

Wisse et al. [21] showed that spherical aberration was reduced significantly (-15.68%) (p < 0.001) at one year after CXL for keratoconus, whereas other corneal HOAs remained unchanged. Greenstein et al. [18] performed a study

involving 96 eyes (64 eyes with keratoconus and 32 eyes with ectasia) and found that the mean pre-CXL total anterior corneal HOAs, total coma, 3rd order coma, and vertical coma were 4.68 μm ± 2.33 μm, 4.40 ± 2.32 μm, 4.36 ± 2.30 μm and 4.04 ± 2.27 μm respectively. One year after the CXL procedure, the mean values decreased significantly to 4.27 ± 2.25 μm, 4.01 ± 2.29 μm, 3.96 ± 2.27 μm and 3.66 ± 2.22 μm, respectively (all $p < 0.001$). There were no significant changes in posterior corneal HOAs. Bozkurt et al. [15] in their study found that total HOAs decreased from 0.54 ± 0.26 before CXL to 0.51 ± 0.28 six months after CXL, coma decreased from 0.45 ± 0.26 before CXL to 0.43 ± 0.26 six months after CXL, and spherical aberration decreased from 0.08 ± 0.06 before CXL to 0.07 ± 0.06 six months after CXL. Kosekahya et al. [19] in their study concluded that RMS total, RMS HOAs, vertical coma, and spherical aberration values significantly decreased after CXL ($p < 0.001$, $p = 0.02$, $p = 0.04$, and $p < 0.001$, respectively). The improvements in HOAs were significant at postoperative 6th months compared to the baseline measurements, while they remained the same between postoperative 6th months and 12th months. We had comparable results in our study (Table 2). It shows that the CXL has a high potential as a treatment method that should be used primarily for treating keratoconus, provided the corneal criteria are met. The aim is not only to stabilize the keratoconus but also to significantly improve the visual function.

Keratoconus patients are, in general, thought to have much larger amounts of astigmatism. The Zernike terms are significantly higher among these patients: oblique astigmatism (Z_2^{-2}), trefoil (Z_3^{-3} and Z_3^{3}), vertical coma (Z_3^{-1}), as well as secondary astigmatism (Z_4^{-2} and Z_4^{2}). Vertical coma and spherical aberration showed the highest values among higher-order Zernike coefficients. In our study, negative vertical coma was present in 16 of 19 keratoconus eyes studied. Our study showed that vertical coma and spherical aberration improved significantly after CXL. That led to a functional improvement of the eye, i.e., BCVA increased continuously from 0.43 ± 0.15 before CXL to 0.56 ± 0.18 one month and 0.71 ± 0.19 six months after surgery. At all times, the visual acuity improvement was highly significant ($p < 0.001$). Other studies confirm these results and associate BCVA improvement with the reduction of coma and keratometry values [20, 22].

Limitations

The present study has some limitations due to the retrospective design and the limited number of eyes treated. There was no formal assessment of the quality of vision, such as a standardized quality of life visual function questionnaire or assessment of contrast sensitivity as part of the routine clinical workup.

Implications for further research

In the future, a longer follow-up period is planned for further analysis, which should show the dynamics of CXL on the cornea in keratoconus eyes as a long-term minimally invasive therapy option.

Conclusion

Our study confirms previous preliminary studies showing that standard epithelium off CXL in keratoconus treatment improves BCVA and topographic parameters and significantly reduces corneal wavefront aberrations, especially vertical coma, spherical aberration, and RMS.

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This research received no external funding.

Data availability statement

The data presented in this study are available on request from the authors; the datasets, in particular, are archived in the clinics treated. The data are not publicly available as they contain information that could compromise the privacy of the participants.

Conflicts of interest

The authors declare no conflict of interest.

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Early postoperative results analysis of standard and mini-incision posterolateral approach in total hip arthroplasty

Analiza ranih postoperativnih rezultata standardnog i minimalno incizionog posterolateralnog pristupa kod totalne artroplastike kuka

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Abstract

Background/Aim. Total hip arthroplasty is the replacement of the hip joint with an artificial one. Standard surgical procedures involve a long skin incision and extensive dissection of healthy tissue. Mini-incision surgery is a modification of standard operative approaches. In addition to a significantly smaller skin incision, the main difference is based on much less damage to soft tissues, especially the muscles that move the hip. The aim of this study was to compare the early results of the mini-incision and a standard approach in total hip arthroplasty and to determine the advantages and disadvantages of the mini-incision surgical technique. **Methods.** A retrospective study analyzed data based on 63 patients who underwent total hip arthroplasty with a mini-incision and standard approach at the Institute of Orthopaedic Surgery "Banjica", Belgrade from 2004 to 2010. All the patients suffered from primary coxarthrosis. All operations were carried out by the same surgical team. All patients were clinically evaluated before and after the surgery using the Harris Hip Score (HHS). **Results.** The group of patients operated on with the mini-incision approach included 32 patients, while 31 patients made up the group of patients operated on with the standard approach. Comparing these groups did not reveal a statistically significant difference in age, body mass index, surgery duration, and HHS before the surgery. A statistically significant difference was determined by comparing intraoperative blood loss, the amount of drainage fluid after the surgery, and the HHS after the surgery. **Conclusion.** The mini-incision posterolateral approach, compared to the standard approach, apart from an esthetically more acceptable scar, achieves significantly less intraoperative blood loss and better hip function with almost the same risk of complications.

Key words:
arthroplasty, replacement, hip; intraoperative complications; orthopedic procedures; postoperative complications; treatment outcome.

Apstrakt

Uvod/Cilj. Totalna artroplastika kuka predstavlja zamenu zgloba kuka veštačkim zglobom. Standardni operativni postupci podrazumevaju dugačak kožni rez i ekstenzivnu disekciju zdravog tkiva. Minimalno inciziona hirurgija predstavlja modifikaciju standardnih operativnih pristupa. Pored značajno manjeg reza kože, glavna razlika je mnogo manje oštećenje mekih tkiva, posebno mišića pokretača kuka. Cilj rada bio je da se uporede rani rezultati minimalno incizionog i standardnog pristupa u totalnoj artroplastici zgloba kuka i utvrde prednosti i nedostaci minimalno incizione hirurške tehnike. **Metode.** Retrospektivnom studijom analizirani su podaci o 63 bolesnika kojima je urađena totalna artroplastika kuka minimalno incizionim i standardnim pristupom na Institutu za ortopediju „Banjica" u Beogradu u periodu od 2004. do 2010. godine. Svi bolesnici imali su primarnu koksartrozu. Sve operacije je uradio isti hirurški tim. Svi bolesnici su klinički procenjivani, pre i posle operacije, korišćenjem bodovnog sistema po Harisu. **Rezultati.** U grupi bolesnika operisanih minimalno incizionim pristupom bila su 32 bolesnika, a u grupi operisanih standardnim pristupom, 31 bolesnik. Poređenjem tih grupa nije utvrđena statistički značajna razlika u životnom dobu, indeksu telesne mase, trajanju operacije i Harisovom skoru pre operacije. Poređenjem intraoperativnog gubitka krvi, količine drenažne tečnosti posle operacije i Harisovog skora posle operacije, utvrđena je statistički značajna razlika. **Zaključak.** Pored estetski prihvatljivijeg ožiljka, minimalno incizionim posterolateralnim pristupom se, u odnosu na standardni pristup, postiže i značajno manji intraoperativni gubitak krvi i bolja funkcija kuka, uz skoro isti rizik od komplikacija.

Ključne reči:
artroplastika kuka; intraoperativne komplikacije; ortopedske procedure; postoperativne komplikacije; lečenje, ishod.

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Introduction

Total hip arthroplasty (THA) is the replacement of a hip joint with an artificial implant. Artificial joints are designed to enable joint function close to natural. Nowadays, THA is one of the most successful operations in orthopedic surgery. For many years, it has enabled the recovery of normal joint function and the return of the patients to normal life activities [1]. The operative procedures by which total hip arthroplasty is conducted are numerous and quite different. Over time, they have undergone significant changes in order to reduce morbidity and increase the longevity of implants [2]. Furthermore, the implants themselves have undergone significant changes in every respect: metal alloys, design, chemical and physical characteristics of polyethylene and cement have been improved, the contact surfaces are made porous, which ensures biological fixation, etc. All this has made THA a highly successful surgical procedure [3, 4].

Standard surgical procedures involve a long skin incision and extensive dissection of healthy tissue to approach the diseased joint. Apart from leaving large scars, such approaches cause unnecessarily extensive soft tissue damage; thus, all operative and postoperative risks, especially the risk of infection, are increased. As a result, there is a need for an operative technique that will give the same or even better operative results through a smaller skin incision and with less soft tissue dissection [5]. Although the standard approach to hip arthroplasty provides a good improvement in joint function, with long-lasting components and a low percentage of complications, surgeons constantly try to improve the existing techniques [6]. Minimally invasive surgery (MIS) is a modification of standard surgical approaches to hip arthroplasty. In addition to a significantly smaller skin incision, which is up to 30 cm in the standard posterolateral approach, while in the minimally invasive one is 10 cm maximum, the main difference is in much lesser (minimal) damage to soft tissues, especially hip muscles. The incision length mostly depends on the size of the acetabular component in order to ensure its correct placement [7]. The Institute of Orthopedic Surgery "Banjica" in Belgrade has been applying the Dorr posterolateral mini-incision approach since 2004 [8].

The introduction of MIS procedures into total hip arthroplasty has led to great controversy in orthopedic circles. The complete definition of 'minimally invasive' is not clearly determined; hence, many authors consider the terms 'minimally incisional' or 'less invasive' as more appropriate [9]. In addition to providing standard implant durability, the expectations from the MIS technique are that, apart from a more acceptable scar, it enables reduction of blood loss during surgery, reduction of soft tissue trauma, reduction of intensity and duration of postoperative pain, reduction of hospital stay, and acceleration of rehabilitation [10]. The realization of these expectations has been disputed by many authors. There are claims that skin trauma, infection rate, and neurovascular damage are higher than in the case of the standard approach surgery [11]. Some authors emphasize the possibility of malposition of components due to limited visibility and tissue interposition in the operative field. They also claim that the MIS should not be widely used until the degree of risk and benefit of this method is well determined and documented [12].

The aim of this study was to compare the early postoperative results of the mini-incision and standard posterolateral approach in total hip arthroplasty, as well as to determine the advantages and disadvantages of the MIS technique.

Methods

A retrospective cohort study analyzed data based on 63 patients who underwent total hip arthroplasty with a standard and MIS posterolateral approach at the Institute of Orthopedic Surgery "Banjica", Belgrade from 2004 to 2010. All patients suffered from primary coxarthrosis. All operations were carried out by the same surgical team with cementless implants. The patients were divided into two groups based on the surgical technique used to perform the operation. The first group of patients underwent a standard procedure surgery (Standard group) and the second group underwent surgery with an MIS technique (MIS group). The following relevant sociodemographic characteristics were also analyzed: gender, age, and body mass index (BMI).

Depending on the physical characteristics, the length of the incision was determined for each patient individually at the time of the surgery. In all cases, the standard posterolateral surgical approach was used, as well as the Dorr MIS approach. It involves a skin incision in the area of the posterior aspect of the great trochanter from the *vastus tubercle* to the apex of the great trochanter, stratification of the fibers of the *gluteus maximus*, and lifting of the *gluteus medius* and *gluteus minimus*, as well as L incision of the capsule to the *quadratus femoris* [8]. Dedicated Hohmann retractors with long handles and various angulations were used, distancing the assistant's hands from the operative field. Implants were positioned according to Lewinnek's "safe zone" [13] and anatomical references without intraoperative fluoroscopic control. In all patients, the fascia, subcutaneous tissue, and skin were closed by a standard procedure, and the wounds were drained with an aspiration drainage system for a maximum of 24 hrs.

Standard antibiotics and thromboembolic prophylaxis were used: first-generation cephalosporins were administered *iv* for a total of 24 hrs, with the first dose an hour before the surgery. Low molecular weight heparins were administered *sc* until the 35th postoperative day, with the first dose 12 hrs before surgery. The length of the operative incision was measured with a ruler. During the operation, the blood loss was monitored as the sum of the volume of blood in the bottle of the aspiration apparatus and the weight of the gauze used during the procedure. The time from the beginning of the incision to the complete closure of the operative wound was taken as the duration of the operation. After the operation, the volume of drainage fluid in the drainage system was monitored. The patient's condition was assessed daily in the first postoperative week, as well as on an outpatient basis two months after the operation. The follow-up of the patient's condition lasted on average 51 months, the shortest

34, and the longest 63 months. All the patients were clinically evaluated before and two months after the surgery using the Harris Hip Score (HHS) [14].

The two groups of patients were compared according to HHS before and after surgery, considering the blood loss during surgery, the amount of drainage fluid, and the duration of the surgery. Central tendency measures (arithmetic mean and median) and variability measures (standard deviation and minimum and maximum values) were used to describe the data. Student's *t*-test was used for the intergroup comparison of the differences.

Results

The average age of the patients was 53 years. In total, there were 10 (16%) men and 53 (84%) women. Thirty-three patients underwent a right hip joint surgery, while thirty underwent a left hip joint surgery. In the group of patients with the standard approach, there were 31 (49%) patients, while there were 32 (51%) patients in the group treated by the MIS approach. Sociodemographic characteristics of the patients are shown in Table 1.

Comparing these groups did not reveal a statistically significant differences in age, BMI, duration of the surgery, and HHS before the surgery ($p > 0.05$). The BMI in the Standard group was 24.6 kg/m^2, while in the MIS group, the average BMI was 25.6 kg/m^2. The average duration of the surgery in the Standard group was 58 min, while this time in the MIS group was 60 min. The mean value of the HHS before the surgery in the Standard group was 46.77, while in the MIS group, the average value of this score was 44.97. The average length of the incision in the Standard group was 14.4 cm, compared with the MIS group, where it was 7.8 cm.

A statistically significant difference between the two groups was determined when comparing the intraoperative blood loss, the amount of drainage fluid after the surgery, and the HHS after the surgery ($p < 0.01$). In the group with the mini-incision technique, the average blood loss during the operation was 296.6 mL, while this value in the standard approach group was 428.1 mL. By measuring the volume of the drainage fluid after the surgery, it was determined that its average value in the Standard group was 335 mL, while in the MIS group, this value was 220 mL. After the operation in the Standard group, the mean value of the HHS was 94.93, while this value in the MIS group was 97.56. There was also a highly statistically significant difference between pre- and postoperative HHS in both groups of patients ($p < 0.01$). All intra- and postoperative parameters are shown in Table 2.

The complications that occurred in the group of patients operated on using the standard technique were one luxation that occurred one year after the surgery and one verified pulmonary embolism. In the MIS approach group, a deep vein thrombosis of the leg was observed in one patient. There was no delayed wound healing or superficial or deep infection.

Discussion

Over the last four decades, total hip arthroplasty has become one of the most successful operations in terms of improving the quality of life of patients with hip joint disease. Although new advances in anesthesia and faster rehabilitation have had the effect of reducing mortality and morbidity, the surgical approach and technique have changed little during the last few decades [15]. The rationality in introducing less invasive surgical procedures, i.e., the mini-incision technique, is that they represent less destructive surgery with a more acceptable cosmetic result. The possible benefits include less intraoperative blood loss, less postoperative pain, shorter hospital stays, and shorter rehabilitation. However, there are concerns not only in terms of indications that large-

Table 1

Demographic data

Parameter	Standard group	MIS group
Number of patients	31	32
Age (years)	51.3 ± 10.3	54.7 ± 11
BMI (kg/m^2)	24.7 ± 3.8	25.6 ± 3.5
Gender		
male	7 (22.6)	3 (9.4)
female	24 (77.4)	29 (90.6)
Operated side		
right	12 (38.7)	21 (65.6)
left	19 (61.3)	11 (34.4)

All values are expressed as mean ± standard deviation or number (percentages).
MIS – mini-incisional surgery; BMI – body mass index.

Table 2

Operative and postoperative parameters

Parameter	Standard group	MIS group	*p*
Operation duration (min)	58.4 ± 8.9	59.7 ± 9.5	> 0.05
Intraoperative blood loss (mL)	428.1 ± 93.9	296.6 ± 108.1	< 0.01
Operative wound drainage (mL)	335.5 ± 90.6	220.9 ± 82.0	< 0.01
Incision length (cm)	14.4 (13–16)	7.8 (7–9)	< 0.01
Preoperative HHS	44.8 ± 7.9	45.0 ± 6.8	> 0.05
Postoperative HHS	94.9 ± 4.5	97.6 ± 1.9	< 0.01

All values are expressed as mean ± standard deviation or median (minimum-maximum).
MIS – mini-incisional surgery; HHS – Harris Hip Score.

ly depend on body weight but also in the accuracy of implant positioning and, most importantly, the risk of complications [16]. Minimally invasive techniques can be successfully used only by experienced orthopedic surgeons, i.e., the surgeons who have already performed a large number of these operations with standard surgical procedures. For the technique to become widely accepted, it must show an obvious advantage over the standard method of total hip arthroplasty without increasing the frequency of accompanying complications [17].

The justification of the term 'minimally invasive', which should mean a minor intraoperative tissue trauma accompanied by minor bleeding and pain, is questioned by many. The term 'minimally invasive' is increasingly being replaced by the term 'minimally incisional' [18]. Namely, it should be borne in mind that the 'minimally incisional' technique does not have to be a 'minimally invasive' operation [19]. Numerous approaches have been developed in minimally invasive hip surgery that can be classified into two groups: those that save the muscles and those that are mini-incisional. In the case of the techniques that save muscle fibers, the cutting of muscle bodies or separation of their attachments is avoided – an example is the MIS technique with two incisions. Mini-incisional techniques involve shorter skin incisions and less muscle damage compared to their standard equivalents [20]. Under the skin, hip resection, acetabulum, and femoral canal treatment, and even the endoprosthesis itself are the same as in the case of standard surgical procedures. However, it is indisputable that the soft tissues, especially the muscles and the joint capsule, are significantly less damaged, and the blood loss is lesser. As a result, the postoperative pain is less, and rehabilitation is easier and faster. In addition, the patients prefer accepting a small operative scar and increasingly require this type of surgery. The choice of patients is one of the most important factors on which the success of this surgical procedure depends, and it represents the greatest limitations of its application. Most authors believe that patients should not have a BMI greater than 30. Other contraindications are a high degree of hip dysplasia, previous operations on that joint, revision surgery, as well as very pronounced joint contractures [21].

Our results showed that the average value of BMI, HHS before the surgery, and the duration of the operation did not differ in the two observed groups. In the group with the mini-incision approach, a statistically significant lower intraoperative blood loss and a smaller volume of drainage fluid were observed. The length of the incision was reduced by 46% (from 14.4 cm to 7.8 cm). Pavone et al. [22], who analyzed 46 patients with incisions of 8 cm and 15 cm in a randomized prospective study, concluded that there was significantly less blood loss and less wound drainage in the group with a shorter skin incision. On the other hand, Wright et al. [23] did not find any statistically significant difference in blood loss and hospital stay in their patients.

Statistically significantly better HHS was observed after the surgery in the MIS group. The data in the references are not consistent with this score. Chung et al. [16] found no statistically significant difference in their study, while Goosen et al. [24] found that there was a significant difference in their patients, with a significantly better score in the mini-incision group. Dorr et al. [25], as well as Wenz et al. [26], showed in their studies that their patients operated on with the mini-incision technique began to walk earlier and with less need to use orthopedic aids compared to those operated on with the standard approach.

Component positioning is one of the most important aspects of hip surgery. Woerner et al. [27] state a significant impact of the reduced visibility of the operative field due to a smaller incision on the implant placement. Other authors, such as Ogonda et al. [15], disagree with this statement, believing that it largely depends on the individual experience of the surgeon. In their randomized study of 219 patients, the MIS and standard techniques were compared, after which they did not notice a significant difference between the two approaches.

Tan et al. [28] compared the standard with the MIS approach with piriformis muscle preservation in a group of 100 patients. After ten years of follow-up, the authors did not find a difference in hip joint functionality between these two approaches, with almost the same complication rate in both groups.

There were a total of three postoperative complications in our study. In the group that was operated on with the standard approach, there was one luxation and one pulmonary embolism, while in the other group, there was only one thrombosis of the deep veins of the leg. There were no other postoperative complications in the form of components malposition, early infection, or intraoperative periprosthetic fractures. Leg length discrepancies in booth groups were less than 1 cm measured from the superior anterior iliac spine to the medial malleolus. Taking into consideration that the total number of complications is not significant, such rare complications fit the results of other authors [7].

A limitation of this study was the fact that only early postoperative functional results were analyzed, excluding the results obtained during the entire follow-up period.

Conclusion

The attractiveness of the mini-incision technique is obvious due to lower morbidity and faster recovery, which are just some of the advantages. The MIS approach compared to the standard, apart from an aesthetically acceptable scar, achieves significantly less intraoperative blood loss and better hip function in the early postoperative period with almost the same risk of complications. The choice of the patients is one of the key factors on which the success of this operative procedure depends. The results of this study emphasize the benefits that patients have from the MIS and indicate its clear advantages over the standard posterolateral surgical approach. Randomized prospective controlled clinical trials, as well as long-term follow-ups, are needed to fully understand and demonstrate the advantage of this technique over the standard approach.

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Fabry disease in Serbia – current status and future perspectives

Fabrijeva bolest u Srbiji – trenutno stanje i buduće perspektive

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Key words:
fabry disease; genetic diseases, inborn; diagnosis; signs and symptoms; therapeutics; serbia.

Ključne reči:
fabrijeva bolest; nasledne bolesti; dijagnoza; znaci i simptomi; lečenje; srbija.

Introduction

Fabry disease (FD) (OMIM 301500) is a rare, X-chromosome-linked, slowly progressive lysosomal storage disorder [1]. The estimated prevalence of FD is very hard to establish and affects 1 in 40,000–117,000 newborns worldwide [2]. FD was first described in 1898 by two dermatologists, William Anderson and Johannes Fabry. It is hence sometimes referred to as Anderson-Fabry disease [3, 4].

The aim of this paper was to shed some light on this disease, which is profoundly under-recognized in our country, and to provide insight into the current status of FD in Serbia as well as to give future perspectives on this matter (planning of screening procedures and management issues).

Fabry disease – basic characteristics

In FD, as a consequence of a genetic variation in the *GLA* gene, an impairment of enzyme α-galactosidase A (AGAL) activity occurs. It leads to the accumulation of glycosphingolipids in various cell types. The finding of globotriaosylceramide (Gb3) in biopsy specimens from affected organs is essential from a diagnostic point of view. For this purpose, it is necessary to perform an electronic microscopic examination of the affected tissue, which demonstrates Gb3 accumulations as intracellular inclusions ("myeloid bodies" or "zebra bodies"). On the other hand, globotriaosylsphingosine (Lyso-Gb3), the deacylated form of Gb3, is very important not only as a diagnostic but also as a prognostic biomarker in FD, which should be measured in bodily fluids during the treatment of FD patients [5]. In contrast to other X-chromosome-linked diseases in which females can only be carriers, a disease of different severity can develop in FD females. The clinical picture of female patients with pathologic genetic variation largely depends on how the X chromosome is inactivated. In addition, it is a consequence of mosaicism of wild-type *GLA* gene and pathologic type *GLA* gene in their cells [6, 7]. Due to the manner of inheriting FD, all children of affected mothers have a 50% chance of inheriting the genetic variation. However, in the case of an affected father, daughters and not sons will inherit the pathologic genetic variation.

There are more than 1,000 genetic variations in the *GLA* gene (The Human Gene Mutation Database, www.hgmd.cf.ac.uk). Many of these genetic variations are pathogenic and lead to classic or late-onset forms of FD. Other genetic variations have uncertain clinical significance, and some variations are probably benign [5, 7, 8]. We can assort FD as an attenuated lysosomal storage disorder by knowing the fact that patients can live well into adulthood. Nevertheless, it poses a substantial burden on the lives of FD patients, with an estimated lifespan of 15–20 years shorter in classic hemizygous male patients and 5–10 years in classic heterozygous female patients. The leading causes of death in FD patients nowadays are cardiovascular disorders [9, 10].

There are two major forms of FD: classic form and late-onset variants.

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In patients with a classic form of the disease, the accumulation of glycosphingolipids starts practically *in utero*. The classic form of FD is characterized by absent or residual enzyme activity (AGAL < 1%) and abundant accumulation of Gb3 in various cells. Typically, the disease clinically manifests itself in patients during childhood with acroparesthesia. Neuropathic pain is the most frequent clinical symptom of FD, occurring in over 80% of males and 60% of females. The pain is usually aggravated during exercise, in febrile states, or a warm environment. It can last for days and sometimes be so excruciating that it disables the patient (so-called Fabry crises). That is often coupled with sweating disturbances, usually hypo- or anhydrosis, which makes this condition even worse. In addition, during childhood, eye changes can ensue, with *cornea verticillata* being the most prominent but still nonspecific for FD. Diagnosis is quite simple, with a slit lamp examination. It does not impair vision. A premature cataract is also one of the eye manifestations of FD, accompanied by tortuosity of retinal blood vessels on fundoscopic examination. During childhood and teenage years, skin changes so that angiokeratoma becomes prominent. Its localization is mainly around the umbilicus and inner thighs ("bathing trunk region"); it represents small red to purple papules composed of surface blood vessels (dilated capillaries). Gastrointestinal dysmotility is another important part of the FD spectrum. Usually, it manifests as postprandial pain, bloating, cramping, diarrhea, or constipation [11]. Bearing in mind all the aforementioned, rather nonspecific symptoms and signs, all FD patients generally experience a diagnostic odyssey before establishing a proper diagnosis. Initially, more than 25% of FD patients have a wrong diagnosis, ranging from different psychiatric disorders, across irritable bowel syndrome and fibromyalgia to multiple rheumatologic conditions [12]. During adulthood, patients with FD may experience serious target organ damage [11].

Kidneys are rather important target organs for FD patients since most patients with the classic form of FD have a kidney disorder, and untreated patients with classic FD usually develop end-stage renal disease (ESRD) into their 50s [13]. Accumulation of intracellular Gb3 inclusions in various kidney cells starts very early. Podocytes are terminally differentiated cells with restricted capability for regeneration. Their loss eventually leads to glomerulosclerosis [14]. Albuminuria, as the first sign of kidney disease, can be found very early, during childhood. Later overt proteinuria ensues but is rarely above 1 g/day. Some authors have found that podocyturia significantly impacts the diagnosis of Fabry nephropathy since it commences before proteinuria and can be an early sign of disease [15]. Ultrasound examination of kidneys is a crucial part of nephrology workup. With this technique, we can find the existence of parapelvic cysts in FD patients [16]. They are nonspecific for FD but can allege us to think about FD in our patients. Urine cytological examination is usually underestimated in our clinical practice but can be important, even though nonspecific. In FD patients, we can find mulberry cells (distal tubular epithelial cells in which Gb-3 has accumulated) or Maltese crosses (with polarized light microscopy – glycosphingolipid laden epithelial cells) in urinary sediment specimens that can lead us to FD diagnosis [14, 17]. Kidney biopsy is a vital part of the diagnostic and prognostic workup for many kidney diseases, including Fabry nephropathy. An essential part of kidney biopsy examination should be electronic microscopy (EM). Characteristic EM findings on kidney biopsy are myeloid bodies, also called "zebra bodies", which resemble the accumulation of glycosphingolipids in renal cells. These changes are characteristic of FD but can also be found in patients who are on certain medications, such as amiodarone, antimalarials, fluoxetine, and other drugs, or can be related to lithium ingestion [18]. The most usual finding on light microscopy examination in the case of FD is the one that resembles focal segmental glomerulosclerosis, a nonspecific consequence of podocyte loss. Before that, vacuoles in podocytes and distal tubular cells can be found [19].

Cardiac involvement in FD patients is a very important issue since 40–60% of FD patients will have some form of cardiac involvement during the course of their disease. The most prominent cardiac manifestation is left ventricular hypertrophy (LVH) (Figures 1 and 2), which is partly a

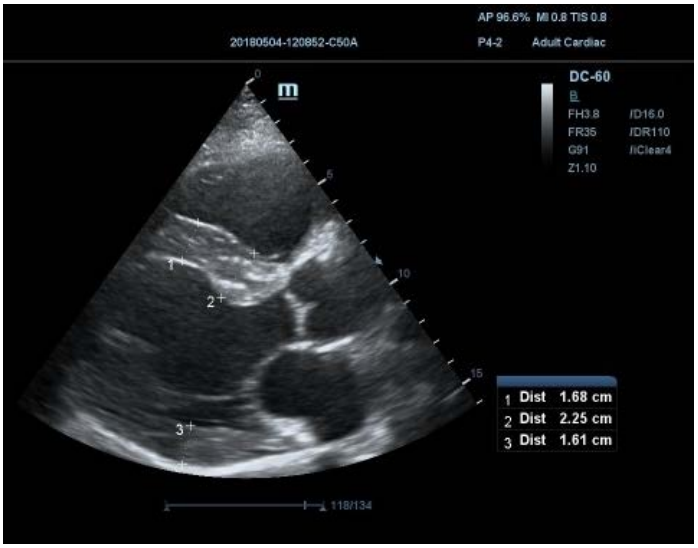

Fig. 1 – Echocardiographic image of Fabry disease patient – interventricular septum (IVS) in diastole 1.68 cm, posterior left wall (PLW) d 1.61 cm: hypertrophic cardiomyopathy.

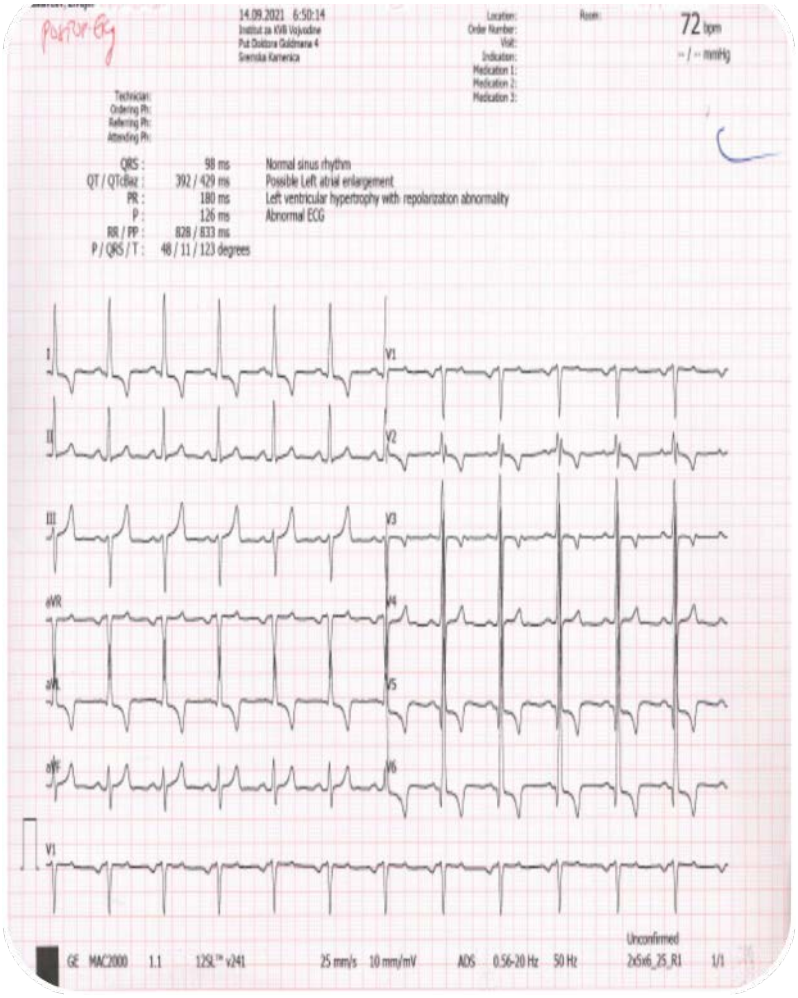

Fig. 2 – Electrocardiogram of Fabry disease patient (left ventricular hypertrophy).

consequence of glycosphingolipid accumulation, but some other mechanisms are also responsible for hypertrophic changes in the myocardium. Accumulated Gb3 triggers processes that lead to some signaling pathways affecting the functional impairment of myocytes. Aside from that, other mechanisms like inflammatory changes in the heart tissue can be responsible for the prominent hypertrophy of the myocardium [20]. It is usually concentric and leads to an increase in left ventricular mass over time. Along LVH, patients with FD may have conduction abnormalities, bradyarrhythmias, supraventricular and ventricular tachyarrhythmias, valvular disorders, dysfunction of cardiac microcirculation, and cardiac fibrosis which is best visualized with magnetic resonance imaging [21]. The posterolateral wall of the left ventricle is the predominant site for fibrosis development. Patients with FD usually suffer from heart failure with preserved ejection fraction, but the global longitudinal strain is impaired. Arrhythmias in FD patients can often be intermittent, so the preferred way of diagnosis is to perform a 48-hour ECG Holter monitoring [22].

Besides the peripheral and autonomous nervous system, another important site for FD manifestations is the central nervous system (CNS). The main CNS manifestations are cerebrovascular ("cerebral vasculopathy"), psychiatric, and cognitive disorders and vestibulocochlear nerve dysfunction. Cerebrovascular manifestations usually affect patients aged 15–55 years and range from ischemic strokes, transitory ischemic events, cerebral hemorrhage, cerebral venous thrombosis, dissection of cervical arteries, and white matter lesions (cerebral microangiopathy) (Figure 3). Stroke is one of the leading causes of death in FD patients. In addition, affection of large blood vessels, predominantly of the vertebrobasilar region [dolichoectasia (Figure 3), calcifications], is often found in FD patients. Psychiatric disorders comprise a spectrum of manifestations that vary from developmental difficulties (learning disabilities) and stress problems due to chronic pain and multisystemic affection to high rates of depression with suicidal intentions and neuropsychologic involvement with dementia. The affection of the eighth cranial nerve is manifested in vertigo, tinnitus, and deafness [23, 24].

Late-onset variants of FD usually manifest themselves with heart (cardiac variant) and kidney (renal variant) involvements that are similar to those in classic FD patients but manifest clinically somewhat later in the life of patients than in the classic form of FD. These patients generally lack all aforementioned signs and symptoms of the classic form of FD, aside from the affected organs [11].

Fig. 3 – Computed tomography (CT) finding: white matter lesions (arrows) and ectasia of the right basilar artery (red asterisk).

Fabry disease in Serbia

According to the last census, Serbia has a population of 7,186,862 inhabitants [25] and only 17 established cases of FD so far, 7 males and 10 females, from 7 families. One adult male patient receives treatment with enzyme replacement therapy (ERT) at the University Clinical Center of Serbia in Belgrade, and three patients receive ERT at the University Clinical Center of Vojvodina in Novi Sad. Four patients have received approval for ERT from the Ministry of Health of the Republic of Serbia and are waiting for the therapy. According to the known epidemiological data, this is not a proper number of FD patients.

There are many reasons for such a low number of registered FD patients in Serbia. Patients with the same genetic variation from the same family can have a completely different phenotype of the disease. For instance, male and female patients can have very different clinical presentations because female patients experience the phenomenon of X chromosome inactivation. Therefore, our male patient with classic FD genetic variation, c.871G>C (Ala291Pro), has a full-blown disease presentation with acroparesthesia, premature cataract, proteinuric end-stage renal disease, left ventricular hypertrophy, basilar artery ectasia, and white matter lesions. On the other hand, his four years older sister with the same genetic variation of the *GLA* gene only has mild proteinuria with preserved renal function, mild left ventricular hypertrophy, and *cornea verticillata*. Furthermore, patients of the same gender can have a very heterogeneous phenotype of the disease, and that can be a diagnostic problem on its own. However, in our opinion, the main problem in establishing a diagnosis of FD in Serbia is the lack of awareness among our physicians regarding rare diseases in general, among them FD. A particularly complex problem is represented by the existing difficulties in testing possibilities for FD in Serbia. One of the ways we could raise awareness about FD undeniably is to present this rare disease to the scientific and physician auditorium with papers like this. We should all bear in mind the clinical picture of classic or late-onset FD patients described in the previous text and think about it in differential diagnosis while taking care of our patients. As we already mentioned, there are some essential rules in diagnosing an FD patient. It relies largely on the gender of our patients.

The most common way of finding a new FD patient is high-risk population screening. It stands for screening among patients on renal replacement therapy, especially for those with unknown causes for ESRD and younger than 50 years of age (female patients regardless of age), but also among patients with unknown causes of chronic kidney disease of any grade [26, 27]. Moreover, high-risk population screening is implicated in patients with hypertrophic cardiomyopathy of unknown origin [28] and patients with cerebrovascular accidents [29] under 55 years of age (so-called cryptogenic cerebrovascular accidents).

In previous years in our country, there have been only a few sporadic attempts at hemodialysis population screening for FD. Those attempts include only a few hundred hemodialysis patients without any success in finding a patient with FD (unpublished data, personal communication). So

far, there has been no published data on screening for FD in Serbia, and most of the FD patients in Serbia have been diagnosed on a clinical suspicion basis. There are numerous publications on screening programs for FD in a high-risk group population, with different results depending on the type of population screened and the types of genetic changes that were established. In a meta-analysis by Doheny et al. [30], they established that the prevalence of FD in hemodialysis patients is 0.21% of males and 0.15% of females, in cardiac screening, 0.94% males and 0.9% of females, and in stroke patients, 0.13% of males and 0.14% of females. Family screening is the most important part of the process of discovering FD patients. It should be done for every index FD case due to the estimation that for every new FD patient, we could expect to find other 3–5 patients through family screening [5]. Ideally, genetic counseling should be a vital part of managing FD patients and their families. Most of their work should be based on genetic testing *per se* but also on the commentary of the finding and prenatal family consultations. In our country, there is a possibility for genetic counseling.

The first patient was diagnosed in 2009 in Novi Sad with a classic FD genetic variation c.334C>G (R112G). After establishing the diagnosis of FD, he moved to Australia, where he received ERT until 2018 when he moved back to Serbia. In the first 6 months after coming back to Serbia, he received ERT through a donation of agalsidase alfa from the pharmaceutical industry and, after that time, through the approval of the Ministry of Health Commission for Rare Diseases of the Republic of Serbia. This patient has 3 relatives with established FD who are living abroad. The second patient from Novi Sad has already been described, with classic FD genetic variation, c.871G>C (A291P). This patient has been on agalsidase beta therapy since 2017 after approval from the Ministry of Health Commission for Rare Diseases of the Republic of Serbia. He experienced cadaveric kidney transplantation at the beginning of 2019 without a break in ERT treatment, and he received ERT regularly after the successful kidney transplantation. Several patients have been diagnosed with FD after a family screening was performed upon establishing FD diagnosis in proband cases. The number of detected FD patients is certainly below the real one.

Under the auspice of the Nephrology Association of Serbia, high-risk population screening among the hemodialysis patient population of Serbia is underway. There are 63 dialysis centers in our country, with around 6,500 patients on chronic hemodialysis programs [31]. We intend to test male dialysis patients with unknown causes of ESRD below 50 years of age and female dialysis patients with unknown causes of ESRD regardless of their age. According to the last annual report of the Registry of Kidney Disease Patients in Serbia [30], 8.1% of our patients on renal replacement therapy have an unknown cause of ESRD, in 19.1% of them, the cause of ESRD is some form of glomerulonephritis, while 13.5% of our patients on renal replacement therapy are categorized as "other" for the cause of their ESRD (Table 1). We should all bear in mind that kidney presentation of FD is proteinuria and slowly progressive chronic kidney disease. A gold standard for a proteinuric kidney disorder is ultrasound-guided percutaneous kidney biopsy. Due to the lack of performance of electronic microscopy of biopsied tissue, there is a great risk of misinterpreting findings as focal and segmental glomerulosclerosis and missing some FD diagnoses by reporting these patients to have glomerulonephritis as the cause of their ESRD. Looking back on all this data, the importance of dialysis population screening in Serbia is only getting stronger.

Table 1

Prevalence per million population (PMP) and percentage of the causes of end-stage renal disease (ESRD) in Serbia [31]

Diagnosis	PMP	%
Hypertension	195.3	24.4
Glomerulonephritis	153.1	19.1
Diabetes mellitus	131.8	16.5
Other	107.7	13.5
Pyelonephritis	84.8	10.6
Unknown	64.7	8.1
Polycystic kidney disease	53.6	6.7
Renovascular disease	8.6	1.1

The first step in the diagnostic algorithm for male patients suspected of FD is enzyme (AGAL) activity testing. Usually, affected hemizygous males with classic FD have absent or substantially reduced enzyme activity levels (< 1%), while in heterozygous female patients with classic FD, due to random X-chromosome inactivation, enzyme activity levels may be just slightly reduced or even normal. In order to establish the exact genetic variation of the *GLA* gene, the next step for male patients with reduced enzyme activity and all female patients suspected of having FD is genetic testing. The genetic study is "*conditio sine qua non*" for suspected female FD patients. Measurement of biomarkers in the blood (Lyso-Gb3), urine (Gb3), or affected tissue (Gb3) can help in establishing the diagnosis and in the management and prognosis of FD patients. In classic FD patients (males more than females), levels of these biomarkers are substantially elevated. The easiest way to test for FD is with dried blood spot (DBS) testing, in which a few drops of blood are enough for the enzyme, biomarker, and genetic testing.

With this screening program, we hope to find some new FD patients. The most important result of our screening program should be the acknowledgement of new, previously unrecognized patients with FD through family screening that will follow (Figure 4). In these patients, implementing adequate and timely therapy could prevent or slow down target organ damage. Moreover, having a genetic variation running in the family would allow for proper genetic counseling of future parents from the affected families.

Contemporary FD therapy is directed toward enzyme replacement. Nowadays, there are two forms of agalsidase enzyme on the market, agalsidase alfa and agalsidase beta.

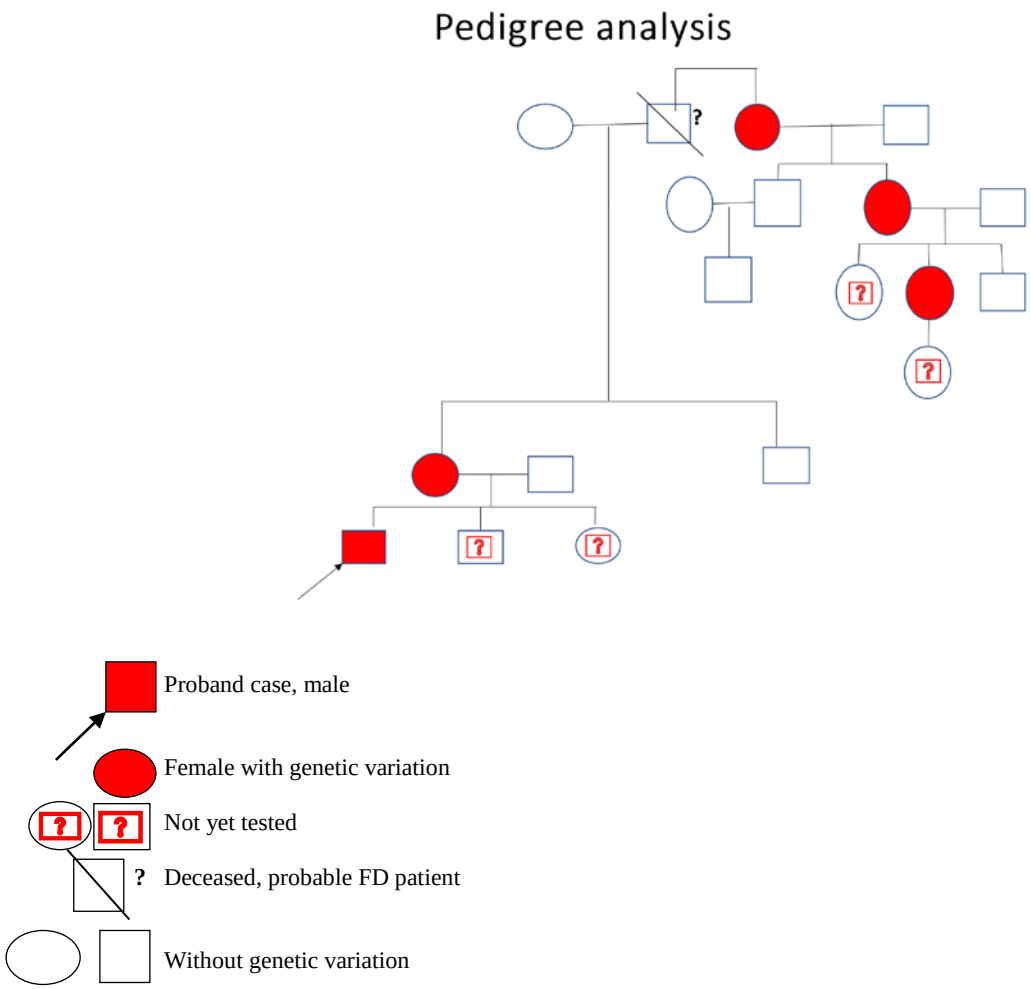

Fig. 4 – Pedigree analysis in Fabry disease.

Both enzyme forms have a place in the treatment of FD patients and are administered as *iv* infusions every 14 days. The dosage for agalsidase alfa is 0.2 mg/kg of body weight, and for agalsidase beta, 1 mg/kg of body weight. For some amenable genetic variations in the *GLA* gene, we can also use chaperone therapy with oral medication, migalastat [32]. Nevertheless, we cannot ignore adjunctive and supportive treatment for FD patients, such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, pain control medications, anti-arrhythmic drugs or devices, renal replacement therapy, etc.

Since 2017, enzyme replacement therapy has been available for treating FD patients in Serbia. Both preparations, agalsidase alfa (Replagal®, Takeda) and agalsidase beta (Fabrazyme®, Sanofi Genzyme), are available and registered in our country. For every FD patient eligible for enzyme replacement therapy, physicians need to prepare adequate documentation that comprises a complete enzyme, biomarker, and genetic workup and the complete clinical phenotype of a patient as well. The physicians then present it to the Ministry of Health Commission for Rare Diseases of the Republic of Serbia, which approves therapy for patients in need.

Conclusion

FD is an orphan disease, but patients with it should not be the ones. Predominantly, the paper's role is to promote awareness about this rare, X-chromosome-linked, slowly progressive lysosomal storage disorder that can equally affect males and females. Major target organs in FD are the kidneys, heart, and nervous system. Until now, 17 FD patients have been diagnosed in Serbia, but the number of affected people is probably larger. There are many reasons for such a low number of registered FD patients in Serbia. One of the main reasons for such a situation is certainly a very heterogeneous phenotype of FD. However, the main problem in diagnosing this disease is the lack of awareness among physicians regarding rare diseases in general and especially difficulties in testing possibilities for FD in Serbia. A high-risk population screening program among the hemodialysis patient population of Serbia is underway. The aim of the program will be to detect new patients, give more objective data regarding the prevalence of FD in Serbia, and establish the basis for family screening. In newly detected patients, implementing adequate and timely therapy could prevent or slow down the occurrence of target organ damage.

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CASE REPORTS

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Leukemic infiltration of the ovary as an initial presentation of chronic myeloid leukemia in the chronic phase

Infiltracija jajnika kao inicijalna prezentacija hronične mijeloidne leukemije u hroničnoj fazi

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Abstract

Introduction. Extramedullary sites of leukemic proliferation, harboring an adverse outcome, are rare and usually found in the blastic phase of chronic myeloid leukemia. We report a case of a newly diagnosed patient with chronic myeloid leukemia in the chronic phase, with leukemic infiltration of the right ovary on disease presentation. **Case report.** The patient presented with abdominal pain, leukocytosis, and anemia. A peripheral blood smear indicated chronic myeloid leukemia, and cytoreductive treatment was started. Due to the worsening of the abdominal pain, computed tomography was done. It revealed a cystic tumor of the right ovary. The tumor was surgically removed. Bone marrow examination confirmed the diagnosis of chronic myeloid leukemia in the chronic phase. Immunohistochemical analysis of the ovarian tumor showed leukemic infiltration with 5% of blasts. The patient was treated with imatinib for one year. Due to treatment failure, imatinib was switched to nilotinib. Allogeneic stem cell transplantation was considered. **Conclusion.** This case highlights the critical role of the multidisciplinary team approach and close treatment monitoring to achieve the best possible outcome in these patients.

Key words:
drug therapy; leukemia, myelogenous, chronic, bcr-abl positive; neoplasm invasiveness; ovary.

Apstrakt

Uvod. Ekstramedularna proliferacije ćelija u hroničnoj mijeloidnoj leukemij je retka, najčešće se javlja u fazi blastne transformacije i znak je loše prognoze bolesti. Prikazujemo slučaj bolesnice sa leukemijskom infiltracijom desnog jajnika, kao inicijalnom prezentacijom hronične mijeloidne leukemije u hroničnoj fazi. **Prikaz bolesnika.** Bolesnica je hospitalizovana zbog bolova u trbuhu, leukocitoze i anemije. Pregledom razmaza periferne krvi posumnjano je na hroničnu mijeloidnu leukemiju i započeta je citoreduktivna terapija. Zbog pogoršanja abdominalnih bolova urađena je kompjuterizovana tomografija abdomena i male karlice. Ovom metodom je pokazano postojanje cističnog tumora desnog jajnika. Tumor je hirurški uklonjen. Pregledom kostne srži postavljena je dijagnoza hronične mijeloidne leukemije u hroničnoj fazi. Imunohistohemijskim pregledom tumora jajnika potvrđena je leukemijska infiltracija sa 5% blasta. Bolesnica je lečena imatinibom godinu dana. Usled nezadovoljavajućeg odgovora na terapiju imatinibom, lečenje je nastavljeno nilotinibom. Razmatra se alogena transplantacija. **Zaključak.** Multidisciplinarni pristup i praćenje terapijskog odgovora su neophodni za najbolji ishod lečenja ovih bolesnika.

Ključne reči:
lečenje lekovima; leukemija, mijeloidna, hronična, bcr-abl pozitivna; neoplazme, invazivnost; jajnik.

Introduction

Chronic myeloid leukemia (CML) is a chronic myeloproliferative disorder characterized by a clonal proliferation of the hematopoietic stem cell. The proliferation is a consequence of the reciprocal translocation of chromosomes 9 and 22, which results in the formation of a shortened chromosome 22 called the Philadelphia (Ph) chromosome. As a result of the translocation, BCR and ABL genes get into close contact, and the product of their transcription is a highly active tyrosine kinase that causes the uncontrolled proliferation of myeloid cells. The disease has a

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chronic, accelerated, and blastic phase. Most patients are diagnosed in the chronic phase (CP), characterized by hepatosplenomegaly, leukocytosis, and fatigue. Extramedullary sites of leukemic proliferation are rare and usually found in the blastic phase of the disease, harboring an adverse outcome [1].

Case report

A 24-year-old woman was referred to the doctor due to a sudden onset of abdominal pain and weakness. The pain was localized in the right lower quadrant. She had no significant previous medical history but reported that she lost around 5 kg over the last four months. Routine blood work showed leukocytosis and anemia. After a brief hospitalization in a regional hospital, she was transferred to a tertiary center. Upon admission to the Clinic of Hematology, the physical examination showed tenderness in the lower right quadrant and a palpable spleen 5 cm below the left costal margin. Her last period was fifteen days prior to admission.

Initial blood count showed an extreme leucocytosis with anemia and slight thrombocytosis [hemoglobin 69 g/L (reference range – RR: 120–160 g/L), white blood count 603 × 10^9/L (RR: 4–10 × 10^9/L), platelets 464 × 10^9/L (RR: 140–400 × 10^9/L]. A peripheral blood smear was indicative of CML in CP (blasts 0.01, promyelocytes 0.01, myelocytes 0.04, metamyelocytes 0.07, bands 0.07, neutrophils 0.69, eosinophils 0.01, basophils 0.04, lymphocytes 0.04, monocytes 0.02, nucleated red blood cells 2/100). Intravenous hydration with saline, low molecular weight heparin, and cytoreductive treatment with hydroxyurea was started. Further hematological diagnostics were postponed due to the worsening of the abdominal pain and the development of an acute abdomen on the second day of hospitalization. Computed tomography of the abdomen and pelvis revealed a large, partially cystic tumor of the right ovary 53 × 53 × 63 mm [anteroposterior (AP), laterolateral (LL), craniocaudal (CC), respectively] in close contact with the uterus and bladder, enlarged liver (AP 150 mm, CC 185 mm) and spleen (140 × 110 × 180 mm AP, LL, CC, respectively) (Figure 1). The patient underwent an emergency laparotomy. A ruptured cyst macroscopically resembling a cyst of corpus luteum and hematoperitoneum was found intraoperatively. The cyst was enucleated. The postoperative course was complicated with the development of a hematoma of the front abdominal wall and pelvis. For that matter, the patient underwent further three surgical drainages. After stabilization, a bone marrow tap and trephine biopsy were performed. The result was a hypercellular bone marrow with the predomination of mature granulocytes; the percentage of blasts was less than 5% in concordance with the diagnosis of CML-CP. Cytogenetics revealed the presence of the Ph chromosome (46, XX, t(9:22)(q34:q11)) in all 30 analyzed metaphases, and quantitative PCR showed 49% of the ratio BCR-ABL to ABL on the international scale. She was classified as low risk according to Sokal score (0.64), Hasford (513.6), EUTOS score (48 points), and EUTOS long-term survival (ELTS) score (1.0497). Pathohistological examination of the intraoperatively enucleated tumor showed infiltration of ovarian tissue with atypical myeloid cells in all states of differentiation (Figure 2). The number of $CD34^+/CD117^+$ blasts was around 5%. One month after surgery, treatment with imatinib was started. A hematologic response was achieved after 2 months. The patient did not reach a complete cytogenetic response after six months of imatinib treatment (Table 1). An optimal cytogenetic and molecular response according to European LeukemiaNet (ELN) guidelines was not reached even after one year of imatinib treatment (Table 1) [2]. Gynecological follow-up showed a normal finding. Due to treatment failure with imatinib, nilotinib 800 mg/day was introduced. Human leukocyte antigen (HLA) typification was performed only for the patient because she did not have siblings. We plan to repeat PCR and cytogenetics after six months of treatment with nilotinib and decide about allogeneic transplantation from unrelated matched donors.

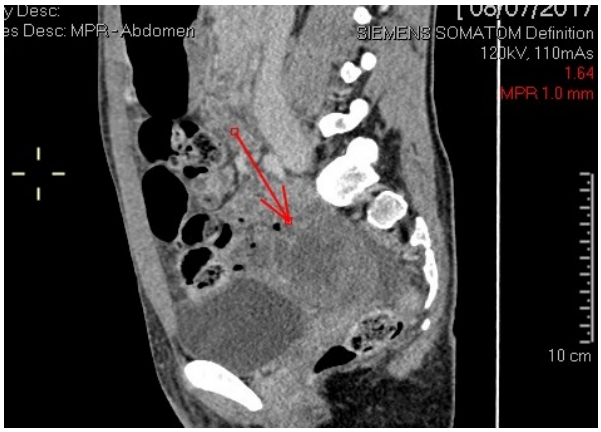

Fig. 1 – A sagittal computed tomography (CT) scan (abdomen and pelvis) showing a huge ovarian tumor (red arrow).

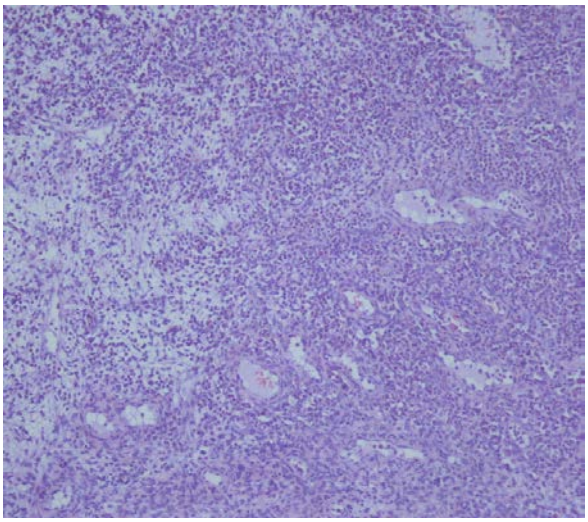

Fig. 2 – Histopathology of the excised ovarian tumor showing infiltration of myeloid cells in different stages of differentiation (hematoxylin and eosin staining, ×100).

Table 1

Results of treatment response monitoring

Duration of imatinib treatment	PCR (BCR-ABL to ABL)	Cytogenetics
Start	49%	46,XX, t(9:22)(q34:q11) / 30 metaphases
3 months	22%	Not done
6 months	1.5%	46,XX, t(9:22)(q34:q11) / 2 metaphases
1 year	0.69%	46,XX, t(9:22)(q34:q11) / 2 metaphases

PCR – polymerase chain reaction.

Discussion

Leukemic infiltration was previously described in CML, although the most common sites were lymph nodes, liver, spleen, and bones [1, 3]. Ovaries and other parts of the genital system are rarely affected by CML, especially in young females. The finding of leukemic infiltration in the ovarian mass was quite surprising in our patient. Mostly, complex cystic masses in young females are hemorrhagic cysts, endometriomas, a tubo-ovarian abscess, or ectopic pregnancy [4]. Solid masses can be benign or malignant ovarian tumors [4].

Pathohistological examination of the tumor is crucial for obtaining the correct diagnosis. Only morphological examination with hematoxylin and eosin stain can give an incorrect diagnosis [1]. Immunohistological positivity for myeloperoxidase, CD34, and CD117 was essential to determine cell origin and the number of blasts [1]. Fluorescent *in situ* hybridization (FISH) should be performed to prove the clonal origin of CML. In our case, it was not done due to technical difficulties.

In general, extramedullary leukemic infiltration indicates poor prognosis since it commonly represents an extramedullary blast crisis. Extramedullary blast crisis is most common in acute leukemia and the blastic phase of CML [1, 3]. Usually, it happens simultaneously or precedes marrow blast crisis by a few months [3]. It is very rare as an initial presentation of CML and in CML-CP [3]. Tyrosine kinase inhibotors (TKI) revolutionized the treatment and outcome of CML, inducing around 85% of major cytogenetic responses after 12 months of treatment with imatinib [5]. The response rate is even higher on treatment with nilotinib [6]. However, imatinib does not improve the prognosis of blast crisis dramatically [1]. Cytotoxic treatment followed by an early allogeneic stem cell transplantation is needed to induce remission in these patients [1].

In our opinion, our patient was not in an extramedullary blast crisis since the number of blasts in the infiltrated tissue was around 5%. That was very important for the treatment decision. Due to our country's regulations, imatinib was the only first-line treatment option. Nilotinib was introduced because of imatinib treatment failure after one year. We continued TKI treatment because the patient was still in CML-CP but decided to do HLA typification. The decision about the timing of allogeneic transplantation will be based on the results of PCR and cytogenetics after six months on nilotinib.

Another dilemma about CML and the ovarian localization of extramedullary hematopoiesis is fertility and childbirth. There is no solid evidence that TKI treatment impairs fertility, but medication should be stopped before pregnancy because of teratogenicity [7]. In the case of treatment with stem cell transplantation, fertility preservation methods should be used. Despite seldom reports of ovarian extramedullary tumors of CML origin, there is a possibility of gonadal infiltration by leukemic cells in hematologic malignancies. The critical question is the risk of disease relapse after in vitro fertilization. If the collected follicles carry leukemic residues, there might be a possibility of disease relapse during hormonal stimulation and the reimplantation of the follicles [8, 9]. Pregnancy itself could contribute to disease relapse [9]. One study on this topic showed that although leukemic cells were found in the ovarian tissue of patients with leukemia after complete remission, no leukemic relapse was detected after the tissue was transplanted into murine hosts [10].

Conclusion

Leukemic infiltration of the ovary is seldom found in patients with CML-CP, especially as an initial presentation. The finding or exclusion of extramedullary blast crisis is essential for decision-making concerning the treatment of these patients.

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CASE REPORT

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Avascular necrosis of the femoral head following an occult femoral neck stress fracture

Avaskularna nekroza glave femura nakon okultnog stres preloma vrata femura

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Abstract

Introduction. Osteonecrosis (ON) of the femoral head (FH) – (ONFH) is an intractable disease that causes progressive femoral head collapse, severe pain, and gait disturbance. We report a case of avascular necrosis of the femoral head following an occult femoral neck (FN) stress fracture. **Case report.** A 55-year-old woman presented to our department with a chief complaint of low back pain that radiated into the left anterolateral thigh over the period of two months. Her left anterolateral thigh became progressively more painful over the past two weeks. No abnormal findings indicative of ONFH or an occult fracture of the FN were detected by X-ray or computed tomography, but an occult insufficiency fracture of the left FN was identified on magnetic resonance imaging (MRI). The diagnosis of FN stress fracture was delayed, resulting in femoral head necrosis. The fracture was treated with total hip arthroplasty, and the resected FH was subjected to histopathology (HP). Based on the HP findings, the final diagnosis of this case was ONFH with an occult fracture of the left FN. Clinical symptoms were relieved postoperatively. **Conclusion.** An early MRI examination is recommended in patients presenting with a suspected stress fracture of the FN to avoid FH necrosis due to a delayed diagnosis.

Key words:
arthroplasty, replacement, hip fracture; fracture, stress; femur head necrosis; magnetic resonance imaging; surgical procedures, operative; osteonecrosis.

Apstrakt

Uvod. Osteonekroza (ON) glave femura (GF) – (ONGF) je bolest koju je teško kontrolisati, a izaziva progresivni kolaps GF, praćen jakim bolom i otežanim hodom. Prikazana je bolesnica sa avaskularnom nekrozom GF, nastalom usled okultne stres frakture vrata femura (VF). **Prikaz bolesnika.** Žena stara 55 godina javila se na Odeljenje, žaleći se na bol u leđima koji se širio u levu butinu u prethodna dva meseca. Bol u levoj butini se progresivno pojačavao tokom prethodne dve sedmice. Na radiografskim snimcima, kao i snimanjem kompjuterizivanom tomografijom, nije utvrđen patološki nalaz indikativan za ONGF ili okultnu frakturu VF, ali je snimanjem magnetnom rezonancom (MR) utvrđena okultna insuficijentna fraktura levog VF. Dijagnoza stres frakture VF nije postavljena, što je rezultiralo nekrozom GF. Bolesnici je urađena totalna artroplastika kuka, a uzorak GF je upućen na histopatološku (HP) analizu. Na osnovu HP nalaza, postavljena je konačna dijagnoza ONGF sa okultnom frakturom levog VF. Simptomi bolesnice su se povukli posle operacije, što je rezultiralo njenim kliničkim oporavkom. **Zaključak.** Kod bolesnika koji se prezentuju stanjem koje je sumnjivo na stres frakturu VF, preporučuje se rano snimanje MR, da bi se, usled odložene dijagnoze, izbegla nekroza GF.

Ključne reči:
artroplastika kuka; kuk, prelom; prelomi usled zamora; femur, nekroza glave; magnetska rezonanca, snimanje; hirurgija, operativne procedure; osteonekroza.

Introduction

Osteonecrosis (ON) of the femoral head (FH) – ONFH is an intractable disease that causes progressive FH collapse, severe pain, and gait disturbance [1–3]. The most common risk factors for ONFH are previous trauma, long-term corticosteroid use, and alcohol abuse; however, idiopathic ONFH, in which the patient has no known risk factors, is not an infrequent finding [4, 5]. The collapse of a necrotic FH often occurs in subchondral bone, but an occult insufficiency fracture

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of the femoral neck (FN) is relatively rare in cases of ONFH. As the pathogenesis of ONFH is unclear, it is necessary to investigate its clinical manifestations to improve diagnosis, treatment, and prognosis. In this report, we describe a case of avascular necrosis of the FH following an occult FN stress fracture. The fracture was spontaneous, and there was no trauma or unusual activity. No signs of an occult FN fracture or ONFH were found by X-ray or computed tomography (CT). The entire FH was necrotic following the occult FN stress fracture. Written informed consent was obtained from the patient to publish this case report and any accompanying images.

Case report

A 55-year-old woman (height, 160 cm; weight, 55 kg; body mass index 21.4 kg/m^2) presented to our Department with a chief complaint of low back pain that had radiated into the left anterolateral thigh over the past two months.

The patient had experienced progressive pain in the left anterolateral thigh for about two weeks. The pain was relieved when she lay down on a bed but was exacerbated by standing or walking. She had no history of trauma, alcohol abuse, corticosteroid use, or smoking.

The physical findings were thoracolumbar spine range of motion of approximately 80° flexion, 10° extension, and 15° lateral flexion bilaterally. The lumbar paraspinal musculature was tender to the touch at all levels, and there was a negative Lasegue sign and no lower extremity numbness. Deep left groin area tenderness and a positive Patrick sign were observed on the hip examination.

Routine laboratory tests were normal. Lumbar spine magnetic resonance imaging (MRI) demonstrated intervertebral disc degeneration at the L4/5 levels. The bone mineral density (BMD) of the left hip measured by dual X-ray absorptiometry was 0.772 g/cm^2 (T score, -1.9). The BMD of the lumbar spine was 0.900 g/cm^2 (T score, -2.5). No abnormal findings were detected on a plain radiograph of the left hip (Figure 1). No bone necrosis or fracture signs were found on a CT scan (Figure 2), but T1-weighted MRI revealed a large, low-intensity area in the left FH and FN. An occult insufficiency fracture of the left FN was found (Figure 3A) on the T2-weighted image, and there was a large area of bone marrow edema (BME) in the left FH and FN (Figure 3B).

Fig. 1 – No significant findings were observed in the anteroposterior view.

Fig. 2 – Coronal computed tomography (CT) images of the left hip did not reveal bone necrosis or fracture signs.

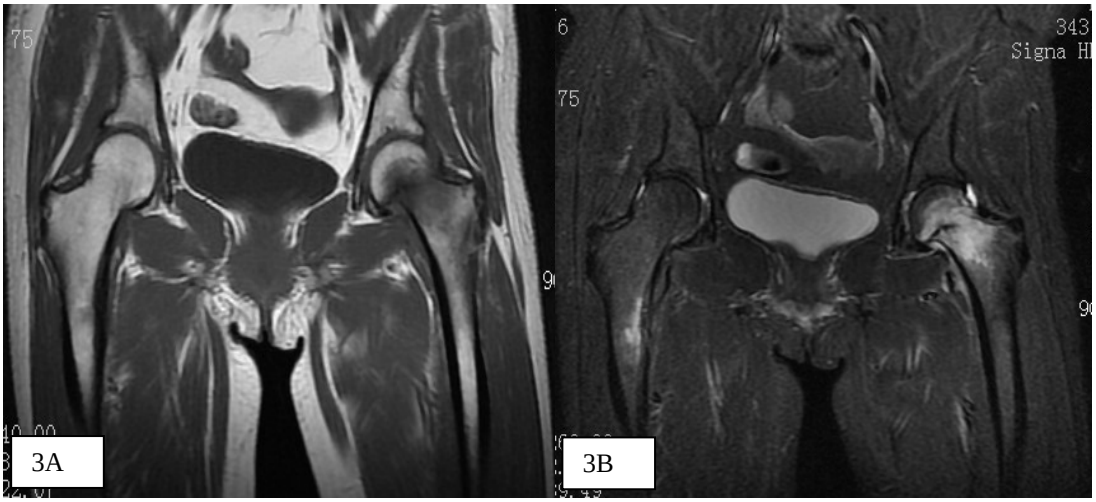

Fig. 3 – A) T1-weighted magnetic resonance image (MRI) showing a large low-intensity area in the left femoral head (FH) and femoral neck (FN) and an occult insufficiency fracture of the left FN; B) T2-weighted MRI showing a large area of bone marrow edema in the left FH and FN and an occult insufficiency fracture of the left FN.

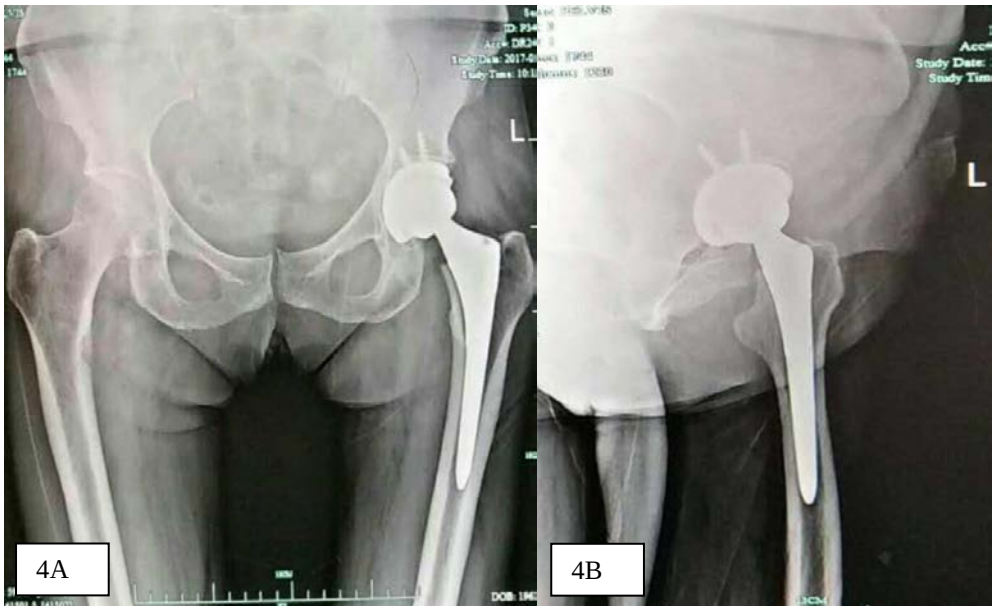

Fig. 4 – Anteroposterior (4A) and lateral (4B) X-ray of the left total hip arthroplasty.

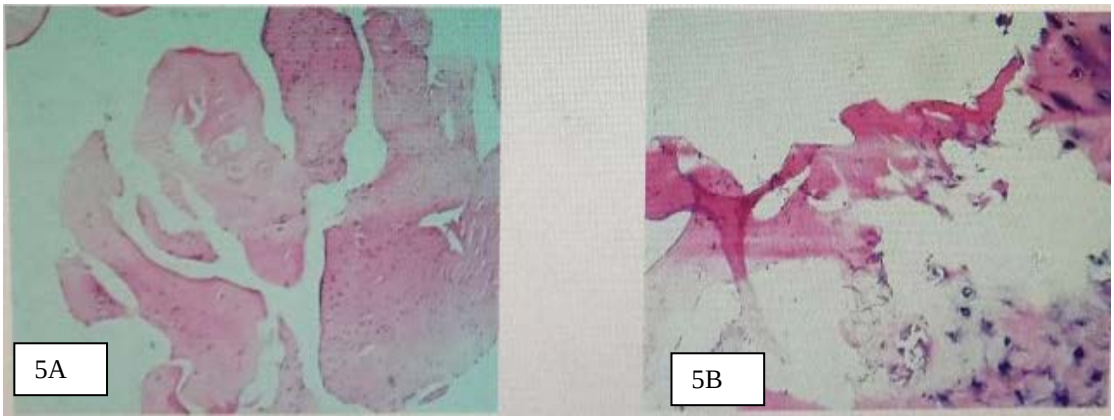

Fig. 5 – Histological examination reveals dead osteocytes in the trabeculae of the femoral head (5A), which is consistent with the pathological diagnosis of osteonecrosis (5B). (hematoxylin and eosin, ×100).

The diagnosis was an occult fracture of the left FN based on the MRI findings. The occult fracture may have been caused by osteoporosis. The diagnosis of FN stress fracture was delayed, resulting in FH necrosis. The fracture was treated using total hip arthroplasty (Figure 4), and the resected FH was observed histopathologically. The histological examination revealed a typical pattern of dead osteocytes in the trabeculae of FH, consistent with a pathologic diagnosis of osteonecrosis (Figure 5). Based on these histopathological findings, the final diagnosis, in this case, was ONFH with an occult fracture of the left FN. The clinical symptoms were relieved postoperatively.

Discussion

ONFH often leads to FH collapse and disabling secondary osteoarthritis of the hip joint, which destroys the hip. In the United States, 20,000–30,000 patients are diagnosed with osteonecrosis every year [6]. The most important risk factors for ONFH in the United States are alcohol misuse (20–40%), corticosteroid therapy (35–40%), and idiopathic diseases (20–40%) [7]. Idiopathic (primary or spontaneous) ONFH is the fourth leading cause of ONFH and has no overt risk factors. The most common clinical manifestation of ONFH is progressive hip pain. The clinical presentation can vary and may involve spine or knee symptoms, with normal hip physical examination findings or onset of pain in the pelvis, buttocks, groin, and lower limbs [8]. Our patient had no history of trauma, alcohol abuse, corticosteroid use, or smoking, so it was not intuitive to suspect occult FN stress fracture or ONFH based on the medical history and clinical manifestations. In particular, no signs of occult FN fracture or ONFH were found by X-ray or CT, which delayed the diagnosis of a stress fracture of the FN and thus promoted the progression to FH necrosis.

ONFH often occurs in the weight-bearing area of the FH, i.e., in the subchondral bone, and rarely involves the FN. An occult insufficiency fracture of the FN is relatively rare in

cases of ONFH. In our patient, T1-weighted MRI demonstrated a large low-intensity area in the left FH and FN, and an occult insufficiency fracture of the left FN was found on the T2-weighted image, in association with a large area of BME in the left FH and FN. However, BME should be distinguished from osteonecrosis because it is a secondary phenomenon resulting from FH collapse that resolves spontaneously [9–11]. No typical clinical pattern was found in our patient, and the quality of the bone was insufficiently poor for a spontaneous fracture of the FN to occur. Subchondral insufficiency fracture of the FN may occur in elderly patients with osteoporosis; spontaneous necrosis of the FH may occur first, followed by a stress fracture of the FN. In this case, the resected FH was observed histopathologically and showed a zonal pattern comprised of an area of bone infarction, reparative granulation tissue, and viable tissue. Based on these findings, the final diagnosis, in this case, was ONFH with an occult fracture of the left FN. Subcapital fractures in cases of ONFH rarely have good outcomes. Consequently, these patients should be treated with hip arthroplasty. We treated our case with total hip arthroplasty. Fukui et al. [12] reported a case of occult fracture of the FN due to extensive ONFH in a 60-year-old man with hip pain. Yoon et al. [13] reported an unusual case of ONFH that was misdiagnosed as a stress fracture. However, in our patient, the delayed diagnosis of FN stress fracture resulted in FH necrosis.

Conclusion

Although the clinical relevance of an occult fracture of the FN in cases of ONFH remains unclear, an early MRI examination is recommended in a patient with a suspected stress fracture of the FN to avoid FH necrosis due to a delayed diagnosis.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Conflict of interest

No potential conflicts of interest relevant to this article are reported.

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CASE REPORT

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Laparoscopic hysterectomy as a treatment modality for gestational trophoblastic neoplasms: a report of two cases

Laparoskopska histerektomija u lečenju bolesnica sa gestacijskim trofoblastnim neoplazmama: prikaz dva slučaja

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Abstract

Introduction. Measuring the serum levels of human chorionic gonadotropin beta isoform (β-hCG) remains a crucial marker for diagnosing gestational trophoblastic neoplasms (GTNs). Choriocarcinoma is commonly diagnosed due to extremely high levels of β-hCG, but the presence of distant metastasis is not uncommon. Placental site trophoblastic tumors and epithelioid trophoblastic tumors remain an enigma because the levels of β-hCG are usually low. **Case report.** The first case report describes a 44-year-old woman, P2G3, admitted to the Clinic under the suspicion of molar pregnancy. She had vaginal bleeding with variable intensity, and her β-hCG was 1,837,787 mIU/mL. After two explorative curettages, the level of β-hCG declined, and a partial hydatidiform mole (HM) was diagnosed histopathologically. The patient was admitted to the Clinic on two occasions due to the increasing values of β-hCG. Since β-hCG failed to drop after two explorative curettages, a hysteroscopic biopsy, and one chemotherapy cycle, along with the suspicious ultrasonographic feature of metastatic GTN, and the fact that the patient has refused further chemotherapy, a total laparoscopic hysterectomy was performed. Choriocarcinoma was diagnosed after a histopathological exam was done. The second patient, a 50-year-old woman, P2G4, was admitted to the Clinic under the ultrasonographic suspicion of molar pregnancy. She was complaining of pelvic discomfort and frequent urination. Initial levels of β-hCG were 128,359 mIU/mL. Instrumental revision of the uterine cavity was performed, and partial HM was diagnosed histopathologically. Because of the increasing levels of β-hCG, ultrasonographical suspicion of the development of GTN in the uterine corpus, in accordance with the patient's age and the fact that she has regular menstrual cycles, total laparoscopic hysterectomy was performed, and a histopathological exam made the diagnosis of the placental site trophoblastic tumor. **Conclusion.** Laparoscopic hysterectomy could be a treatment of choice for the chemotherapy-resistant GTNs but also for choriocarcinoma in patients who have finished their reproductive activity and refuse to be treated with chemotherapeutics.

Key words:
choriocarcinoma; gestational trophoblastic disease; hysterectomy; laparoscopy; trophoblastic neoplasms; trophoblastic tumor, placental site.

Apstrakt

Uvod. Određivanje koncentracija beta izoforme humanog horionskog gonadotropina (β-hCG) u serumu predstavlja značajan marker gestacijskih trofoblastnih neoplazmi (GTN). Zbog izrazito visokih vrednosti β-hCG-a, horiokarcinom se obično dijagnostikuje, ali prisustvo udaljenih metastaza nije neuobičajeno. S druge strane, trofoblastni tumor placentnog ležišta i epitelioidni trofoblastni tumor ostaju velika enigma, s obzirom na to da su koncentracije β-hCG-a kod tih tumora često niske. **Prikaz bolesnika.** U prvom prikazu opisana je žena od 44 godine, P2G3, koja je primljena na Kliniku zbog sumnje na molarnu trudnoću. Žalila se na vaginalno krvarenje varijabilnog intenziteta. Inicijalna vrednost serumskog β-hCG-a iznosila je 1 837 787 mIU/mL. Nakon dve eksplorativne kiretaže došlo je do pada β-hCG-a, a histopatološki je dijagnostikovana parcijalna hidatidna mola (HM). Zbog ponovnog rasta β-hCG-a, bolesnica je u dva navrata hospitalizovana. S obzirom na to da nije došlo do pada vrednosti β-hCG-a posle dve instrumentalne revizije materične duplje, histeroskopije sa ciljanom biopsijom, jednog ciklusa hemioterapije, uz ultrazvučni nalaz visoko sumnjiv na metastatsku GTN u zidu uterusa, kao i činjenice da je bolesnica odbila dalju hemioterapiju, urađena joj je totalna laparoskopska histerektomija, sa konzervacijom adneksa. Histopatološkom analizom dijagnostikovan je horiokarcinom. U drugom prikazu je opisana žena od 50 godina, P2G4, upućena na Kliniku zbog sumnje na molarnu trudnoću, postavljenu na osnovu ultrazvučnog nalaza. Žalila se na nelagodnost u maloj karlici i često mokrenje. Inicijalna vrednost β-hCG-a iznosila je 128 359 mIU/mL. Urađena joj je instrumentalna revizija materične duplje, a histopatološki je dijagnostikovana parcijalna HM. Zbog rastućih vrednosti

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β-hCG-a, sumnje na perzistirajuću GTN, postavljene na osnovu ultrazvučnog nalaza, a shodno godinama bolesnice i činjenici da je imala redovne cikluse, urađena joj je totalna laparoskopska histerektomija, sa konzervacijom jajnika. Histopatološkom analizom dijagnostikovan je trofoblastni tumor placentnog ležišta. **Zaključak.** Laparoskopska histerektomija bi mogla biti tretman izbora u lečenju bolesnica sa GTN rezistentnih na hemioterapiju, kao i bolesnica sa horiokarcinomom koje su završile svoju reproduktivnu aktivnost i koje odbijaju lečenje hemioterapijom.

Ključne reči:
horiokarcinom; trofoblastne bolesti, gestacijske; histerektomija; laparoskopija; neoplazme, trofoblastne; trofoblastni tumor, posteljičnog ležišta.

Introduction

Gestational trophoblastic diseases (GTD) represent a spectrum of abnormal proliferation of trophoblast cells. They include complete and partial hydatidiform mole (HM), sometimes marked as "premalignant GTD", and gestational trophoblastic neoplasia (GTN), which include the following: choriocarcinoma, placental site trophoblastic tumor, epithelioid trophoblastic tumor, and invasive mole [1].

Measuring the serum levels of human chorionic gonadotropin's beta isoform (β-hCG) remains a crucial marker for diagnosing the GTN, but also a valuable indicator of (un)successful therapy [1, 2]. The diagnosis of postmolar GTN is usually made by observing the persistent or increasing levels of β-hCG following the evacuation of the HM. Choriocarcinoma is commonly diagnosed due to extremely high levels of β-hCG, but the presence of distant metastasis is not uncommon [1–3]. At the same time, choriocarcinoma is highly chemotherapy-sensitive; therefore, nowadays, a complete recovery is possible in 90% of cases [3]. On the other hand, placental site trophoblastic tumors and epithelioid trophoblastic tumors remain an enigma because the levels of β-hCG are usually low or even normal, and these tumors are resistant to chemotherapy in most cases [1–3].

Even though modern chemotherapy protocols provide almost complete remission of the disease, cases of chemotherapy-resistant GTNs require surgical treatment [4]. A particular challenge in surgical treatment represents patients who wish to preserve their fertility and those whose ovarian function must be conserved [4]. On the other hand, another challenge represents the group of patients who refuse the chemotherapy treatment method.

We present two patients with GTN with unusual clinical features in which total laparoscopic hysterectomy provided a complete recovery for the patients.

Case report

Case I

A 44-year-old patient, P2G3, was admitted to the Clinic under the suspicion of molar pregnancy. Vaginal bleeding of variable intensity was the only symptom she was complaining about. Levels of β-hCG were 1,837,787 mIU/mL (Figure 1). On the first and the fourth hospital day, explorative curettages were performed. From the obtained samples, a histopathological diagnosis of partial HM was made. After the procedures, the levels of β–hCG were in decline (Figure 1). After one month, she was once again admitted to the Clinic. The levels of β-hCG were increasing (from 630 to 1,263 mIU/mL). A suspicious mass in the right uterine cornu, intimately beside the uterine cavity, was seen on the ultrasonographic exam (Figure 2A). A diagnostic hysteroscopy was performed. Hysteroscopically, no residual tissue nor trophoblastic protrusion was seen, and the histopathological exam of the tissue obtained from the endometrial biopsy revealed a secretory endometrium. After diagnostic hysteroscopy and one chemotherapy cycle, the serum levels of β-hCG dropped

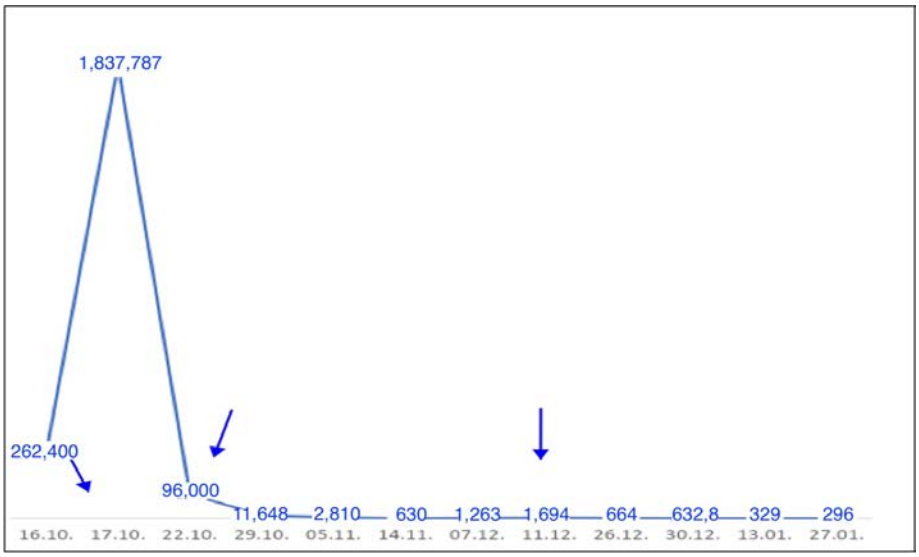

Fig. 1 – Levels of serum β-hCG (mIU/mL) in the first patient; interventions are marked with an arrow.

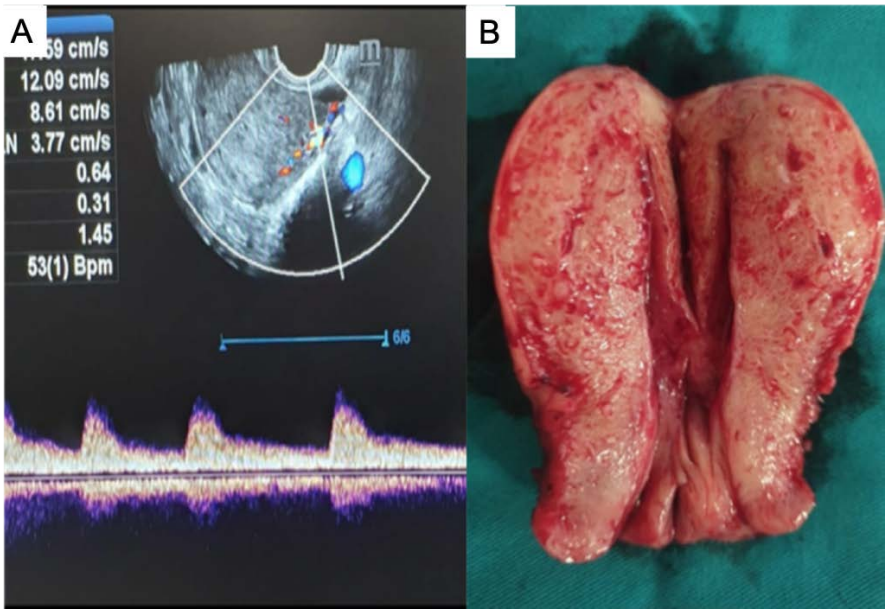

Fig. 2 – A) Gynecologic ultrasonographic image of the first patient reveals a suspicious mass in the right uterine cornu, intimately beside the uterine cavity; B) Uterus after the hysterectomy.

(from 1,694 to 664 mIU/mL) (Figure 1). After more than one month, she was admitted again to the Clinic, given that β–hCG levels were persisting (329 and 296 mIU/mL) (Figure 1). The patient refused further chemotherapy treatment, and it was decided to perform a total laparoscopic hysterectomy with preservation of the ovaries (Figure 2B). A histopathological exam of the obtained tissue revealed choriocarcinoma. The levels of β-hCG significantly dropped, and the patient was discharged.

Case 2

A 50-year-old woman, P2G4, was admitted to the Clinic under the ultrasonographic suspicion of molar pregnancy. She was complaining of pelvic discomfort and frequent urination. Serum levels of β–hCG were 128,359 mIU/mL (Figure 3). Since the ultrasonographic exam confirmed the suspicion of molar pregnancy, explorative curettage of the uterine cavity was performed. Histopathological diagnosis from the obtained sample of the partial HM was made. Levels of serum β–hCG were in decline after the intervention (31,661 mIU/mL) (Figure 3). Since the concentration of β–hCG saw a trend of increase (from 430 to 598 mIU/mL) (Figure 3), the patient was once again admitted to the Clinic. When the patient's age, the fact that she had regular menstrual cycles, and her serum levels of β–hCG were taken into account, along with the suspicion of the development of GTN in the uterine corpus (Figure 4A), it was decided to perform a total laparoscopic hysterectomy with the conservation of the one ovary (Figure 4B). Histopathologically, the decidual remains and

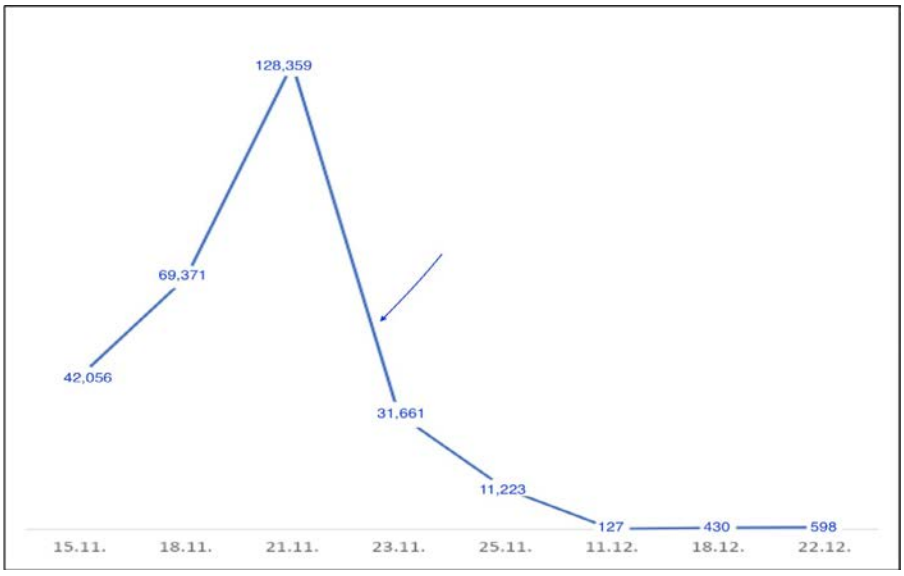

Fig. 3 – Levels of serum β–hCG (mIU/mL) in the second patient; intervention is marked with an arrow.

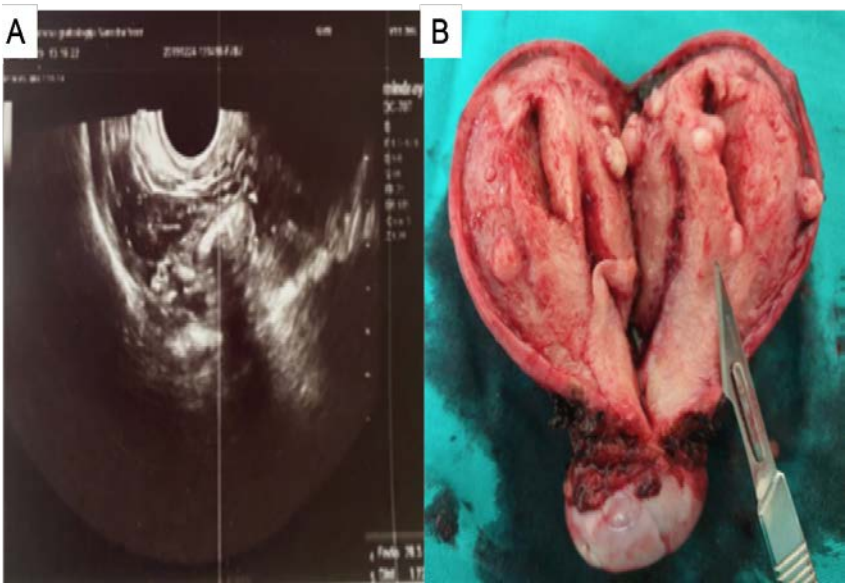

Fig. 4 – A) Ultrasonographic image in the second patient before the operation: the suspect development of a gestational trophoblastic neoplasm (GTN) in the uterine corpus; B) Uterus after the hysterectomy.

the several intermediate trophoblastic cells of the placental site in the endometrium were seen. Chorionic villi were not present. The diagnosis of the placental site trophoblastic tumor was made. A few days after the surgery, serum β-hCG was negative, and the patient was discharged.

Discussion

Complete HMs usually present with high levels of serum β-hCG [5]. It was shown that in more than 50% of patients with complete HM, the levels of β-hCG before evacuation exceeded 100,000 mIU/mL [5]. On the other hand, only 10% of the patients with partial HM had such high serum β-hCG levels [5]. Our patient, whose final diagnosis was choriocarcinoma, was initially diagnosed with partial HM, along with β-hCG levels of 1,837,787 mIU/mL, a concentration unusually high for this type of GTD. Moreover, it has been reported that 15–20% of complete HMs progress into an invasive mole or other forms of GTN [5]. However, only 0.5–2.0% of partial HMs transform into some form of GTN [2, 5]. In both of our patients with GTN, the initial diagnosis was partial HM.

About 50% of choriocarcinomas arise based on complete HM, 25% after a normal pregnancy, and about 25% after a miscarriage or ectopic pregnancy [6]. Only a few reports in the literature regarding choriocarcinomas occurred based on partial HMs [6, 7]. One of the main features of choriocarcinoma is extremely high β-hCG levels [1, 6]. In our first case, the levels of β–hCG persisted in the range from 329 to 1,694 mIU/mL, which is unusual for this type of GTN. Choriocarcinomas are chemotherapy-sensitive [6], which can explain the significant drop in β-hCG levels after one chemotherapy cycle. When there is no distant metastasis, an individualized chemotherapy protocol for each patient is made, and the chemotherapy itself is usually the treatment of choice [5, 6].

Placental site trophoblastic tumor is extremely rare, and the epithelioid trophoblastic tumor accounts for about 0.2–3.0% of GTNs [8]. A special challenge for the correct diagnosis and proper treatment of these tumors is relatively low levels of β-hCG, often nonspecific clinical features, and chemotherapy resistance [8]. Even though the data is limited, it has been reported that placental site trophoblastic tumor occurs in 61% after a normal pregnancy, in 12% after a molar pregnancy, in 9% after a spontaneous miscarriage, in 8% after induced abortions, and in 3% after ectopic pregnancy [8, 9]. The remaining 7% does not have a clear etiology [8, 9]. This tumor arises exclusively from the proliferation of the intermediate trophoblasts [8–10]. The absence of syncytiotrophoblasts is exactly the reason why the levels of β-hCG in these tumors are usually low or even normal. Therefore, the measurement of human placental lactogen (hPL) is a good marker for diagnosing these tumors [8, 9]. These tumors usually present with irregular bleeding and an invasion of the myometrium and endometrium [9]. In our case, the invasion of the tumor into the uterine walls was not present. In almost all cases of this tumor, the treatment of choice was total hysterectomy with the preservation of the adnexa, except in those cases with a family history of ovarian cancer and postmenopausal women [10]. To the best of our knowledge, there are no reports of laparoscopic hysterectomy as a treatment method for the placental site trophoblastic tumor.

Conclusion

Even though gestational trophoblastic diseases have been known for a long time, they can still be a mystery for modern gynecological practice. Owing to everyday progress in chemotherapy, they are often marked as completely curable diseases. Although chemotherapy is usually the treatment of choice, there are types of GTN that are resistant to chemo-

therapy and require surgical treatment. Laparoscopy, on the other hand, brings many advantages for the patients, from less intraoperative bleeding to shorter postoperative recovery. With these two cases, we have shown that laparoscopic hysterectomy could become a treatment of choice for chemotherapy-resistant GTNs, but also for the treatment of choriocarcinomas in patients who have finished their reproductive activity and refuse the treatment with chemotherapeutics.

Conflict of interest

The authors declare no conflict of interest.

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CASE REPORT

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Can propylthiouracil induce autoimmune-related immunotoxicity?

Može li propiltiouracil izazvati autoimunski posredovanu imunotoksičnost?

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Abstract

Introduction. The use of propylthiouracil can be associated with mild adverse reactions, but severe complications such as agranulocytosis and vasculitis can also be seen. Direct toxicity and immune-mediated induction of anti-neutrophile cytoplasmic antibodies have been described as possible mechanisms responsible for agranulocytosis. The majority of vasculitis is antimyeloperoxidase antibodies associated, but the exact mechanism for anti-neutrophile cytoplasmic antibodies-associated vasculitis as an adverse effect of propylthiouracil treatment is still unclear. **Case report.** We presented a 61-year-old female patient with Graves' disease who experienced a fever and throat pain two weeks after propylthiouracil therapy was initiated. Agranulocytosis alongside basal left-sided pneumonia was noted. Propylthiouracil was discontinued, and the treatment with broad-spectrum antibiotics was started, as well as Lugol's solution, methylprednisolone, and granulocyte-colony stimulating factor. Further course of treatment was complicated by the occurrence of a generalized erythematous-papillomatous rash. The patient was diagnosed with agranulocytosis and antimyeloperoxidase, anti-neutrophile cytoplasmic antibodies positive vasculitis as an adverse effect of propylthiouracil. **Conclusion.** Patients presenting with concomitant agranulocytosis and anti-neutrophile cytoplasmic antibodies-associated vasculitis as a complication of propylthiouracil therapy for Graves' disease are rare in clinical practice. Prompt discontinuation of the antithyroid drug is of great importance to reduce damage to target organs. Similarities in the pathogenesis of both conditions could be the potential explanation for these two adverse events occurring at the same time, which points out the need for a deeper understanding of this topic.

Key words:
agranulocytosis; anti-neutrophil cytoplasmic antibody-associated vasculitis; drug-related side effects and adverse reactions; graves disease; propylthiouracil.

Apstrakt

Uvod. Propiltiouracil može biti povezan sa blagim neželjenim efektima, ali se u praksi mogu videti i ozbiljne komplikacije, poput agranulocitoze i vaskulitisa. U literaturi je kao mogući mehanizam nastanka agranulocitoze opisana direktna toksičnost i imunski posredovana aktivacija antineutrofilnih i anticitoplazmatskih antitela. Većina vaskulitisa je udružena sa antitelima na mijeloperoksidazu, ali nije poznat tačan mehanizam nastanka vaskulitisa udruženih sa antineutrofilnim citoplazmatskim antitelima kao neželjenim dejstvom terapije propiltiouracilom. **Prikaz bolesnika.** Prikazana je bolesnica sa Bazedovljevom (Grejsovom) bolesti, stara 61 godinu, koja se javila lekaru zbog povišene telesne temperature i bolova u grlu, dve nedelje nakon započinjanja terapije propiltiouracilom. Verifikovana je agranulocitoza kao i levostrana bazalna pneumonija. Obustavljena je primena propiltiouracila, a započeta terapija antibioticima širokog spectra, uz Lugolov rastvor, metilprednizolon i faktor stimulacije kolonije granulocta. Dalji tok se komplikovao pojavom generalizovane, eritematozno-papulomatozne ospe. Kod bolesnice je postavljena dijagnoza agranulocitoze i vaskulitisa udruženog sa antitelima na mijeloperoksidazu i antineutrofilnim citoplazmatskim antitelima, kao neželjenim dejstvima propiltiouracila. **Zaključak.** Bolesnici koji imaju Grejvsovu bolest, sa istovremenom agranulocitozom i vaskulitisom udruženim sa antineutrofilnim citoplazmatskim antitelima kao komplikacijom terapije propiltiouracilom, su retki u kliničkoj praksi. Hitan prekid terapije antitiroidnim lekom je od velikog značaja za smanjenje oštećenja ciljnih organa. Sličnost u patogenezi oba stanja mogla bi objasniti istovremenu pojavu ta dva neželjena dejstva, što zahteva dalja istraživanja.

Ključne reči:
agranulocitoza; vaskulitis, povezan sa antineutrofilnim antiplazmatskim antitelima; lekovi, neželjeni efekti i neželjene reakcije; gušavost, egzoftalmička; propiltiouracil.

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Introduction

Propylthiouracil (PTU) is a thiourea antithyroid drug (ATD) used in the treatment of hyperthyroidism. It acts as an inhibitor of thyroid hormone synthesis by competitive inhibition of the enzyme peroxidase and conversion of thyroxine (T4) to triiodothyronine (T3) [1–3]. Even though systemic complications like gastric intolerance, pruritus, and arthralgia can be found in only about 1% to 5% of patients, severe complications like agranulocytosis and vasculitis have also been identified [1–4]. Patients with autoimmune thyroid diseases may have other autoimmune conditions and are more prone to the development of drug-induced autoimmune diseases [5, 6]. Direct toxicity and immune-mediated induction of anti-neutrophile cytoplasmic antibodies (ANCA) have been described as possible mechanisms responsible for agranulocytosis. It mostly occurs within the first 3 months of the ATD treatment in 0.2–0.5% of patients with Graves' disease and is potentially life-threatening due to an increased susceptibility to infection [7]. The exact pathogenesis of ANCA-associated vasculitis as an adverse effect of PTU treatment remains a matter of debate, even though it is widely known that the majority of these vasculitides are anti-myeloperoxidase (MPO) antibodies associated [8]. The prevalence of ANCA positivity in patients treated with PTU varies across literature between 4% and 64%, with a median prevalence of 30% [9–13]. It is hypothesized that these antibodies are induced by frequent and prolonged PTU therapy and occur more frequently in women, probably due to a higher prevalence of thyroid dysfunction in women [9, 14–16]. It should be emphasized that some patients have ANCA positivity even prior to PTU therapy [5].

This case report describes a patient who experienced two adverse events secondary to PTU treatment: agranulocytosis and ANCA-associated vasculitis. Similarities in the pathogenesis of both conditions could be the potential explanation for these two adverse events occurring at the same time, which points out the need for a deeper understanding of this topic.

Case report

A 61-year-old female with Graves' disease was admitted to the Department of Endocrinology with a 3-day history of fevers, throat soreness, arthralgia, myalgia, and fatigue.

Table 1

Biochemical analyses of the presented patient with Graves' disease

Parameter	Normal range	On admission	After therapy
Leukocytes, $\times 10^9$/L	4.0–10.0	0.51	10.43
Erythrocytes, $\times 10^{12}$/L	3.9–5.4	4.0	3.83
Hemoglobin, g/L	120–160	117	118
Hematocrit, L/L	0.370–0.470	0.346	0.369
Thrombocytes, $\times 10^9$/L	140–400	126	184
Neutrophils, $\times 10^9$/L	2.0–7.0	0.04	5.88
Urea, mmol/L	2.2–7.1	11.1	4.2
Creatinine, µmol/L	49–97	85	63
Uric acid, µmol/L	154–357	291	244
Total protein, g/L	60–83	51	68
Albumin, g/L	35–55	26	41
Total bilirubin, µmol/L	3.0–21.0	25.8	6
Direct bilirubin, µmol/L	0.1–4.2	12.3	3.4
AST, U/L	1–31	8	16
ALT, U/L	5–50	17	19
GGT, U/L	3–38	18	23
K, mmol/L	3.5–5.1	3.7	3.5
Na, mmol/L	135–150	134	139
Cl, mmol/L	96–112	101	109
CRP, mg/L	< 5.0	325.6	9.4
Fibrinogen, g/L	2.20–4.96	6.0	2.98
PCT, ng/mL	< 0.05	41.33	0.06
APTT, R	0.83–1.30	1.28	0.91
PT, R	0.83–1.30	2.03	1.16
FT3, pmol/L	2.6–5.7	4.4	14.9
FT4, pmol/L	9.0–19.0	25.7	31.9
TSH, mIU/L	0.35–4.94	< 0.01	< 0.01
ESR, mm/h	< 37	39	15
C3, g/L	0.75-1.75	0.87	-
C4, g/L	0.15-0.45	0.18	-
Anticardiolipin IgG, GPLU/mL	< 12	8.7	-
Anticardiolipin IgM, MPLU/mL	< 12	6.4	-
Anti β2 glycoprotein IgG, AU/mL	< 12	4.5	-
Anti β2 glycoprotein IgM, AU/mL	< 12	< 3	-
Anti dsDNA IgG, IU/mL	< 20	12.6	-
Anti PR3, AU/mL	< 12	7.5	2.3
Anti MPO, AU/mL	< 12	30.2	78.6
ANA	negative	Positive, homogenous, mild intensity	negative

AST – aspartate aminotransferase; ALT – alanine aminotransferase; GGT – gamma-glutamyl transferase; CRP-C – reactive protein; PCT – procalcitonin; APTT – activated partial thromboplastin time; PT – prothrombin time; FT3 – free triiodothyronine; FT4 – free thyroxine; TSH – thyroid stimulating hormone; ESR – erythrocyte sedimentation rate; Anti dsDNA IgG – anti-double stranded DNA IgG antibodies; Anti PR3 – anti-proteinase-3 antibodies; Anti MPO – anti-myeloperoxidase antibodies; ANA – antinuclear antibody.

She was diagnosed with Graves' disease 13 years prior to this admission and was initially treated with thiamazole followed by two doses of radioactive iodine, after which her symptoms resolved, and she had not used any ATD since. Thirteen years later, during a regular visit, the patient presented with palpitations, excessive sweating, and mild hand thrill. Her eye finding was unremarkable. She had been diagnosed with recurring hyperthyroidism, and PTU treatment was started. On a regular check-up after two weeks, the patient complained of fever and throat pain. Laboratory tests showed agranulocytosis. The patient was admitted to the hospital. On initial examination, the patient presented with tachycardia, normal breathing rate, and normal body temperature. The mucosa of the soft palate was edematous and hyperemic, and the tonsils were swollen and covered with a white coating. Lung crackles were present in the lower right lobe. There was no evidence of thyroid enlargement, vasculitis, or any other skin and joint lesions. Her family history revealed one of her sisters had primary hypothyroidism. Previous medical history and lifestyle habits were unremarkable, and the patient reported no allergies.

PTU was stopped immediately, and the patient was put on empirical antibiotic therapy.

Additional biochemical analyses were done, and they are presented in Table 1. A basal right-sided pneumonia with a small pleural effusion was described on the chest radiograph (Figure 1). Treatment included broad-spectrum antibiotics for community-acquired pneumonia (ampicillin-sulbactam, 1.5 g *iv* every 8 hrs, q8hr), Lugol's solution (15 drops, q8hr), and methylprednisolone (1 mg/kg). Exogenous granulocyte-colony stimulating factor (G-CSF) (Zarzio®, 30 MU/0.5 mL) was administered on the fifth day. The response was satisfactory, which was confirmed by the normalization of white blood cell count, proinflammatory markers, and thyroid function tests (Table 1). However, the following course of treatment was complicated by the occurrence of a generalized erythematous-papillomatous rash with areas of urticaria (Figure 2), which was

Fig. 1 – Chest X-ray of the presented patient reveals basal right-sided pneumonia with a small pleural effusion. PA – posterior-anterior projection.

Fig. 2 – Generalized erythematous-papillomatous rash with areas of urticaria.

initially resolved by an escalation of methylprednisolone dosage together with antihistaminic treatment. As soon as the methylprednisolone dose was reduced, the generalized annular exanthematous rash and fever worsened again. Elevation of serum level of inflammatory markers was not seen. Microbiological testing, including blood, urine, and nasopharyngeal swab cultures, was performed again and came back negative; nevertheless, immunological testing revealed positive ANCAs with positivity for MPO. Antinuclear antibodies were identified as positive, homogenous, and of mild intensity. All other immunological testing was negative. Diagnosis of ANCA-positive vasculitis with a preserved kidney function was proposed probably as a side-effect of PTU treatment. Methylprednisolone and local treatment of skin lesions resulted in a resolution of skin changes and an overall improvement of the patient status. Primary hyperthyroidism was eventually treated surgically. The patient was discharged from the hospital and continued with ambulatory check-ups.

Biopsy of skin lesions was done too late, i.e., after the vasculitis-like lesion resolved and turned into simple hyperpigmentation. The reason for the late biopsy lies in prolonged prothrombin time at the time of florid skin lesions.

At follow-up visits, the patient was stable, with no recurrence of the skin rash. Substitute levothyroxine therapy was continued with a gradual reduction of corticosteroid therapy.

Discussion

This case report describes a patient who experienced concomitant agranulocytosis and ANCA-associated vasculitis as an adverse effect of PTU treatment for Graves' disease. Only 0.1–1.75% of patients with Graves' disease receiving ATDs are estimated to develop agranulocytosis, mostly within the first 12 weeks of the start of treatment, as was the case with our patient [17–20]. However, according to some reports, it can also occur up to eight courses of treatment later (with either the same or a different ATD) [20, 21]. Even though it is defined as an absolute neutrophil count less than 500/μL, most patients present with a count < 100/μL [22]. PTU-induced activation of circulating antibodies against differentiated granulocytes might be held accountable for the development of agranulocytosis, which is a possible explanation of an underlying immune-mediated mechanism [23]. ANCAs are the most commonly found antibodies. ANCAs react against neutrophil granules, induce complement-mediated opsonization and cytotoxicity, reduce neutropoiesis, and induce apoptosis [7, 24], but neither has been clearly proven. Various cytokines, sometimes facilitated by an underlying bacterial infection, may lead to an increase in ANCA expression and translocation of these antibodies from the intracellular region to the plasmatic membrane of neutrophils [25, 26]. It should be noted that vasculitis could be associated with acute infection, but the underlying mechanism is not fully understood [27].

The most frequent symptoms are fever, which is present in almost 80% of cases, and sore throat, reported in 72.8% of cases, followed by a lower incidence of skin and gastrointestinal infections [28]. Immediate discontinuation of the drug is imperative in order to prevent further damage, followed by *iv* administration of broad-spectrum antibiotics. In some cases, hematopoietic growth factors such as G-CSF might be used. Yet, the reports on the benefit of this treatment vary [29]. A recent study by Yang et al. [27] showed that recovery time in the G-CSF-treated group did not differ from that in the group not treated with G-CSF. However, in our patient, a complete recovery was identified after the administration of the drug. Surgery or radioactive iodine are effective options for further treatment of primary hyperthyroidism [18, 30]. Due to having already received radioactive iodine, our patient successfully underwent a total thyroidectomy.

It is now also widely known that certain drugs are responsible for the development of ANCA-associated vasculitis, with PTU, hydralazine, sulfasalazine minocycline, penicillamine, and interferon being the most common [31, 32]. However, it should be mentioned that 2.9% of patients have ANCA positivity prior to PTU therapy, whereas 22% on PTU are ANCA positive [5]. PTU-induced ANCA vasculitis is characterized by the recognition of multiple target antigens, including MPO, proteinase 3, cathepsin G, lactoferrin, neutrophil elastase, and azurocidin [33, 34]. The exact mechanism of MPO-ANCA-associated vasculitis is still unclear. Possible mechanisms for this phenomenon include degranulation and apoptosis of neutrophils caused by PTU or its metabolites, which, in return, produce oxygen radicals and interact with MPO enzyme activity [14]. Production of p-ANCA is thought to occur through a direct effect of PTU itself or its metabolites [15, 16]. PTU might also serve as an MPO substrate, which may result in an autoimmune response of activated lymphocytes toward self-material [35]. According to Harper et al. [13], PTU-induced ANCA vasculitis might also be a result of polyclonal B cell activation. Some studies have shown that patients with PTU-induced ANCA vasculitis had significantly higher titers and avidity of MPO-ANCA antibodies than those patients without clinical vasculitis. These antibodies are less pathogenic, which might partly explain why the severity of PTU-induced vasculitis is usually milder than in primary vasculitis [36].

Upon initiating PTU treatment, vasculitis usually develops several months after the treatment. Nonetheless, a great variety has been reported ranging from a single week up to 13 years [3, 14]. Patients typically present with systemic symptoms, such as fever, myalgia, arthralgia, sore throat, and malaise [1, 14, 37]. However, in PTU-associated ANCA vasculitis, severe, even life-threatening symptoms, including pulmonary hemorrhage and acute kidney injury, might also occur [37, 38]. If skin involvement is present, it affects subcutaneous parts of the skin and mostly consists of acral, purpuric plaques or nodules defined as "retiform purpura" [39–42]. Similarly, our patient exhibited purpuric urticarial plaques on the lower extremities. Skin lesions in our patient are most probably the consequence of PTU-induced ANCA vasculitis since the period between the cessation of the drug and the occurrence of the skin lesions was very short.

Most patients affected by this condition achieve the resolution of most symptoms upon withdrawal from PTU therapy. In some cases in which severe impairment of one or

multiple organs is present, other treatment modalities must be started as well, with corticosteroid and immunosuppressive agents (especially cyclophosphamide) being feasible options. No consensus on the duration of immunosuppressive therapy has been achieved yet, especially for maintenance therapy. Normally, once PTU is discontinued and remission of vasculitis is achieved, relapses most commonly do not occur [43]. Our patient underwent a skin biopsy too late, so the diagnosis was not pathologically proven. The biopsy was performed after the correction of prolonged prothrombin time which could be explained by changes in coagulation and fibrinolysis on the ground of hyperthyroidism itself [44]. Pathologists described chronic inflammatory infiltrates, and at the time of the biopsy, only hyperpigmentation was present in the affected areas of the skin.

Conclusion

This is an exceedingly rare case of a patient presenting with concomitant agranulocytosis and anti-neutrophile cytoplasmic antibodies-associated vasculitis as a complication of propylthiouracil therapy for Graves' disease. We would like to emphasize the importance of prompt discontinuation of antithyroid drugs in order to reduce end-organ damage and the possible mutual pathogenesis responsible for the occurrence of these adverse events.

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Delovi rada su: **naslovna strana, apstrakt sa ključnim rečima, tekst** rada, zahvalnost (po želji), literatura, prilozi.

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a) Poželjno je da naslov bude kratak, jasan i informativan i da odgovara sadržaju, podnaslove izbegavati.

b) Ispisuju se puna imena i prezimena autora sa oznakama redom: *, †, ‡, §, ‖, ¶, **, ††, ...

c) Navode se puni nazivi ustanove i organizacijske jedinice u kojima je rad obavljen mesta i države za svakog autora, koristeći standardne znake za fusnote.

d) Zaključak može da bude posebno poglavlje ili se iznosi u poslednjem pasusu diskusije.

e) Podaci o autoru za korespodenciju.

2. ***Apstrakt i ključne reči***

Na drugoj stranici nalazi se strukturisani apstrakt (250-300 reči za originalne članke i meta-analize) sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **Uvod/Cilj** rada, osnovne procedure **– Metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi **– Rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **Zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt za kazuistiku (do 250 reči), sadrži podnaslove **Uvod, Prikaz bolesnika** i **Zaključak**). Ispod apstrakta, „Ključne reči“ sadrže 3–10 ključnih reči ili kratkih izraza koje ukazuju na sadržinu članka.

3. ***Tekst članka***

Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Uvod.** Posle uvodnih napomena, navesti cilj rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo važne podatke iz literature a ne opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

Metode. Jasno opisati izbor metoda posmatranja ili eksperimentnih metoda (ispitanici ili eksperimentne životinje, uključujući kontrolne). Identifikovati metode, aparaturu (ime i adresa proizvođača u zagradi) i proceduru, dovoljno detaljno da se drugim autorima omogući reprodukcija rezultata. Navesti podatke iz literature za uhodane metode, uključujući i statističke. Tačno identifikovati sve primenjene lekove i hemikalije, uključujući generičko ime, doze i načine davanja. Za ispitivanja na ljudima i životinjama navesti saglasnost nadležnog etičkog komiteta.

Rezultate prikazati logičkim redosledom u tekstu, tabelama i ilustracijama. U tekstu naglasiti ili sumirati samo značajna zapažanja.

U **diskusiji** naglasiti nove i značajne aspekte studije i izvedene zaključke. Posmatranja dovesti u vezu sa drugim relevantnim studijama, u načelu iz poslednje tri godine, a samo izuzetno i starijim. Povezati zaključke sa ciljevima rada, ali izbegavati nesumnjive tvrdnje i one zaključke koje podaci iz rada ne podržavaju u potpunosti.

Literatura

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

Primeri referenci:

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Tabele

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

Ilustracije

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

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

Skraćenice i akronimi

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

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

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