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and the Department of the Serbian Medical Society in Niš

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ANTIBACTERIAL ACTIVITIES OF FRUITS EXTRACTS OF THREE MULBERRY SPECIES (*MORUS ALBA L., MORUS RUBRA L. AND MORUS NIGRA L.*) AND BILBERRY (*VACCINIUM MYRTILLUS L.*)

Vojkan Miljković[1], Goran Nikolić[2], Tatjana M. Mihajlov-Krstev[3], Biljana Arsić[4]

Delphinidin is a dominant anthocyanidin in bilberry. Antimicrobial activity of methanol extracts of the genus *Morus* showed that *M. nigra L.* extract was more active than extracts of other two species (*M. alba L.* and *M. rubra L.*). Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract was in the range of MIC/MBC = 15.75-252.00 mg/mL. Antimicrobial effect of the tested extracts was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *S. epidermidis, S. pyogenes. P. mirabilis* and *S. aureus*.

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Key words: *Morus alba L., Morus rubra L., Morus nigra L., Vaccinium myrtillus L., antimicrobial activity*

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Introduction

Bilberry (*Vaccinium myrtillus L.*) is a shrub growing to 50 cm with elliptical leaves. Fruits are berries, globular, dark purple, juicy and sour (1). In some European countries, bilberry is one of the most economically important wild berry species (2). It is classified as a Class 1 herb by the American Herbal Products Association (3), which means that it can be safely consumed when it is used appropriately. *Morus alba L.* is a native tree in India, China, and Japan. It came to Europe a few centuries ago. The tree was introduced to America for silkworm cultivation in early colonial times and it was naturalized and hybridized with the local red mulberry. Red mulberry or American mulberry originates from the eastern part of the USA and black mulberry came from Asia. Red mulberry fruits arrived in Europe before Roman times. Black mulberry is distributed in Asia, Europe, North and South America and Africa (4, 5).

Various *Vaccinium species* (*V. myrtillus, V. vitisidaea, V. macrocarpon*) are used in phytomedicine and pharmacy. Fruits of these species may have additional health benefits because they are rich in phytochemicals such as anthocyanins responsible for their red, purple and blue colors. Bilberry fruits contain up to 10% tannins, anthocyanins, organic acids, and pectins. It contains high quantity of anthocyanins (five anthocyanidins-delphinidin, cyanidin, petunidin, peonidin, and malvidin are combined with three sugar types-galactose, glucose, arabinose), flavonols (quercetin, myricetin, rutin), phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, ellagic acid, gallic acid) and stilbene (trans-resveratrol) (6, 7). Bilberry has higher anthocyanin content compared to other types of berries, such as strawberry, cranberry, elderberry, sour cherry, and raspberry (8-11). In traditional medicine, fruits of V. myrtillus are used as antidiarrheal (12).

There are papers on phytochemical analysis of leaves and fruits of bilberry plant (13, 14). There are, however, only a few reports on the effect of frozen storage on berry phenolics and their composition (15-17), although this knowledge is important because most Nordic berries are frozen due to the short harvesting season. Mulberry phenolics are investigated qualitatively (18), and our further investigations will be directed towards the quantitative definition of mulberry phenolics.

The control of human GI tract pathogens by diet or by natural medicinal components is actively examined (19-22). Minimal use of antibiotics is re-

commended due to the threat of the spread of antibiotic resistance among normal human GI tract microbial flora, and therefore alternative antimicrobial compounds are sought (21, 23). The antimicrobial activity of berry compounds drew the attention because of the recent studies which show that anthocyanins may protect against human pathogenic bacteria (24-30). Several mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of the cytoplasmic membrane, permeabilization of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism and deprivation of the substrates required for microbial growth. Antimicrobial activities of berries may also be related to antiadherence of bacteria to epithelial cells, which is a prerequisite for colonization and infection of many pathogens (31). Extracts from common Finnish berries inhibited the growth of Gram-negative, but not Gram-positive bacteria. Other authors reported that there was no correlation between Gram-positive or Gram-negative bacterial status and susceptibility to the berries (24).

Aims

The aims of our paper were investigations of anti-bacterial activities of fruits of three mulberry species (*Morus alba L., Morus rubra L. and Morus nigra L.*), and bilberry (*Vaccinium myrtillus L.*).

Material and methods

Extraction

Bilberries (*Vaccinium myrtillus L.*) were sampled at the fully ripe stage in July 2011 from woods from Koroska and Skofja Loka. They were stored at -20 0C for one week when extracts were prepared. The extraction method was modified and improved according to the already reported method (6). Frozen samples (600 g) were firstly homogenized in 2 L ice-cold deoxygenated methanol that had previously been flushed for a few minutes with nitrogen. The homogenate was extracted for 1h by shaking on mag netic stirrer IKA REO Basic C (Konigswinter, Germany) at room temperature. The extract was filtered by vacuum through the technical filter paper. The residue was extracted again in 1 L ice-cold deoxygenated methanol for 0.5h and the suspension was filtered as before. The third time the residue was extracted as described before. Finally, all three filtrates were pooled, flushed with nitrogen for a few minutes, and then stored at -20^{0}C until analysis.

Ultra high-performance liquid chromatography diode array-electrospray ionization mass spectrometry analysis

The liquid chromatography (UHPLC) runs were carried out using Dionex Ultimate 3000 UHPLC + system equipped with diode array (DAD) detector and also connected with LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, USA). The separations were performed on Hypersil gold C18 column (50 x 2.1 mm, 1.9 µm) of the same producer, at 25^{0}C. The mobile phase consisted of (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. The next linear gradient program at flow rate of 0.250 ml/min has been applied: Method I: 10 % to 30 % (B) for the first two minutes, then 40 % to 50 % (B) for 5-7 min and 80 % to 90 % (B) from 9 to 11 min, followed by isocratic run at 90 % (B) from 11-12 min and from 90-100 % (B) from 12 to 12.1 min, and finally the isocratic run with 10% (B) to 20th min; Method-II: 20 % to 50 % (B) for the first five minutes, then 70 % to 90 % (B) for 5-7 min, followed by isocratic run at 90 % (B) from 7-9 min and from 90-20 % (B) from 9 to 9.1 min, and finally the isocratic run with 20 % (B) to 15th min.

Absorption UV-VIS spectra were recorded on DAD-detector (with a total spectral range between 200 nm and 800 nm). MS analysis was performed using LCQ 3D-ion trap mass spectrometer with electrospray ionization (ESI) in the negative, as well as in positive ion mode. The ESI-source parameters for negative mode were set as follows: source voltage 4.5 kV, capillary voltage -41 V, tube lens voltage -95 V, capillary temperature 350^{0}C, sheath and auxiliary gas flow (N2) 32 and 8 (arbitrary units), respectively. On the other hand, the ESI-source parameters for positive ion mode were: source voltage 4.5 kV, capillary voltage 19 V, tube lens voltage 95 V, capillary temperature 275^{0}C, sheath and auxiliary gas flow (N2) 32 and 8 (arbitrary units), respectively. MS-spectra (both modes) were obtained by full range acquisition of m/z 130-900. For fragmentation study (MS/MS), a data-dependent scan was performed by deploying the collision-induced dissociation (CID). The normalized collision energy of the CID cell was set at 15 and 25 eV, for the negative and positive mode, respectively.

Micro-well dilution assay

Bacterial strains

Antimicrobial activity of investigated extracts was evaluated against laboratory control strains from ATCC collection,

Gram (+) bacteria:
- Staphylococcus aureus ATCC 6538,
- Staphylococcus epidermidis ATCC 12228,
- Streptococcus pyogenes ATCC 19615,
- Enterococcus faecalis ATCC 19433,
- Propionibacteri-um acnes ATCC 11827,

Gram (-) bacteria:
- Escherichia coli ATCC 9863,
- Pseudomonas aerugino-sa ATCC 9027,
- Acinetobacter baumannii ATCC 196060,
- Proteus mirabilis ATCC 12453,
- Klebsiella pneumoniae ATCC10031,

and against related strains isolated from human wound swabs.

Micro-well dilution method

Minimum inhibitory (MIC) and minimum bacteri cidal concentrations (MBC) of extracts were deter-

mined by employing the broth micro-well dilution method with some modifications (32). An overnight culture of tested bacterial strains was used for the preparation of suspensions (0.5 McFarland standard turbidity). A serial doubling dilution of the extracts (in 10% aqueous Dimethyl sulfoxide - DMSO) was prepared in a 96 well microtiter plate with inoculated Mueller Hinton broth (MHB) at concentrations ranging from 0.02-100.00 mg/mL (*Morus alba L.*), 0.13 - 270.50 mg/mL (*Morus rubra L.*), 0.12 - 251.00 mg/mL (*Morus nigra L.*), and 0.06-126.00 mg/*mL (Vaccinium myrtillus L.*). The final volume was 100 µL and the final concentration of bacterial suspensions was 106 CFU/mL in each well. The plates were incubated for 24h at 37^0C. Metronidazole, Doxycycline, Ciprofloxacin, and Gentamicin were used as positive control (Sigma Aldrich, St Louis, MO, USA), and dilutions were prepared at concentrations ranging from 0.01 to 100 mg/mL. All determinations were performed in triplicates. Microbial growth was determined by adding 20 µL of 0.5 % triphenyl tetrazolium chloride (TTC) aqueous solution in microtitar plates. MIC was defined as the lowest concentration of the extracts at which the microorganisms showed no visible growth. In order to determine MBC, the broth was taken from each well and inoculated on Mueller Hinton agar (MHA) for 24h at 37^0C. The MBC is defined as the lowest concentration of the extracts at which 99.9 % of inoculated bacteria were killed.

Results and Discussion

The content of extracted anthocyanins was determined, and their quantities are presented in Table 1. The obtained results indicated that delphinidin is a dominant anthocyanidin in bilberry. Burdulis et al. (31) found cyanidin as a dominant anthocyanidin in their bilberry samples. Meanwhile, Moze et al. (6) identified 15 anthocyanins using LC - MS/MS from seven different locations in Slovenia, which contents were 1210.3 ± 111.5 mg CGE/100 g FW. The content of total anthocyanins in the Slovak Republic in bilberry was in the range from 5578 mg/kg to 2887.75 mg/kg (33).

Table 1. Individual anthocyanins in bilberries and their extract.

	Anthocyanins	[1]Extract concentration mg/L	[2]Mass concentration g/100 g	Percentage %
1.	delphinidin 3-galactoside	692.0 ± 1.1	153.4 ± 0.3	15.6
2.	delphinidin 3-glucoside	654.8 ± 1.5	145.1 ± 0.3	14.8
3.	cyanidin 3-galactoside	506.7 ± 1.6	112.3 ± 0.4	11.5
4.	delphinidin 3-arabinoside	595.6 ± 1.7	132.0 ± 0.4	13.5
5.	cyanidin 3-glucoside	495.5 ± 0.8	109.8 ± 0.2	11.2
6.	petunidin 3-galactoside	153.0 ± 1.0	33.9 ± 0.2	3.5
7.	cyanidin 3-arabinoside	298.3 ± 0.4	66.1 ± 0.1	6.7
8.	petunidin 3-glucoside	298.4 ± 7.7	66.1 ± 1.7	6.7
9.	peonidin 3-galactoside	38.6 ± 0.3	8.6 ± 0.1	0.9
10.	petunidin 3-arabinoside	95.5 ± 0.4	21.2 ± 0.1	2.2
11.	peonidin 3-glucoside	158.2 ± 0.3	35.1 ± 0.1	3.6
12.	malvidin 3-galactoside	88.2 ± 0.2	19.5 ± 0.0	2.0
13.	peonidin 3-arabinoside	14.7 ± 0.1	3.3 ± 0.0	0.3
14.	malvidin 3-glucoside	269.9 ± 0.8	59.8 ± 0.2	6.1
15.	malvidin 3-arabinoside	63.5 ± 1.5	14.1 ± 0.3	1.4

Data were quantified as mg of standard cyanidin 3-glucoside equivalents per [1]L of bilberry extract or [2]100 g of fresh bilberries.
Data were expressed as mean ± SEM, a number of independent measurements was n=3.

The obtained results (Table 2a, 2b) for antimicrobial activity of methanol extracts of the genus *Morus* showed that M. nigra extract was more active than extracts of other two species. This extract had MIC = 31.26-125.05 mg/mL, and MBC = 125.05-251.00 mg/mL. The highest tested concentration of the extract showed no bactericidal activity against most strains isolated from wounds such as *S. aureus, S. pyogenes, E. faecalis, E. coli, P. aeruginosa* and *K. pneumoniae*, as well as against the ATCC strains, *P. acnes* and *K. pneumoniae*. The best activity of the extract was observed against strains *S. epidermidis* ATCC 12228 and *P. mirabilis* from swabs of wounds, MIC / MBC = 62.52/125.05 mg /mL, and against *S. pyogenes* ATCC 19615, MIC / MBC = 31.26/251.00 mg/mL. *M. alba* extract has a weak inhibitory activity at the highest tested concentration against 73 % of investigated strains. The bactericidal activity was present only against *S. aureus, S. epidermidis* and *S. pyogenes* (MIC = MBC = 100

mg/mL). Extract of *M. rubra* had no inhibitory or bactericidal effect against all tested bacterial strains.

Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract (Table 2a, 2b) was in the range of MIC/MBC = 15.75 - 252.00 mg/mL. The best activity was against *S. epidermidis* ATCC 12228, (MIC = MBC = 15.75 mg/mL) and *S. Epidermidis* isolated from wound swabs (MIC/MBC = 15.75/31.50 mg/ml), against *E. faecalis* from wound (MIC = MBC = 31.50 mg/mL) and against *S. Pyogenes* ATCC 19615 and *P. mirabilis* ATCC 12453 (MIC / MBC = 31.50/63.00 mg/mL, respectively).

Table 2a. Antimicrobial activity of methanol extracts of *Morus species* and *Vaccinium myrtillus L*. against pathogenic bacterial strains (MIC/MBC in mg/mL)

Bacterial strains		**Methanol extracts**			
Isolated and ATCC strains		*Morus nigra* L.	*Morus alba* L.	*Morus rubra* L.	*Vaccinium myrtillus* L.
Gram (+)	**Source**				
Staphylococcus aureus	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	63.00/63.00
Staphylococcus aureus	ATCC6538	62.52/251.00	100.00/100.00	>270.50/>270.50	63.00/63.00
Staphylococcus epidermidis	Wound swabs	62.52/251.00	100.00/100.00	>270.50/>270.50	15.75/31.50
Staphylococcus epidermidis	ATCC12228	62.52/125.05	100.00/>100.00	>270.50/>270.50	15.75/15.75
Streptococcus pyogenes	Wound swabs	31.26/>251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Streptococcus pyogenes	ATCC19615	31.26/251.00	100.00/100.00	>270.50/>270.50	31.50/63.00
Enterococcus faecalis	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	31.50/31.50
Enterococcus faecalis	ATCC19433	125.05/251.00	>100.00/>100.00	>270.50/>270.50	63.00/126.00
Propionibacterium acnes	ATCC11827	125.05/>251.00	100.00/>100.00	>270.50/>270.50	126.00/126.00
Gram (-)	**Source**				
Escherichia coli	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	63.00/126.00
Escherichia coli	ATCC9863	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Pseudomonas aeruginosa	Wound swabs	125.05/>251.00	>100.00/>100.00	>270.50/>270.50	31.50/252.00
Pseudomonas aeruginosa	ATCC9027	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/126.00
Acinetobacter spp.	Wound swabs	125.05/125.05	100.00/>100.00	>270.50/>270.50	252.00/252.00
Acinetobacter baumannii	ATCC196060	125.05/251.00	100.00/>100.00	>270.50/>270.50	63.00/63.00
Proteus mirabilis	Wound swabs	62.52/125.05	>100.00/>100.00	>270.50/>270.50	63.00/63.00
Proteus mirabilis	ATCC12453	125.05/251.00	>100.00/>100.00	>270.50/>270.50	31.50/63.00
Klebsiella spp.	Wound swabs	125.05/>251.00	100.00/>100.00	>270.50/>270.50	126.00/252.00
Klebsiella pneumoniae	ATCC10031	125.05/>251.00	>100.00/>100.00	>270.50/>270.50	126.00/126.00

Table 2b. Referent antibiotics against pathogenic bacterial strains (MIC/MBC in mg/mL)

Bacterial strains		**Antibiotics**			
Isolated and ATCC strains		Metronidazole	Doxycyclin	Ciprofloxacin	Gentamicin
Gram (+)	**Source**				
Staphylococcus aureus	Wound swabs	3.91/15.62	7.81/7.81	1.26/1.26	0.60/0.60
Staphylococcus aureus	ATCC6538	15.62/31.25	7.81/15.61	1.26/2.52	0.60/0.60
Staphylococcus epidermidis	Wound swabs	7.81/31.25	3.91/3.91	0.63/0.63	0.30/0.30
Staphylococcus epidermidis	ATCC12228	7.81/62.50	3.91/7.81	0.63/0.63	0.30/0.30
Streptococcus pyogenes	Wound swabs	7.81/7.81	0.06/0.12	0.16/0.16	0.30/0.30
Streptococcus pyogenes	ATCC19615	0.98/15.62	0.06/0.12	0.16/0.16	0.30/0.30
Enterococcus faecalis	Wound swabs	7.81/62.50	0.25/0.25	0.30/0.30	0.08/0.08
Enterococcus faecalis	ATCC19433	3.91/62.50	0.25/0.49	0.30/0.30	0.16/0.16
Propionibacterium acnes	ATCC11827	0.98/15.62	15.61/15.61	2.50/ >20.00	2.50/ >20.00
Gram (-)	**Source**				
Escherichia coli	Wound swabs	15.62/31.25	7.81/7.81	2.50/2.50	2.50/20.00
Escherichia coli	ATCC9863	31.25/31.25	7.81/15.61	2.50/2.50	2.50/20.00
Pseudomonas aeruginosa	Wound swabs	15.62/62.50	7.81/15.61	0.02/0.63	2.50/10.00
Pseudomonas aeruginosa	ATCC9027	31.25/31.25	15.61/15.61	0.02/0.63	2.50/10.00
Acinetobacter spp.	Wound swabs	1.95/62.50	15.61/15.61	10.00/20.00	10.00/20.00
Acinetobacter baumannii	ATCC196060	15.62/15.62	15.61/15.61	10.00/20.00	10.00/20.00
Proteus mirabilis	Wound swabs	7.81/7.81	7.81/15.61	10.00/20.00	1.25/5.00
Proteus mirabilis	ATCC12453	62.50/125.00	7.81/15.61	10.00/20.00	5.00/10.00
Klebsiella spp.	Wound swabs	31.25/125.00	15.61/15.61	0.63/20.00	2.50/10.00
Klebsiella pneumoniae	ATCC10031	31.25/62.50	15.61/15.61	10.00/20.00	10.00/20.00

Minimal inhibitory and bactericidal concentration of *V. myrtillus* methanol extract (Table 2a, 2b) was in the range of MIC/MBC = 15.75-252.00 mg/mL. The best activity was against *S. epidermidis* ATCC 12228, (MIC = MBC = 15.75 mg/mL) and *S. Epidermidis* isolated from wound swabs (MIC/MBC = 15.75 / 31.50 mg/ml), against *E. faecalis* from wound (MIC = MBC = 31.50 mg/mL) and against *S. pyogenes* ATCC 19615 and *P. mirabilis* ATCC 12453 (MIC / MBC = 31.50/63.00 mg/mL, respectively).

Conclusion

Methanol extracts of *V. myrtillus* exhibited better antimicrobial activity compared to the methanolic extract of *M. nigra*, whereas the extracts of *M. alba* and *M. rubra* had weak or no effect. In general, the antimicrobial effect of the tested extracts was less potent against strains from wounds compared to ATCC strains as well Gram (-) bacteria compared to Gram (+) bacteria. The most sensitive strains were *S. epidermidis, S. pyogenes. P. mirabilis* and *S. aureus* and therefore the fruit extracts of the investigated plant species may find use as additives in creams for skin care and protection.

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ANTIBAKTERIJSKE AKTIVNOSTI EKSTRAKATA PLODOVA TRI VRSTE DUDA (*MORUS ALBA L., MORUS RUBRA L.* I *MORUS NIGRA L.*) I BOROVNICE (*VACCINIUM MYRTILLUS L.*)

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Delfinidin je dominantni antocijanidin u borovnici. Antimikrobna aktivnost metanolnih ekstrakata roda *Morus* je pokazala da je *M. nigra L*. ekstrakt aktivniji nego ekstrakti druge dve vrste (*M. alba L.* i *M. rubra L*.). Minimalna inhibitorna i baktericidna koncentracija *V. myrtillus L*. metanolnog ekstrakta bila je u rasponu MIC/MBC = 15,75-252,00 mg/mL. Antimikrobni efekat testiranih ekstrakata je bio slabiji prema sojevima iz rana u poređenju sa ATCC sojevima, kao i Gram (-) bakterijama u poređenju sa Gram (+) bakterijama. Najsenzitivniji sojevi su bili *S. epidermidis, S. pyogenes., P. mirabilis* i *S. aureus*.

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Ključne reči: *Morus alba L, Morus rubra L, Morus nigra L, Vaccinium myrtillus L, antimikrobna aktivnost*

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QUALITY OF LIFE OF SURGICALLY TREATED PATIENTS WITH FRACTURES OF FACIAL BONES

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Patients with fractures of facial bones often have a poorer quality of life after a fracture, as well as some form of psychological morbidity.

The aim of this paper is to assess the quality of life of patients with surgically treated fractures of facial bones.

Thirty patients with fractures of the facial bones and jaw were included in this prospective clinical study, treated at the Department of Maxillofacial Surgery in Nis and the Department of Otorhinolaryngology and Maxillofacial Surgery in Podgorica, of both sexes, aged 18 to 65. The standardized questionnaire of the quality of life in relation to health, (UW QoL v.4), was used.

Women, as compared to men, had higher level of anxiety. Patients were mostly male (> 90%), while patients younger than 50 years old had a higher level of anxiety than the older ones. During the one month monitoring period, 60% of the operated patients had a good quality of life. Mood swings and feelings of depression were present in approximately half of the patients. A third of them stated those factors as the most annoying ones, which was cited as the most common cause of the poor quality of life in other studies, too.

Facial fractures have a major impact on the quality of life of patients soon after the injury in terms of altered appearance, inability to perform activities and recreation and mood swings, as well as presence of pain. It is important to understand the impact of maxillofacial trauma for each patient individually, physically and mentally.

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Key words: *quality of life, fracture, facial bones*

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Introduction

One of the constantly present public-health problem in developed countries, as well as in developing countries are the fractures of facial bones and jaw (1). Patients with fractures of some of the facial bones often have a lower quality of life after the fracture, as well as some form of psychological morbidity. In studies related to this topic, the presence of specific psychosocial factors such as: depression, anxiety, changes in the perception of one's own body looks after the surgery on the bones of the face and jaw, low self-esteem and poor social relationships is mentioned (2-6). Violence is mentioned as a major factor in studies of the dominant causes of maxillofacial injuries (3, 6, 7).

Some authors state that injuries of the fractures of facial bones have a huge impact on the quality of life of patients, as measured by various tests on the quality of life. Furthermore, the surgeon must pay attention to a variety of psychological and physical needs of patients (8-11).

Surgical treatments of the face area are associated with a specific and strong fear (12-14). According to some studies, there is a clearly expressed psychological morbidity (such as anxiety and depression) in 30% of the patients immediately after fractures of the face and after the surgical procedure (9, 15). Depressive symptoms (which may also be associated with pain) may increase immediately after the surgical procedures on facial bones and jaw, as well as be present throughout the entire period of postoperative monitoring of the patient (3, 1, 16). Recent studies show that in maxillofacial injuries, the rate of post-traumatic stress disorder (PTSD) reaches the value of 27% (while some studies find even 47%), with the possibility of becoming a chronic condition if it is not recognized and treated

in due time (5, 17, 18, 9). So, it is very important to pay attention to the long-term consequences of maxillofacial injuries at the very beginning of their treatment (19).

Attention must be paid to the psychological symptoms that are caused by an injury of the patients with fractures of facial bones and jaw apart from the restitution of anatomical integrity and function (2, 20).

The studies on the quality of life have relatively recently begun to gain momentum in our country, while the studies that deal with the issues of the quality of life (with a special emphasis on psychosocial factors) after maxillofacial injuries are extremely rare. In our literature there is almost no study that has examined the quality of life of patients with fractures of facial bones, while this is a particularly current issue worldwide, which highlights the importance of such an issue (2).

The literature states that the poor quality of life measured in the initial (baseline) period of treatment anticipates the occurrence of depression during the control periods (1). This indicates the potential value of a life quality questionnaire and a screening test of the possible occurrence of any psychological morbidity (e.g. depression, post-traumatic stress disorder) in the subsequent periods of treatment, which can often be unnoticed and which can develop into a chronic condition (16).

A specific questionnaire on the quality of life of patients with fractured facial bones and jaw has not been made so far, although there is a need for such a questionnaire (20-22).

We believe that the insights obtained from this study will help us in the development of a specific life quality questionnaire of the patients with fractures of facial bones, since as far as we know, such a questionnaire has not been made although such a need exists (20-22).

Aim

The aim of this study is to assess the quality of life of the patients with surgically treated fractures of facial bones, to determine whether there is psychological morbidity before and after surgical intervention, as well as to analyse the information collected by the instruments of the quality of life.

Material and methods

This prospective clinical study was devoted to examining the quality of life of patients with fractures of the facial bones and jaw, after being treated. Patients were diagnosed by: fracture of the mandible (symphysis, body, ramus, condylar processus, coronoid processus, angulus), fracture alveolar processus of the mandible, fracture alveolar processus of the maxilla, fracture of the maxilla (Le Fort I, II, III), racture walls orbit (superior, inferior, lateral, medial), fracture complexus nasofrotoorbitalis, fracture tabulae externae sinusis frontalis, fracture of the zygomatic bones.

The study involved the patients from the Department of Maxillofacial Surgery of Nis. A survey was conducted in Podgorica and at the Department of Otorhinolaryngology and Maxillofacial Surgery, for one of the above mentioned diagnoses, of both sexes, aged 18 to 65. Ethical evaluation and organizational licenses to perform the study was obtained from the Ethics Committee of the Dental Clinic in Niš.

The study included 30 patients with fractures of the face, who were followed for one month after surgery. The following data were recorded: name, sex, age, occupation of the patient, the cause and type of fracture.

The criteria for the participation in the study were:

- Clinically confirmed fracture of some of the facial bones, of any etiology;
- Minimum age 18;
- The patients had to sign informed consent for the participation in the study, after reading the information on the study.

The criteria for the exclusion from the participation in the study were:

- The existence of any diagnosed malignant disease;
- Clinically confirmed presence of dementia;
- The presence of any other clinically significant diseases which in the opinion of the surgeon-researchers of the study may influence the ultimate goals of the study.

Patients were divided on the basis of injury by groups:

Group 1: patients with a fracture of the mandible and mandibular alveolar processes;

Group 2: patients with a fracture of the maxilla (including Le Fort fractures) and alveolar processus of the maxilla, zygomatic bones, or medial, lateral wall and floor of the orbit;

Group 3: patients with a fracture of the anterior wall of the frontal sinus, complexus nasofrotoorbitalis or the roof of the orbit.

The standardized questionnaire on the quality of life in relation to health, (U QoL v.4), was used in our study and modified and translated into Serbian. This questionnaire was originally designed for patients with malignant tumors of the head and neck, but according to the published literature it was also used for the patients with fractured facial bones (19).

The patients completed the questionnaire themselves, and on average it took about 5 to 10 minutes to fill it in (24). To show the existence of anxiety and depressive symptoms in our patient population (which literature overview suggests), those parts of UW v.4 QoL questionnaires related to psychological factors were used.

All measuring instruments were translated into Serbian.

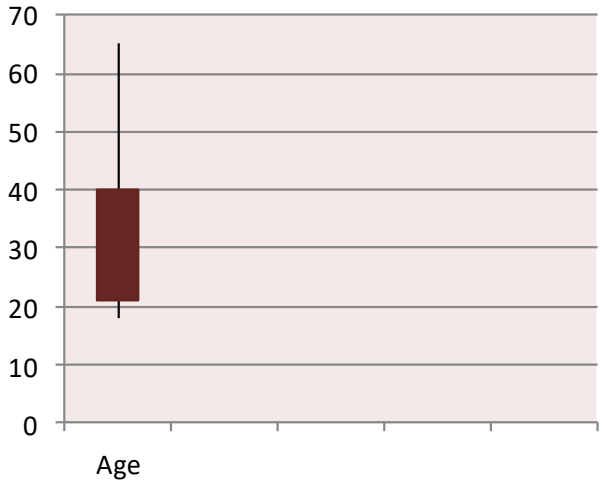
The patients filled out the questionnaires themselves, but if they needed any help or were not capable of completing it themselves, the assistance of researchers or health personnel was possible.

Statistical analyses

MS Access, SPSS v11 programs were used in this study and the processing of the obtained data was performed using standard statistical methods, parametric and nonparametric statistical methods. The statistical significance of $p < 0.05$ was used.

Results

Graph 1 shows the distribution of patients by age, from 18 to 65 years, with the highest incidence in the third and fourth decade, average age 32 years.

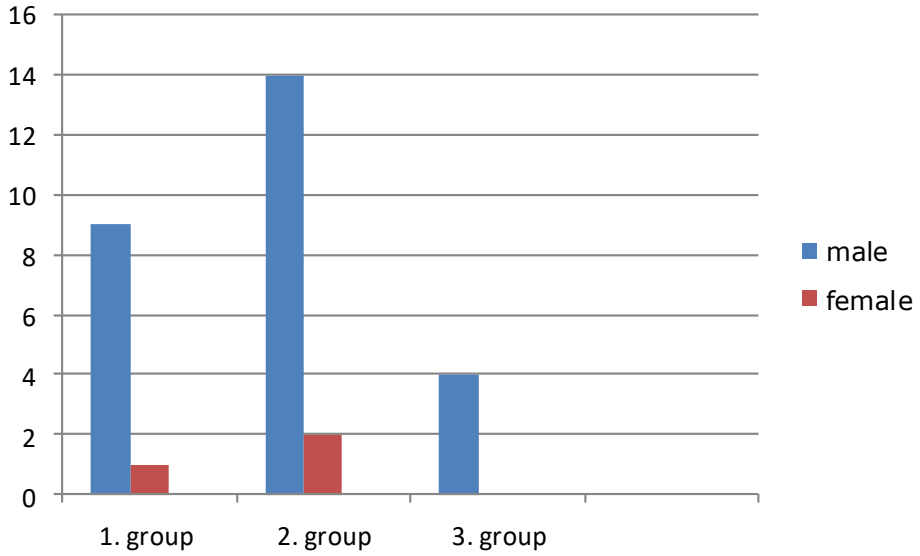


Graph 1. The distribution of patients by age

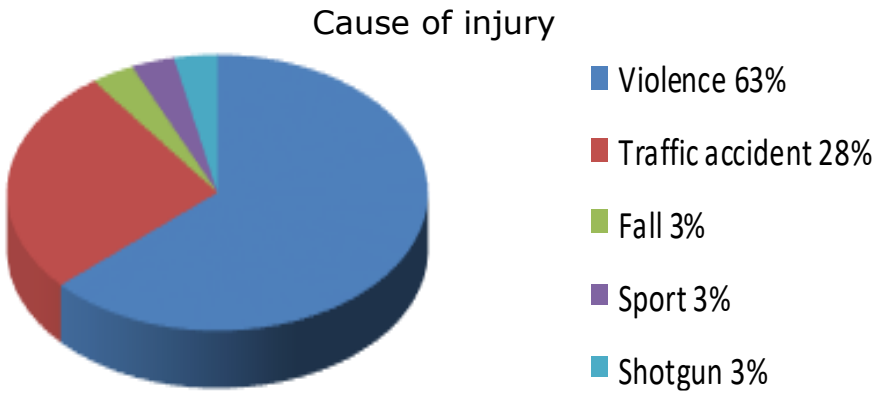
The patients were in the 90% male, presented in Table 1 and Graph 2, such that the ratio of male to female was 9:1. The violence is the most common cause of injury (63%), followed by traffic accidents (28%), rarely fall, sport and schotgun (3%), shown in Graph 3.

Table 1. Representation of fractures of facial bones

Fracture	Male	Female	In total
1. Group			**10**
Mandible	8	1	9
Alveolar processus of the mandible	1	0	1
2. Group			**16**
Zygomatic bone	1	1	2
Maxilla	2	0	2
Alveolar processus of the maxilla	1	0	1
Zygomatic bone+ maxilla	6	1	7
Le Fort	3	0	2
The medial, lateral wall or floor orbit	2	0	2
3. Group			**4**
Fractura tabulae externae sinusis frontalis	2	0	2
Fractura complexus nasofrotoorbitalis	1	0	1
Fractura parietis superior orbit	1	0	1
In total	**27**	**3**	**30**



Graph 2. The frequency of patients by sex, depending of injury by groups



Graph 3. Distribution of etiological factors in injuries

Based on the results obtained in Table 2, it can be seen that of 30 patients with a fracture of some of the facial bones, most of them claimed that they felt pain, change in appearance, reduced activity and recreation, changes in chewing, swallowing and speech, problems with shoulders, altered taste, a change in the secretion of saliva, mood swings and depression.

When it comes to pain, only 3 patients (10%) did not highlight the feeling of pain, while the others in this group stated to have a variety of strong pain, and 10 patients (33.33%) stated to have strong pain that cannot be controlled by medication.

In the mentioned group, 29 patients (96.67%) were not satisfied with their appearance, of whom 5 (16.67%) claimed that due to their appearance they could not be with people.

Reduced activity was stated by 26 patients (86.67%), while 9 of them (30%) stated that the activity was so reduced that they mainly sat or lay down and did not go out of the house.

A total of 25 patients (83.33%) mentioned to have reduced recreation, while 7 of them (23.33%) did not do anything with pleasure.

Ingestion and taste were altered in 26 patients (86.67%), of whom 10 (33.33%) emphasized that they could not swallow, and 16 patients (53.33%) said they could not try any food. Chewing was modified in 22 patients (73.33%), and 8 patients (26.67%) stated they could not chew solid food.

The speech was changed in 27 patients (90%), and 15 (50%) stated that their speech was not understandable.

Twenty-four patients (80%) stated to have problems with the shoulders, while 10 patients (33.33%) stated to have restrictions while working.

Table 2. Quality of life scores

Quality of life scores									
Domains	0	25	33	50	67	75	100	AS	% Best score (of 100)
Pain	3	8		4		6	10	61.67	33.3
Appearance	1	4		11		9	5	60,83	36.6
Activity	4	2		9		6	9	61,67	30.0
Recreation	5	4		6		8	7	56,67	26.6
Ingestion	4		6		10		10	62.27	36.6
Chew	8			14			8	50.00	46.6
Speech	3		4		8		15	72.27	50.0
Shoulders	6		5		9		10	58.93	53.33
Taste	4		3		7		16	52,27	53.33
Saliva	5		4		7		14	66,70	46.67
Mood	2	3		9		9	7	63,33	30.0
Despondency	6		5		9		10	58,93	36.6

Modified salivation was found in 25 patients (83.33%), and 14 of them (46.67%) stated not to have any saliva.

The mood was reduced in different ways in 28 patients (93.33%), and 7 of them (23.33%) stated to have experienced an extreme depression.

Twenty-four patients (80%) were anxious, and 10 of them (33.33%) were very concerned.

Patients mostly bothered about altered speech, taste with reduced secretion of saliva, chewing and swallowing, while some patients complained of the most prominent altered appearance, pain, depression, moodiness, a reduced activity and recreation.

Table 3. Tabulation of responses to general questions about the quality of health the past month (A), health-quality of life during the past 7 days (B) and overall quality of life during the past 7 days (C)

ANSWERS TO THE QUESTIONS											
	0	20	25	40	50	60	75	80	100	AS	% Best scores
A	5		5		6		5		9	56.67	52,94
B	4	3		6		6		8	3	53.33	56,25
C	4	2		6		7		8	3	54.67	54,87

Best scores:
A: % scoring 50, 75 or 100;
B & C: % scoring 60, 80 or 100

Table 3. shows that a month after the surgery 10 patients (33,33%) stated they felt better, the same applied to 6 other patients (20 %), while 14 patients (46,67%) felt worse.

The patients declared differently regarding the quality of life, 11 patients (36.67%) had a good, 6 (20%) low, 8 (26.67%) bad, and 3 (10%) very bad quality of life. The overall quality of life during the previous 7 days was not good in 18 patients (60%).

Based on Table 4, it can be seen on the basis of the rank, that patients primarily pointed out the pain and the change of appearance, followed by a change of mood, change in activity and recreation, difficulty in swallowing, chewing, depression, problems with shoulders.

Table 4. Only the corresponding selected domains

Domains	N patients who have chosen domains	Valid% of patients who have chosen domains	Rank
Pain	13	43.3	1=
Appearance	13	43.3	1=
Mood	10	33.3	3
Activity	8	26.7	4
Recreation	6	20.0	5=
Ingestion	6	20.0	5=
Chew	3	10.0	7=
Despondency	3	10.0	7=
Shoulders	1	3.3	9
Speech	0	0	10=
Taste	0	0	10=
Saliva	0	0	10=

None of the patients particularly singled out the problem with speech, taste and saliva. Approximately one third of patients suggested that pain and bad mood mainly bothered them.

Discussion

In our study, patients were predominantly male (90%), similar to the research in other countries (Canada, Poland, Nigeria, England, India) in different percentages (23-27). The most common etiological factor was the violence, followed by road traffic injuries, the most common age of 21-40 years, similar to other studies (23-26, 28, 29, 30). The large percentage of maxillofacial trauma is the result of the fact that patients are in the third and fourth decades of active period of life when they act vigorously and move at high speed in traffic. The finding of a lower percentage of women is probably the result of the fact that they do not report violence, and are more moderate as participants in traffic.

A third of the studied patients had a fracture of the mandible, and since it is the only mobile bone of the face and the only one in the lower third of the face it is often exposed to trauma, immediately after the nasal bones. The bones of the middle third of the face (about 50%) were more often broken in the second group of patients in our study. These results are described with other researchers, and this is due to exposure of the bone injuries, especially in violence and traffic accidents (26, 31, 32). A small representation of the fracture of the upper third of the face in our patients (about 13%), and the like is described in the literature (1, 11, 19, 33).

In fractures of facial bones, the pain or discomfort or injury to the olfactory nerve and the appearance of taste and nutrition may occur (4).

Some studies have shown that the degree of anxiety is directly proportional to the size of the injury and the scar on his cheek (2, 25).

In a study of surgical anxiety, which included 600 patients on maxillofacial surgery and 800 control patients, according to their subjective assessment, the patients with maxillofacial surgery had higher levels of anxiety in comparison to the control group (12).

Women had significantly higher levels of anxiety compared to men, but younger and middle-aged patients (< 50 years) were more anxious than older patients (4, 6, 12).

In our study women also had higher levels of anxiety, but our patients were mostly male (> 90%), whereas younger patients also had a higher level of anxiety as compared to the older ones (> 50 years).

The effect was found in relation to the years of formal education, which showed that more educated patients had higher levels of anxiety and that the patients who once had an injury of facial bones treated surgically, had the same level of anxiety as the patients who underwent this type of surgery intervention for the first time (12). In our case, the patients generally underwent this surgery for the first time, and the level of education was not high.

The patients with facial injuries often experienced problems later as a result of face injuries, and had difficulty with activities, as in our study, too, where more than half of the patients stated reduced activity and recreation, which contributed to the poor quality of life (8, 26).

Younger patients had a higher level of stress than it was the case with older patients, and similarly in the work of the author Avinash De Sousa of psychological problems in oral and maxillofacial reconstructive surgery (2). The same study compared the patients who underwent a surgery due to face

cancer with the patients with facial trauma, so the patients with the trauma had higher levels of anxiety, depression and worry about appearance than the patients with the cancer. In our study, younger patients had higher levels of stress and they were the most numerous (3 or 4 decades most often). Since these are people who were generally healthy before the injury, full of life and labor, even small changes in their every day routine and activities led to high levels of stress.

Anxiety, stress, and even worse quality of life were more pronounced in patients who were injured in the violence, in relation to the injuries sustained in traffic or otherwise, but there is no statistical significance ($p > 0.05$). When the fractures in the bones of the person are concerned (divided into 3 groups), poorer quality of life was noted in patients with mandibular fractures (comminuted fractures and multiple fractures), and even those of the middle third of the face (especially Le Fort fractures), but there is no statistical significance ($p > 0,05$). Patients with fracture of some of the bones more complained to the appearance and pains, while patients with a fracture of the mandible complained about the changed appearance, salivation, anxiety and low mood. A small number of patients studied limits the actual evaluation of the quality of life in fractures in the bones of the face.

The assessment of the emotional state of the patient before and after surgery was essential, as it is stated in other studies on the quality of life (2).

During the one month monitoring period of our patients, more than a half of the operated persons who underwent surgery due to the fractures of the facial bones stressed that they did not have a good quality of life.

This was expected, because the injuries in the facial area are accompanied by a certain degree of ugliness, and since the patients were mostly younger, the quality of life is associated largely with the appearance. There has been an improvement in the cited study only in the domain of physical health, but not mental. The overall quality of life includes not only physical and mental health, but also many other factors such as family, friends, spirituality, or personal leisure activities that are important for the enjoyment of life and all that contributes to personal well-being, which in the majority of these patients was not the case.

After the rehabilitation of injuries and during rehabilitation, there may be a fear of the outcome of treatment or change in appearance, especially with extensive facial injuries but with less complicated fractures. This fear, especially if it is for a longer period of time, can cause serious psychological and organic disease. This state of the patient is usually associated with poor quality of life (36, 37).

Mood swings and depression were strongly present in case of these patients, approximately in half of the patients in this group, and a third of them stated those factors as the most annoying ones (37, 38). The above factors were cited as the most common causes of poor quality of life in other studies, too (2, 7).

These results are not surprising and in some way they are expected and mentioned in other studies, as well as in the survey on African American studies regarding the fracture of the jaw, which is associated with the quality of life (3).

The intensity and degree of mental pain due to disfigurement, depends not only on real damage, altered appearance, size and type of scarring, facial deformities, but also on the structure of personality, profession, education, intelligence, social, and marital status, general health, physical appearance, sex and age of the injured (38). Changes in appearance, pain, disfigurement, lead to less activity and communication with other people, and all these factors affect the quality of life of the patient.

Limited sample of patients in this study does not allow valid statistical evaluation, so it is difficult to assess their quality of life in different fractures of facial bones. In order to accurately determine the quality of life of patients with the trauma, there must be a larger sample of patients.

Conclusion

It can be said that injuries with fractures of facial bones have a major impact on the quality of life of patients soon after the injury in terms of altered appearance, inability to do activities and recreation and mood swings, as well as presence of pain.

Adequate communication between the patient and the surgeon is very important for the psychological preparation of the patient. It is necessary to provide patients with detailed information and brochures on the type of the injury, type of the surgery and the very surgical technique, as well as about possible complications and possible outcomes and consequences.

The inclusion of psychologists in this team would contribute to improving the quality of life of patients with facial trauma, and fractures of facial bones.

It is therefore important to understand the impact of maxillofacial trauma for each patient individually, physically and mentally.

The surgeon should not only operate the patient, but he/she needs to provide psychological support as well, because physical and mental components are inseparable for the high quality life of patients.

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KVALITET ŽIVOTA KOD HIRURŠKI TRETIRANIH PACIJENATA SA PRELOMOM KOSTIJU LICA

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Kod bolesnika sa prelomom kostiju lica često postoji slabiji kvalitet života posle preloma, kao i neki od oblika psihološkog morbiditeta.

Cilj ovog rada bio je da se proceni kvalitet života kod hirurški tretiranih bolesnika sa prelomom kostiju lica.

U ovom prospektivnom kliničkom istraživanju uključeno je 30 bolesnika sa prelomom kostiju lica i vilica, lečenih na odeljenju za maksilofacijalnu hirurgiju u Nišu i Klinici za otorinolaringologiju i maksilofacijalnu hirurgiju u Podgorici, oba pola, starosti od 18 do 65 godina. Korišćen je (UW QoL v.4) standardizovani upitnik kvaliteta života u odnosu na zdravlje.

Veći nivo anksioznosti imale su žene u odnosu na muškarce; bolesnici su uglavnom bili muškog pola (> 90%), dok su mlađi od 50 godina imali veću anksioznost u odnosu na starije. Tokom mesec dana praćenja, 60% operisanih nisu imali dobar kvalitet života. Promena raspoloženja i depresivna stanja bili su zastupljeni kod približno polovine pacijenata, a trećina je navodila da im to najviše smeta. Navode se kao najčešći uzroci lošeg kvaliteta života i u drugim studijama.

Prelomi kostiju lica imaju veliki uticaj na kvalitet života bolesnika ubrzo posle povrede, u smislu izmenjenog izgleda, nesposobnosti za aktivnosti i rekreaciju i promena raspoloženja, kao i prisustvo bola. Važno je razumeti uticaj maksilofacijalne traume za svakog bolesnika pojedinačno, fizički i psihički.

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Ključne reči: *kvalitet života, prelom, kosti lica*

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EFFECT OF CAROTID ARTERY STENTING ON COGNITIVE FUNCTION IN PATIENTS WITH INTERNAL CAROTID ARTERY STENOSIS

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Carotid artery stenting (CAS) is an important therapeutic strategy for patients with carotid artery stenosis. High-grade stenosis of the internal carotid artery is associated with cognitive impairment and decline, even in asymptomatic patients. However, the potential influence of CAS on cognitive function in patients with carotid artery stenosis has not been determined. The aim of this study was to investigate the influence of carotid artery stenting (CAS) on the global cognition in patients with high grade internal carotid stenosis, on various cognitive domains and potential factors that may affect changes of cognitive function in these patients.

This study involved 25 patients with symptomatic and asymptomatic carotid artery stenosis and 25 healthy controls. Patients were evalueted 1 day before procedure and 3 months after procedure. Montreal cognitive assessment (MoCA) was used for the evaluation of cognition.

The MoCA scores of the patients before CAS were significantly lower than that of the control subjects. These scores were significantly higher 3 months after CAS. Also significantly improved after CAS from baseline were scores for an attention, executive functions and memory.

CAS can improve global cognitive function, attention, executive functions and memory in symptomatic and asymptomatic patients with high grade carotid artery stenosis. High cholesterol levels is independent risk factor for deteriorated cognitive functions before revascularization and low educational level is independent factor for poor cognitive performance after revascularization.

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Key words: *carotid artery stenosis, carotid artery stenting, cognitive function*

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Introduction

Carotid artery stenosis (CS) is one of the most significant risk factors for ischemic stroke (1, 2). High-grade stenosis of the internal carotid artery is associated with cognitive impairment and decline, even in asymptomatic patients. The pathophysiological causes of cognitive impairment due to carotid artery stenosis include cerebral hypoperfusion and embolic stroke (3, 4). Hypertension, diabetes mellitus, smoking habit, alkohol consumption and cholesterol levels are factors that can predispose to carotid stenosis (5-9).

As a minimally invasive procedure, carotid artery stenting (CAS) is an important therapeutic strategy in carotid artery stenosis (10-14). The effect of carotid artery stenting on cognitive function is unclear. Both cognitive improvement and decline have been reported after CAS (15-20). Reopening a stenotic vessel and restoring blood flow to the brain may improve cognitive dysfunction caused by chronic hypoperfusion. Several authors have therefore suggested that in these patients, carotid revascularisation could imorove cignition (21-23). However, it has also been reported that cognitive function can be negatively affected due to microembolisms caused during the CAS procedure itself, or temporary perfusion defects that may take place during balloon dilatation (15, 24).

Symptomatic status also seems to influence cognitive results in patients after CAS. Some researchers reported that the asymptomatic patients had a poorer cognitive performance after the CAS (25, 26).

The conflicting results of studies testing the ralation betwen carotid revascularisation and changes in cognition have been ascribed to differences between the studies in sample size, type of patients, duration of follow - up, and type of neuropsychological assessment. Little research has been done in this area to date.

The aim of this study was to examine the impact of CAS on global cognition, various domains of cognitive function and the influence of potential factors that might affect cognitive function.

Methods

This prospective observational study was conducted at Radiology Center in Clinical Centre in Niš, between October 2012 and June 2013.

This study involved 25 patients, both symptomatic and asymptomatic who had been diagnosed with carotid artery stenosis (≥ 70%) by Color Doppler ehosonography and MSCT angiography and 25 healthy subjects, who were free of carotid artery stenosis and brain diseases or injuries, as the control .

Patients with symptomatic carotid stenoses had a history of an ipsilateral stroke, at least one transient ischemic attack (TIA) or an episode of amaurosis fugax within previous 6 month. Patients with asymptomatic carotid artery stenosis were defined as having no previous minor stroke or TIA.

Carotid stenosis was diagnosed according to the criteria in the North American Carotid Endarterectomy Trial (NASCET). The decision to treat a given patient was left to Consilium for carotid artery stenosis treatment in Clinical Centre in Nis. Individuals in the control group visited Radiology Center for health screenings during the study period. The healthy participants were selected if they had no history of curent symptoms of ishemic or hemoragic stroke. The participants from control group were matched with pathients in gender, age, educational level, smoking and alcohol consumption. Normal results were shown in all participants from control group for carotid Color Doppler ehosonography. The control group served as a baseline reference for cognitive function, to which patients test group were compared.

Neuropsyhological functions were tested 1 day before and 3 months after CAS and compared with the data of control subjects. Montreal cognitive assessment (MoCA) was used for the evaluation of cognition.

Demographic information was obtained from patients' medical records and by direct interview. Vascular risk factors were estimated in patients and controls following the criteria, which included diabetes mellitus (defined as a glycosylated hemoglobin A1 concentracion > 5.8% or current use of hypoglycaemic agents), hyperlipidemia (total holesrerol concentracion ≥ 220mg/dl or current use of cholesterol-lowering agents), hypertension (defined as systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg or current use of antihypertensive medication), tobacoo smoking and alcohol consumption.

The exclusion criteria included present serious medical, psyhiatric and neurologic disorders.

All the subjects agreed to join the research, gave their written informed consent and had the right to withdraw at any time.

Cognitive assessment

MoCA is a brief screening tool assessing visiuospatial and executive functions, attention, short-term memory, language and orientation, has been translated and adapted into several languages and is available freely on the Internet:

(http://www.mocatest.org) (27).

The MoCA assesses global cognitive function and contains of 10 subtests: an alternating trail test, cube copying, clock-drawing, naming, attention, sentence repeating, verbal fluency, abstraction, auditory-verbal learning test (AVLT)-delayed recall, and orientation. Visuospatial abilities are assessed using a clock-drawing task and a three-dimensional cube copy, short-term memory is tested with two learning trials of five nouns followed by a delayed recall task. Executive functions are assessed using a task adapted from the Trail Making B test, a phonemic fluency task, and a two-item verbal abstraction task. Attention, concentration, and working memory are evaluated using an attention task, a serial sub traction task and digits forward and backward. Language is tested with a naming task with low-familiarity animals (lion, camel and rhinoceros), repetition of two syntactically complex sentences, and the fluency task. Orientation is evaluated by time and place.

The total scores of the MoCA scale is 30 and the higher the score, the better the cognition. In the evaluation, a score of > 26 was regarded normal, and an additional 1 point was added when the duration of education was ≤ 12 years (28). The participants in our study were evaluated with the Serbian MoCA, version 7.1.

CAS procedure

Before CAS, detection of coagulation function, routine blood test and electrocardiography were performed and patients were treated with oral aspirin at 100 mg/d and oral clopidogrel at 75 mg/d 7 days before the procedure. Following focal anesthesia, Seldinger technique was used to puncture the right femoral artery, and a 6F vascular sheath was used, followed by insertion of a 6F catheter. Under the guidance of a wire, the catheter was inserted to the proximal part of the lesioned vessel. Heparin (5000 U) was intravenously injected for systemic heparinization. Under the guidance of a road map, a protective umbrella was carefully inserted through the stenotic site, and a stent was then inserted along the umbrella. After accurately locating the stent, the stent was released. The balloon was selected according to the degree of stenosis, and then the umbrella

was expanded, followed by retraction of the umbrella and performance of radiography. On the day of intervention, aspirin and clopidogrel were administered, and the doses were identical to those before intervention.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 20.0. Quantitative data are expressed as mean ± standard deviation, and qualitative data as proportion (%). Student t test was used to compare two means, but in situations when there was not normally distributed data Z-Mann-Whitney U test was used. Z-Wilcoxon Rang test was used to compare two related samples. Linear regression analisis was used to determinate risk factors. A value of $P < 0.05$ was considered statistically significant.

Results

There were no neurological complications during the procedure or during hospitalization in any patient. The procedure was completed with technical success in all patients. The degree of stenosis was significantly reduced after CAS. All 25 patients completed 3 month follow up.

The demographic data of the stent treatment and control subjects are listed in Table 1. Average age was 69,32 ± 7,59 year, the youngest participant was 57 and the oldest 82 years old. There were no statistical difference in:

- gender (χ^2 = 0,333; p = 0,564),
- age (t = 0,464; p = 0,645) and
- educational level (χ^2 = 2,508; p = 0,285) betwen groups (Table1.). There was no statistical difference in bad habits betwen groups:
- smoking (χ^2 = 1,471; p = 0,225) and
- alcohol consumption (χ^2 = 0,365; p = 0,544)

(Table 2.). 6 patients had hypertension (24%), 13 (52%) had diabetes mellitus and 2 of then were on insulin, 17(68%) patients had high cholesterol levels. In control group 12 participants had hypertension (48%), 5 had diabetes mellitus and one of them was on insulin (4%), 6 participants had high cholesterol levels (24%).

There was no statistical difference in vascular risk factors between groups:

- hypertension (χ^2 = 3,125; p = 0,077) and
- diabetes mellitus (χ^2 = 5,600; p = 0,061),

but there were heir levels of cholesterol among patients than control group (χ^2 = 3,125; p = 0,002) (Table 3.).

Table 1. Demographic data of the stent treatment group and control group

		Patients	Controls	χ^2/t*	P
Gender	**Women n (%)**	16 (64,0)	14 (56,0)	0,333	0,564
	Men n (%)	9 (36,0)	11 (44,0)		
Age	$\bar{x}$±SD	69,32 ± 7,59	68,4 ± 4,95	0,464	0,645
Education	**Elementary n (%)**	11 (44,0)	8 (32,0)	2,508	0,285
	High school n (%)	14 (56,0)	15 (60,0)		
	Faculty n (%)	0 (0,0)	2 (8,0)		

Table 2. Distribution of bad habits in stent treatment and control group

		Patients	Controls	χ^2	P
Smoking	**No n(%)**	19 (76,0)	15 (60,0)	1,471	0,225
	Yes n(%)	6 (24,0)	10 (40,0)		
Alcohol	**No n(%)**	18 (72,0)	16 (64,0)	0,368	0,544
	Yes n(%)	7 (28,0)	6 (36,0)		

Table 3. Comorbidity distribution among groups

		Patients	Controls	χ^2	P
HA	**no n(%)**	19 (76,0)	13 (52,0)	3,125	0,077
	yes n(%)	6 (24,0)	12 (48,0)		
DM	**no n(%)**	12 (48,0)	20 (80,0)	5,600	0,061
	oral diabetic's n(%)	11 (44,0)	4 (16,0)		
	insulin n(%)	2 (8,0)	1 (4,0)		
Cholesterol	**Normal levels**	8 (32,0)	19 (76,0)	9,742	0,002
	High levels	17 (68,0)	6 (24,0)		

There were 15 symptomatic (60%) and 10 asymptomatic (40%) patients. All patients had ≥70% stenosis, 19 had 70-80% (76%) and 6 had subocclusion (24%). 14 patients (56%) had right side stenosis, 8 (32%) had left side stenosis and 3 (12%) had both side stenosis.

The values of the total MoCA scor and various cognitive domains in patients before intervention and the control group are shown In Table 4. There was statistical difference among these functions which were significantly lower in patients with carotid artery stenosis than in control group:

- visuospatial abilities (Z = 3,896; p < 0,001),
- attention (Z = 3,082; p < 0,002),
- language (Z = 5,103; p < 0,001), and
- memory (Z = 5,151; p < 0,001).

Total MoCA score was significantly heir among control group (Z = 5,711; p < 0,001). Comparing separately various cognitive function and total MoCA score among patients before and after intervention statistically significant different were:

- attention (Z = 3,080; p = 0,002),
- executive functions (Z = 2,762; p = 0,006),
- memory (Z = 3,793; p < 0,001) and
- total MoCA score (Z = 4,455; p < 0,001).

All these functions were statistically hair after CAS intervention (Table5.).

Comparing separately various cognitive functions and total MoCa score in asymptomatic patients before and after intervention statistically significant different were:

- attention (Z = 2,070; p = 0,038),
- executive functions (Z = 2,000; p = 0,046),
- memory (Z = 2,810; p = 0,005) and
- total MoCA score (Z = 2,877; p = 0,004).

All of these functions were statistically heir after intervention (Table 6.).

Table 4. Cognitive function and total MoCA score comparing among patients and control group before CAS intervention

Functions	Patients	Controls	Z	P
Visuospatial	4,28 ± 0,89	5,00 ± 0,00	3,896	< 0,001
Attention	4,60 ± 0,87	5,36 ± 0,70	3,082	0,002
Language	1,24 ± 0,78	2,52 ± 0,51	5,103	< 0,001
Executive functions	1,32 ± 0,85	1,76 ± 0,44	1,866	0,062
Memory	2,40 ± 1,04	4,28 ± 0,68	5,151	< 0,001
Orientation	6,00 ± 0,00	6,00 ± 0,00	0,000	1,000
Total MoCA score	23,24 ± 2,65	28,16 ± 0,94	5,711	< 0,001

Z- Mann-Whitney U test

Table 5. Cognitive function and total MoCA score comparing in patients before and after CAS intervention

Cognitive function	Before CAS	After CAS	Z	P
Visuospatial	4,28 ± 0,89	4,28 ± 0,97	0,000	1,000
Attention	4,60 ± 0,87	5,32 ± 0,80	3,080	0,002
Language	1,24 ± 0,78	2,52 ± 0,51	0,789	0,425
Executive functions	1,32 ± 0,85	1,80 ± 0,40	2,762	0,006
Memory	2,40 ± 1,04	3,44 ± 0,87	3,793	< 0,001
Orientation	6,00 ± 0,00	6,00 ± 0,00	0,000	1,000
Total MoCA score	23,24 ± 2,65	25,76 ± 2,22	4,455	< 0,001

Z- Wilcoxon Rang test

Table 6. Cognitive function and total MoCA score comparing among asymptomatic patients before and after CAS intervention

Cognitive function	Before CAS	After CAS	Z	P
Visuospatial	4,20 ± 1,03	4,20 ± 1,03	0,000	1,000
Attention	4,60 ± 0,97	5,50 ± 0,53	2,070	0,038
Language	1,20 ± 0,79	1,30 ± 0,53	0,378	0,705
Executive functions	1,40 ± 0,84	1,80 ± 0,42	2,000	0,046
Memory	2,50 ± 1,08	3,60 ± 0,84	2,810	0,005
Orientation	6,00 ± 0,00	6,00 ± 0,00	0,000	1,000
Total MoCA score	23,30 ± 2,65	25,80 ± 1,93	2,877	0,004

Z- Wilcoxon Rang test

Comparing various cognitive domains and total MoCA scor among symptomatic patiens before and after intrvention statisticaly significant diferent were:

- attention (Z = 2,310; p = 0,021),
- executive functions (Z = 2,070; p = 0,036),
- memory (Z = 2,683; p= 0,007) and
- total MoCA scor (Z = 3,453; p = 0,001).

All functions were significantly hier after intervention (Table 7.).

The results of univariant linear regression of risk factors before intervention are shown in Table 8. Statistically significant independent risk factors were diabetes mellitus (Beta = -0,293; p = 0,039) and cholesterol levels (Beta = -0,439; p = 0,002). Diabetes mellitus and high cholesterol levels are predictors of lower results for total MoCA score before intervention. In multivariante model independent variables were studied: diabetes mellitus and cholesterol levels as significant risk factors for lower levels of MoCA score before intervention. Only cholesterol is statistically significant risk factor in this model for lower cognitive function results before CAS (Beta = -0,383; p = 0,006) (Table 9.).

Table 7. Cognitive function and total MoCA score in symptomatic patients before and after CAS intervention

Cognitive function	Before CAS	After CAS	Z	P
Visuospatial	4,33 ± 0,82	4,33 ± 0,82	0,000	1,000
Attention	4,60 ± 0,83	5,20 ± 0,94	2,310	0,021
Language	1,27 ± 0,79	1,47 ± 0,74	0,828	0,408
Executive functions	1,27 ± 0,88	1,80 ± 0,42	2,070	0,038
Memory	2,33 ± 1,05	3,33 ± 0,89	2,683	0,007
Orientation	6,00 ± 0,00	6,00 ± 0,00	0,000	1,000
Total MoCA score	23,20 ± 2,75	25,73 ± 2,46	3,453	0,001

Z- Wilcoxon Rang test

Table 8. Univariant linear regression of patient's risk factors for total MoCA score before CAS intervention

	Unstandardized Coefficients		Standardized Coefficients	95% CI for B	P
	B	SG	Beta		
Gender	0,750	0,918	0,117	-1,096 - 2,596	0,418
Age	-0,110	0,070	-0,220	-0,251 - 0,031	0,124
Education	1,570	0,789	0,276	-0,016 - 3,157	0,052
HT	0,816	0,936	0,125	-1,067 - 2,6999	0,388
DM	-1,524	0,718	-0,293	-2,968 - 0,079	**0,039**
Smoking	1,728	0,939	0,257	-0,159 - 3,615	0,072
Alcohol	0,441	0,969	0,066	-1,507 - 2,390	0,651
Cholesterol	-2,746	0,818	-0,436	-4,390 - 1,101	**0,002**
Symptomatic	0,120	1,322	0,019	-2,615 - 2,854	0,929
Left/right side	1,678	1,257	0,268	-0,923 - 4,278	0,195
stenosis grade%	0,147	1,516	0,020	-2,990 - 3,284	0,924

CI-Confidence interval

Table 9. Multivariante linear regression of risk factors for total MoCA score before CAS intervention

	Unstandardized Coefficients		Standardized Coefficients	95% CI for B	P
	B	SG	Beta		
DM	-0,947	0,700	-0,182	-2,356 - 0,461	0,183
Cholesterol	-2,415	0,847	-0,383	-4,119 - 0,710	**0,006**

Table 10. Univariant linear regression of risk factors for total MoCA score after CAS intervention

	Unstandardized Coefficients		Standardized Coefficients	95% CI for B	P
	B	SG	Beta		
Gender	0,543	0,930	0,121	-1,380 - 2,466	0,656
Age	-0,157	0,056	-0,447	-0,291 - -0,022	**0,025**
Education	1,676	0,755	0,420	0,116 - 3,237	**0,036**
HT	0,899	0,937	-0,196	-2,838 - 1,040	0,347
DM	-0,466	0,755	-0,128	-2,027 - 1,095	0,543
Smoking	1,509	0,983	-0,013	-2,096 - 1,973	0,119
Alcohol	-0,855	0,927	0,015	1,851 - 1,984	0,353
Cholesterol	0,067	0,818	-0,436	-4,390 - 1,101	0,943
Symptomatic	0,067	0,927	0,015	-1,851 - 1,984	0,943
Left/right side	1,195	0,880	0,272	-0,626 - 3,016	0,188
stenosis grade%	0,096	1,063	0,019	-2,103 - 2,296	0,928

Table 11. Multivariante linear regression of risk factors for total MoCA score after CAS intervention

	Unstandardized Coefficients		Standardized Coefficients	95% CI for B	P
	B	SG	Beta		
Age	-0,947	0,700	-0,182	-2,356 - 0,461	0,183
Education	-2,415	0,847	-0,383	-4,119 - 0,710	**0,006**

The results of univariant linear regression analysis of risk factors for total MoCA score after intervention are shown In Table 10. Statistically significant independent risk factors were:

- age (Beta = -0,447; p = 0,025) and
- education level (Beta = 0,420; p = 0,036).

Older patients and lower education are predictors of lower levels for total MoCA scores after intervention. In multivariante model independent variables were studied: age and educational level as statistically significant risk factors for lower total MoCA score after CAS intervention. Only educational level is statistically significant factor in this model for lower cognitive function after CAS
(Beta = -0,383; p = 0,006) (Table 11.).

Discussion

Examination of the demographic and social characteristics of the patients with high grade stenosis in the present study revealed that high blood pressure was the most common vascular risk factor, followed by diabetes and high cholesterol levels. These findings are in line with those of other studies (29). Scores were evaluated relative to those of healthy individuals matched for age, gender, bad habits and educational level. We found that participants from control group had also hypertension, diabetes mellitus and high cholesterol levels which suggest that control group also had high vascular risk factors. In other studies participants from control group were patients with various levels of carotid artery stenosis and high vascular risk factors or healthy participants without vascular risk factors (25, 29-33).

Patients with high grade carotid artery stenosis had significantly poorer scores on cognitive tests than control subjects. The results in our study showed baseline differences between patients and controls in certain cognitive domains. We found that visuospatial abilities, attention, language and memory are lower in patients than controls. We found no significant change in other cognitive domains.

The primary objective of this observational prospective study was to determine the effect of CAS on cognition in patients with high grade artery stenosis. We found that total MoCA score before and after CAS was significantly different. Three months after intervention patients showed significantly better cognition. The results in our study are in accordance with the results from previous reports that have shown improvements in cognitive function in patients treated with stent placement or surgery for carotid artery stenosis (29-33).

We found that certain domains of cognition improved after revascularization. 3 month after CAS our patients reached better scores on test of attention, executive functions and memory. Other researches also reported improvement in executive functions and memory (34). Most studies failed to demonstrate a clear benefit of CAS on various cognitive functions (29-33).

Our secondary objective in the present study was to determine the factors that may affect changes in cognitive function in these patients. It has

been reported previously that gender, older age, and little educational level are risk factors for cognitive deterioration, whereas hyperlipidemia, diabetes mellitus, smoking or drinking are controversial (33). Other researchers reported that potential risk factors for deteriorated MoCA scores 3 years after CAS were age > 65 y; little education; and hypertension (35, 36). It was reported that carotid atherosclerosis is an independent vascular risk factor for cognitive impairment in nonstroke patients. It can not only impair the subtle general cognitive function but also decrease the specific domain such as memory, motor function, visual perception, attention, and executive function, which are still on studying (37). Linear regression analyses in our study showed that before CAS diabetes mellitus and cholesterol levels were independent risk factors for lower total MoCA scores and that age, educational level and bad habits did not influence on cognition before intervention. After CAS age and educational level were independent risk factors for lower total MoCA scores.

Some studies have investigated side-specific cognitive effects. It is generally assumed that restoration of hemodynamic on the treated side will be more beneficial to the cognitive function of the ipsilateral cerebral hemisphere (25, 26, 29). We found no differences in cognitive functions considering the side of carotid artery stenosis.

Symptomatic status also seems to influence cognitive results in patients after CAS (29). Most of the studies were carried out in patients with symptomatic stenosis, only few of them followed asymptomatic patients (23, 31). The changes in cognitive performance of symptomatic and asymptomatic CS patients were analyzed in this study in a prospective manner by testing their cognitive function be¬fore and after the CAS procedure. Both symptomatic and asymptomatic patients showed better results for attention, executive functions and memory after CAS. Some researchers reported that the asymptomatic patients had a poorer cognitive performance after the CAS (25, 26, 38). We can conclude that symptomatic status does not have a clear impact on the cognition after carotid revascularization.

The discrepancies in literature reports on cognitive function can also be explained by differences in methodological factors such as battery of neuropsychological testing, sample size and use of control population, severity of carotid stenosis and time to post-interventional follow-up.

Conclusion

CAS can improve global cognitive function, attention, executive functions and memory in symptomatic and asymptomatic patients with high grade carotid artery stenosis. There was no positive effect on visuospatial abilities and language but CAS was not associated with a decline in any area of cognitive function. High cholesterol levels is independent risk factor for deteriorated cognitive functions before revascularization and low educational level is independent factor for poor cognitive performance after revascularization . Symptomatic status does not have a clear impact on the cognition before and after carotid revascularization.

Future studies in larger groups of patients are probably needed to fully investigate the long-term effect of CAS on cognition in patients with carotid artery stenosis.

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EFEKTI KAROTIDNOG STENTINGA NA KOGNITIVNE FUNKCIJE KOD BOLESNIKA SA STENOZOM KAROTIDNE ARTERIJE

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Stenting karotidne arterije (CAS) je značajan terapijski modalitet kod pacijenata sa stenozom karotidne arterije. Stenoza unutrašnje karotidne arterije visokog stepena dovodi do poremećaja i deficita kognitivnih funkcija, čak i kod asimptomatskih bolesnika. Potencijalni uticaj stentiranja karotidne arterije na kognitivne funkcije bolesnika sa stenozom karotidne arterije nije dovoljno istražen. Cilj ovog istraživanja bio je da se ispita uticaj karotidnog stentinga na kognitivne funkcije kod bolesnika sa stenozom karotidne arterije visokog stepena, na različite kognitivne domene, kao i na potencijalne faktore koji mogu uticati na kognitivne funkcije kod ovih bolesnika.

U studiju je uključeno 25 bolesnika sa simptomatskom i asimptomatskom stenozom karotidne arterije i 25 zdravih ispitanika. Kognitivne funkcije su evaluirane jedan dan pre procedure i tri meseca nakon procedure. Za evaluaciju kognitivnih funkcija korišćen je Montreal cognitive assessment (MoCA)-test.

Ukupan MoCA skor kod bolesnika pre intervencije bio je značajno niži u odnosu na kontrolnu grupu. Ovaj skor je značajno povišen tri meseca nakon intervencije. Značajano su se popravili rezultati za pažnju, egzekutivne funkcije i pamćenje.

Karotidni stenting može poboljšati ukupne kognitivne funkcije kao i pažnju, egzekutivne funkcije i pamćenje kod simptomatskih i asimptomatskih bolesnika sa stenozom karotidne arterije visokog stepena. Visok nivo holesterola predstavlja nezavisni faktor rizika za deficit kognitivnih funkcije pre revaskularizacije, dok nizak nivo obrazovanja predstavlja nezavistan faktor za nizak nivo kognitivnih funkcija nakon revaskularizacije.

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Ključne reči: *stenoza karotidne arterije, karotidni stenting, kognitivne funkcije*

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EVALUATION OF ENAMEL SURFACE ROUGHNESS AND MORPHOLOGICAL CHANGES AFTER EXPOSURE TO COCA-COLA, ORANGE AND ARTIFICIAL GASTRIC JUICE: AN *IN VITRO* STUDY

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Dental erosion is a pathologic, non-bacterial hard dental tissue loss induced by extrinsic or intrinsic acids. This *in vitro* study aimed to evaluate and compare the morphology and surface roughness of dental enamel after erosive challenge in some extrinsic and intrinsic acidic substances, Coca-Cola, orange and gastric juice.

Enamel samples (n = 48), obtained by preparation of surgical extracted human third molars, were subjected to the erosive challenge of the artificial gastric juice and commercially-available Coca-Cola and orange juice by immersion in 50 ml of erosive solutions for 15 min, three times daily, for 10 days. Between immersions, the samples were kept in filtered saliva. Twenty-four samples were prepared for the surface morphology analysis using scanning-electron microscope, and 24 for the analysis of Ra-surface roughness parameter (using a diamond-stylus-profilometer), including the 12 control samples (which did not undergo the erosion procedure). Results of the surface roughness were analyzed by one-way ANOVA Student-Newman-Keuls post hoc test.

Ultrastructural analysis of enamel surface after immersion in Coca-Cola and gastric juice showed type 1 etching pattern with the typical honeycomb appearance. After the erosive challenge with orange juice, a nonspecific morphological model was established. Profilometric parameter Ra was significantly increased for samples immersed in gastric juice compared to samples immersed in Coca-Cola and orange juice, as well as, in samples with Coca-Cola-erosion compared with orange juice-erosion. Gastric juice had higher erosive potential in relation to Coca-Cola and orange juice, with the most intense morphological changes and the highest roughness on the enamel surface.

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Key words: *Enamel erosion, soft drinks, gastric juice, SEM, surface roughness*

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Introduction

Dental erosion has been defined as pathologic, non-bacterial dental hard tissue loss induced by extrinsic or intrinsic acids or chelators acting on plaque-free tooth surfaces (1). The most important extrinsic source of acid exposure is diet, which could include numerous components and products with complex composition and a potential for erosive damage (carbonated and acidic drinks, acidic food, citrus pastilles, various medicaments), professional exposure to corrosive agents (acid vapors from batteries and other appliances), even exposure to chlorinated water in swimming pools during water sports (2-6). In addition, behavioral factors like eating and drinking habits (holding an acid beverage in the mouth before swallowing, swishing around the mouth or sucking juice through the teeth) contribute to its development (7). Intrinsic factors are the result of endogenous acid, generally gastric acids that contact teeth especially in patients suffering from anorexia, bulimia, chronic vomiting during pregnancy and gastrointestinal disturbances (8-11).

Many laboratory studies have found carbonated drinks, especially carbonated cola drinks, to be associated with erosion, most likely due to their low pH (2, 4, 12, 13). Further, in vitro studies have shown

that fruit juices may also be potentially erosive, due to their high content of titratable acid (2, 4, 12).

On the other hand, acidic stomach contents refluxed into the oral cavity can dissolve tooth structures and cause erosive tooth wear (14, 15) because contact between the hydrochloric acid from the stomach (with pH from 1.5 to 3,5) and the oral cavity occurs for a few seconds, several times a day (16).

The aims of the present in vitro study were twofold: (1) to analyze the experimental models of enamel erosion after exposure to Coca-Cola, orange juice and artificial gastric juice at the ultrastructural level, and (2) to evaluate enamel surface roughness after erosive challenge in the same acidic solutions.

Material and methods

The material for this research included 12 human impacted mandibular third molars (from patients aged 18-25 years) disinfected in 1% thymol solution and kept in 1% sodium hypochlorite for 24 h. Organic debris was removed by carefully using a dentist's set of instruments (17).

After the removal of the roots, at least 2 mm below cementoenamel junction, the crowns were cut (using a diamond saw under water irrigation) from the distal, mesial, buccal, and lingual side. Out of the total of 48 samples, 24 were used for SEM analysis and 24 were used for the analysis of enamel surface roughness (Table 1).

Table 1. Distribution of samples used in experimental protocols.

Samples	Number of samples for SEM analysis	Number of samples for surface roughness analysis	Total
Control	6	6 (3 measurements)	12
Immersed in Coca-Cola	6	6 (3 measurements)	12
Immersed in orange juice	6	6 (3 measurements)	12
Immersed in artificial gastric juice	6	6 (3 measurements)	12
Total	24	24	48

Erosion solutions and human saliva

The erosion models caused by soft drinks were obtained by immersing the samples in Coca-Cola (HBC - Serbia A.D. Zemun) and orange juice (NECTAR' D.O.O. Backa Palanka, Serbia).

In the previous study it was established that Coca-Cola had pH 2.67 ± 0.06 and TA 1.87 ± 0.09, whereas orange juice had pH 3.73 ± 0.03, requiring 5.70 ml of NaOH to reach pH 7.0 (4).

The model of enamel erosion with GERD was created using artificial gastric juice according to the methodology of Stefaniak et al. (18) and it was modified in accordance with the stablished goals of the research. Its initial pH was 2,1 (Table 2.).

Table 2. The contents of artificial gastric juice (primary electrolytes and ionic compounds)

Contents	**Concentration**
Calcium chloride dihydrate ($CaCl_2 \times 2H_2O$)	0.264 g/L
Magnesium chloride hexahydrate hexahydrat ($MgCl_2 \times 6H_2O$)	0.152 g/L
Potassium chloride (KCl)	0.864 g/L
Sodium chloride (NaCl)	2.855 g/L
Hydrochloric acid (HCl)	1.426 (3.38 ml; 36.2%)

Human saliva was collected from healthy volunteers in the morning, 2 hours after fasting. Volunteers rinsed their mouths twice with distilled water before saliva collection (19). Filtrates were obtained with Whatman filter papers grade 1: 11 μm (Sigma-Aldrich, USA).

Erosive Challenge

This study was approved by the institution's Ethics Committee. Tooth samples planned for analysis using SEM, immediately after cutting, rinsing and drying, were distributed into one of three erosive challenges, while the samples planned for analysis using profilometer, before exposure to acidic solutions, were prepared as follows: circular molds of 16 mm in diameter and 3 mm deep were filled by self-cured resin. Each sample was embedded in resin, with labial (oral) surfaces uppermost, and was cleaned with nonfluoridated pumice, rinsed with water and dried with oil-free compressed air.

All of the enamel samples were exposed to acidic solution according to the following protocol (10): immersion in 50 ml acidic solution (Coca-Cola/orange juice/gastric juice) for 15 minutes with occasional shaking, rinsing with distilled water, and immersion in human saliva.

The cycle was repeated three times a day for 10 days. During the night, the samples were placed in human saliva, including the 12 control samples (which did not undergo the demineralization procedure). The experiment was conducted at room temperature.

Preparation of samples for SEM analysis

At the end of the experimental period the samples were dried, fixed to aluminum stubs with a fixing agent (Dotite paint xc 12 Carbon JEOL, Tokyo, Japan), sputter-coated with old/palladium (in the unit JFC 1100E Ion Sputter JEOL), and examined by scanning electron microscopy (SEM) (JEOL-JSM-5300).

Preparation of samples for surface roughness analysis

Surface roughness of the enamel samples was measured using a profilometer (Mitutoyo Surftest SJ-301) (20).

Although four parameters of roughness are registered with the stylus of the Mitutoyo type profilometer, the statistical analysis took into account only one, the most frequently used parameter, Ra, which is defined as the average distance from the profile to the mean line over the length of assessment. A detailed description of the measurement method using a diamond stylus profilometer has already been published in our recent study (4). Statistical analysis was carried out using one-way ANOVA Student-Newman-Keuls post hoc test.

On enamel surfaces not exposed to the erosive challenge by Coca-Cola, orange and gastric juice (control group), the typical structures of sound enamel (grooves and perichimata lines) were apparent. Additionally, small depressions or ditches or grinding marks were observed and they were found to be indicative of the cumulative mechanical effects the teeth have experienced. (Figure. 1)

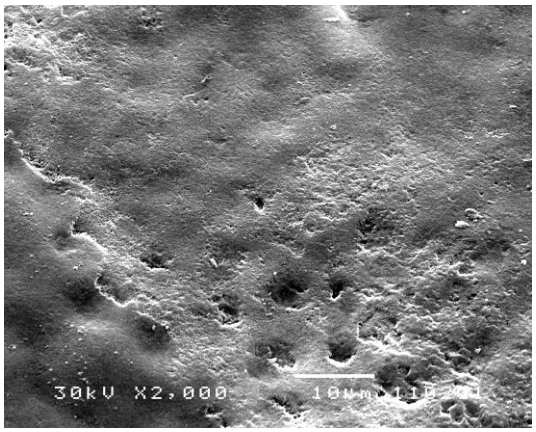

Figure 1. Control samples: the surface of untreated enamel with perikymata.

Results

SEM results are shown in micrographs 1 to 4 and the measurements of enamel surface roughness are shown in Table 3.

Table 3. The values of the enamel surface roughness parameter (Ra) in relation to the tested acid solutions

Roughness parameter	Exposure (min)	Control	I Artificial gastric juice	II Coca-Cola	III Orange juice
R_a	15	0.67 ± 0.02	1.63 ± 0.25[a]	1.49 ± 0.08[a,b]	1.27 ± 0.01[a,b,c]

[a]$p < 0.05$ vs control; [b]$p < 0.05$ vs. artificial gastric juice; [c]$p < 0.05$ vs. Coca-Cola;

The enamel surface of teeth exposed to the acidic solutions clearly demonstrated deep changes in enamel structure: scanning micrographs of enamel samples eroded by Coca-Cola and gastric juice exhibited a distinct pattern, showing hollowing of prism centers with relatively intact peripheral regions, reflecting honeycomb appearance (Figuries 2, 4).

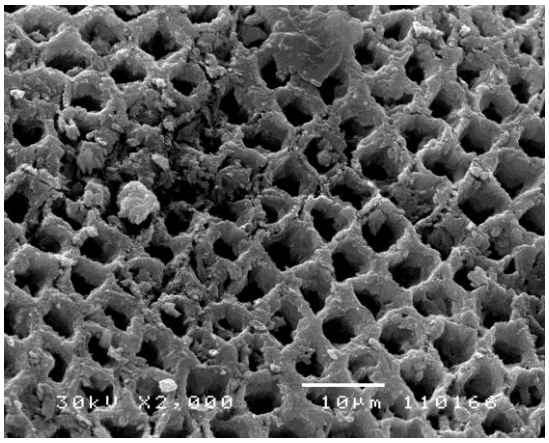

Figure 2. Erosive changes on enamel caused by **Coca Cola**: type 1 erosion, central parts of the prisms are affected and the peripheral parts are relatively preserved.

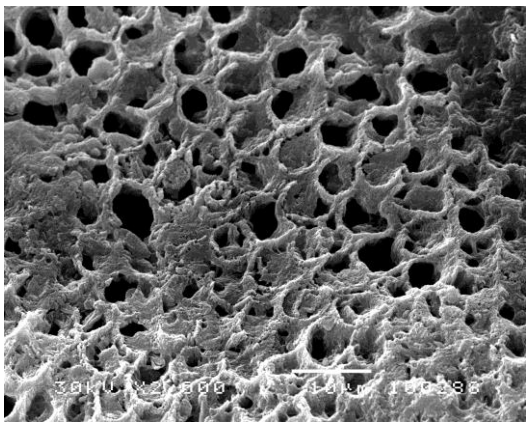

Figure 4. Erosive changes in enamel surface caused by gastric juice with significant type 1 erosion, honeycomb appearance.

In contrast, samples immerged in orange juice showed atypical etching: without prisms, with pitted enamel surfaces, as well as with structures which look like unfinished puzzles, maps, networks. (Figure 3).

The highest value of Ra roughness parameter was observed in the samples immersed in gastric juice, followed by Coca-Cola and, finally, orange juice.

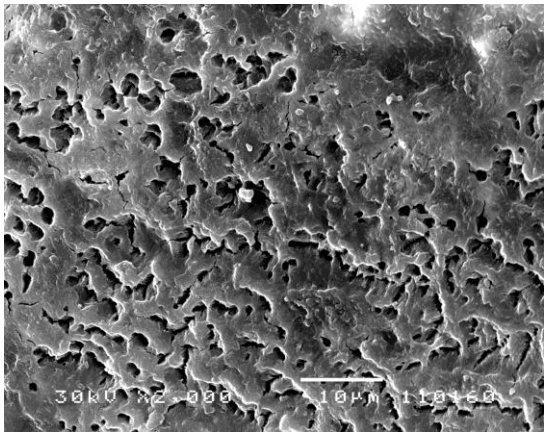

Figure 3. Erosive changes in enamel surface caused by orange juice with irregular erosion type.

There was a statistically significant difference among all of the tested roughness parameter values.

Discussion

Literature data point to an increase in dental erosions in the modern society and they represent a challenge for the researchers. Considering that there is a decreasing tendency in caries instances, erosive tooth wear is becoming a more significant element in planning a long-term model of dental health (2). An increase in the prevalence of various gastrointestinal diseases and eating disorders leads to more frequent contacts between the teeth and gastric acid. Together with increasing use of acidic beverages, these conditions are considered significant risk factors for teeth demineralization.

The goal of our research was to create the ultrastructural experimental models of enamel erosion caused by Coca-Cola, orange juice and artificial gastric acid and to determine differences and possible similarities between the erosions caused by external and internal factors on experimental model. To some extent, the findings could point to the significance of prevention of both internal and external causes of erosive tooth wear.

In order to simulate clinical conditions, the present research used gastric juice formula which, apart from HCl with 2,1 pH, contained only the primary electrolytes and ionic compounds, without organic and amino acids, carbohydrates and pepsin. Calcium, magnesium and sodium salts should act as buffer components which could probably control the erosive potential of gastric juice, similar to in vivo conditions. On the other hand, the majority of the results from laboratory studies regarding the enamel surface changes in reflux disease were obtained based on the use of pure HCl (16, 21, 22, 23, 24). Nevertheless, in studies by Barlet at al. and Braga at al. (9, 10), gastric juice which was aspirated from patients undergoing endoscopy for symptoms of reflux disease was used.

In the present study, the immersion cycles (3 times for 15 minutes) could imitate GERD symptoms

for a shorter period of time (10 days). Similarly, the time of immersion of samples into Coca-Cola and orange juice can imitate the frequency of consumption of soft drinks. On the samples immersed in the Coca-Cola and gastric juice, the following was observed: diffuse demineralization involved the rod core, with decomposition of morphology of prisms: they were severely affected, and a greater prism-core dissolution compared with that in the interprismatic areas gave the enamel a "honeycomb pattern" of etching, similar to the results published by Colombo et al. (25) and Braga at al. (10). Also, Arnold et al. (22) showed that exposure to pure HCl results in four different enamel etching patterns, and the depth of the surface layer was dependent upon the etching time. Our research showed that the degree of destruction of central prism parts varied depending on location, whereby the most prominent changes were observed in the vicinity of cement-enamel junctions. In the present study, samples immerged in orange juice showed atypical etching, which is referred to as type 4 in the literature. (26).

In the current study, surface roughness was measured using a stylus profilometer that overhangs across the surface of the object, registering all of the unevenness at a certain measuring length (17, 27, 28, 29, 30). According to some literature data stylus profilometry shows some disadvantages (the risk of the diamond tip causing damage to the specimens, inability to detect valleys which are narrower than the stylus tip) but nevertheless this technique has a high degree of precision (31). Moreover, the current national standards on measuring surface texture are defined using stylus profilometry (11, 31, 32).

In a number of studies (28, 29), a difference in the surface roughness of the samples examined on various erosive challenges was determined only on the basis of the Ra parameter, where valid conclusions were drawn. The present study showed that this parameter was statistically significantly different among all the tested samples. Likewise, all samples of the experimental groups were also different from the control samples according to the SEM analysis and after analysis of the Ra parameter.

According to the latest literature data, the Ra parameter provides no information on the characteristics of surface irregularities, whereby both maximal and minimal irregularities may show the same Ra values (31, 34). Therefore, this research also included an ultramicroscopic analysis in order to obtain more precise results.

Braga et al. (10) showed that the enamel surface after orange juice had a generalized surface roughening with no apparent evidence of a prism pattern, and the surface was not completely etched. The same authors used atomic emission and FT Raman spectroscopy to analyze the mineral content of enamel after exposure to gastric and orange juice, and they determined that gastric juice has a higher erosive potential than orange juice. Our research analyzed surface roughness of enamel using stylus profilometry following the exposure to the same acidic solutions, as well as to Coca-Cola. It has been determined that gastric juice has a higher erosive effect on the enamel, which is in accordance to the results of the mentioned authors. Roughness parameter showed that, after exposure to gastric juice, the enamel surface had prominent unevenness of the surface which was statistically significant compared to the surface texture of samples exposed to Coca-Cola and orange juice.

According to information from the manufacturers, Coca-Cola contains phosphoric acid, compared to citric acid,phosphoric acid is stronger (33). The effect of phosphoric acid results in a superficial etched zone which might be permanently lost from the tooth surface (26). On the other hand, citric acid may act as a chelator capable of binding the calcium from enamel or dentine, thus increasing the degree of undersaturation and favoring demineralization (35, 36).

In our previous study the erosive potential of various soft drinks was examined by measuring initial pH and titratable acidity and enamel surface roughness using different exposure times. It was found that Coca-Cola had the highest erosive potential in the shortest time interval exposure (15 min), although it had the lowest titratable acidity (4). These results are in accordance with literature data which show that cola-based drinks have a higher erosive potential than orange juices immediately after exposure (12). Profilometric parameters have demonstrated that pure orange juice causes greater enamel erosion during longer exposures. A statistically significant lower degree of roughness compared to Coca-Cola in shorter exposure can be explained by higher initial pH in orange juice compared to Coca-Cola (3.73 vs. 2.67) (4).

In the current study, gastric juice was significantly more erosive to enamel than Coca-Cola and orange juice, and Coca-Cola is more erosive than orange juice. Other studies attest that gastric juice (aspirated from patients undergoing endoscopy) has a greater potential for erosion than orange juice (10) and carbonated drinks (Bartlett and Coward, 2001). Results by Bartlett and Coward reflect the lower pH and titratable acidity of gastric juice compared with the carbonated drink. If this result is extrapolated to the clinical situation, it confirms the suspicion that gastric juice has the potential to produce the severe pattern of erosion found in patients with eating disorders and reflux disease (9).

Conclusion

Despite the limitations characteristic of in vitro studies, it can be concluded that experimental erosion model of enamel surface exposed to Coca-Cola and artificial gastric juice shows type 1 acidic erosion (honeycomb appearance) by SEM analysis. Degree of destruction of central prism parts varied depending on location, whereby the most prominent changes were observed in the vicinity of cement-enamel junctions. Ultrastructural experimental model of enamel surface erosion after exposure to orange juice demonstrates atypical etching with no apparent evidence of a prism pattern. Profilometric parameter Ra was significantly increased for samples immersed in gastric juice compared to samples immersed in Coca-Cola and orange juice, as well as in samples with Coca-Cola-erosion compared with

orange juice-erosion. The results of this study point to a higher erosive potential of gastric juice, compared to Coca-Cola and orange juice, with the most intense morphological changes and the highest roughness on the enamel surface.

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EVALUACIJA HRAPAVOSTI I MORFOLOŠKIH PROMENA NA GLEĐNOJ POVRŠINI POSLE IZLOŽENOSTI COCA-COLI, SOKU OD NARANDŽE I VEŠTAČKOM ŽELUDAČNOM SOKU: *IN VITRO* STUDIJA

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Erozija zuba je patološki gubitak tvrdih zubnih struktura izazvan spoljašnjim i unutrašnjim kiselinama, bez učešća bakterija. Ova *in vitro* studija imala je za cilj da proceni i uporedi morfologiju i površinsku hrapavost gleđi nakon erozivnog izazova nekih eksternih i internih kiselih supstanci kao što su Coca-Cola, sok od narandže i veštački želudačni sok.

Uzorci gleđi (n = 48), dobijeni ekstrakcijom humanih trećih molara, podvrgnuti su erozivnom izazovu veštačkog želudačnog soka i komercijalno dostupnih bezalkoholnih pića (Coca-Cola i sok od narandže) uranjanjem u 50 ml kiselog rastvora u trajanju od 15 min, tri puta dnevno tokom 10 dana. Između potapanja, uzorci su držani u filtriranoj pljuvački. Primljena su dvadeset i četiri uzorka za analizu površinske morfologije korišćenjem skening-elektronskog mikroskopa i 24 za analizu Ra-parametra hrapavosti (korišćenjem stylus profilometra sa dijamantskom iglom), uključujući i 12 kontrolnih uzoraka (koji nisu podvrgnuti proceduri erozije). Rezultati površinske hrapavosti analizirani su pomoću one-way ANOVA Student-Newman-Keuls post hoc testa.

Ultrastrukturna analiza površine gleđi posle potapanja u Coca-Colu i želudačni sok pokazala je tip 1 model nagrizanja sa tipičnom honeycomb strukturom. Nespecifičan morfološki model ustanovljen je nakon erozivnog izazova sokom od narandže. Profilometrijski parametar Ra je značajno povećan kod uzoraka potopljenih u želudačni sok u poređenju sa uzorcima izloženim Coca-Coli i soku od narandže, kao i u uzorcima sa Coca-Cola-erozijom u poređenju sa erozijom izazvanom sokom od narandže. Želudačni sok je pokazao veći erozivni potencijal u odnosu na Coca-Colu i sok od narandže, sa najintenzivnijim morfološkim promenama i najvećom hrapavošću na površini gleđi.

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Ključne reči: *gleđna erozija, bezalkoholna pića, želudačni sok, SEM, površinska hrapavost*

Original article

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ANTERIOR SURGICAL APPROACH IN RESOLVING CERVICAL MYELOPATHY RESULTING FROM A MULTISEGMENTAL DEGENERATIVE PROCESS

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Progressive degenerative diseases of the cervical spine are an increasingly common cause of the development of cervical myelopathy.

The analysis was performed on 67 surgically treated patients with multisegmental degenerative processes of the cervical spine. Thirty-three patients underwent surgical treatment on two levels, 25 on 3 levels and 9 on 4 levels. Muscle strength and control cervical spine X-ray were monitored pre - and postoperatively; control MRI was performed after 6 months of surgery.

All the patients recovered to a higher or lower extent, and the progression of neurological deficits stopped in all of them. In 7 patients, certain swallowing problems were observed. Two patients underwent revision due to the intrusion of titanium grafts into the corpuses and front destabilization. Three patients underwent repeat surgery after one year, whereby segmental approach was expanded from two to three and from three to four levels. In 13 patients, problems with the extent of neck rotation were registered, but this did not affect their normal life activities. The finding resulting from the muscle strength monitoring of the most affected group of muscles indicates a significant improvement with respect to all levels of preoperative motor weakness.

The presence of chronic pain syndrome and the development of neurological deficits in correlation with the MRI finding represent an absolute indication for surgical treatment. The anterior surgical approach not only eliminates the causes of compression of the neurovascular elements, but also provides correction of the intervertebral disc space height, correction of kyphosis and the loss of lordosis.

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Key words: *Cervical myelopathy, cervical disc herniation, anterior approach to the cervical spine*

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Introduction

Degenerative processes in the cervical spine are the result of biochemical, biomechanical and morphological changes in the cervical intervertebral discs (1-4). These processes result in the narrowing of the intervertebral space with the development of a compressive effect on neurovascular elements in intravertebral foramens, and a clinical picture of peripheral neurological deficit (5, 6). Compression of the degenerately altered intervertebral disc on neurovascular elements in the spinal canal leads to the development of a central neurological deficit (7).

Combination of these clinical symptoms forms a cervical myelopathy syndrome. Cervical myelopathy is the most common consequence of the multisegmental degenerative process in the cervical spine. The clinical picture is characterised by a gradual, long-term development of symptoms (8, 9).

The most common approach to treating these patients is conservative, so patients first report neurosurgical examination in the second, and most often in the third and fourth stage of the clinical picture. Good postoperative clinical course is directly related to the preoperative duration of ailments and the stage at which patients undergo surgery.

Material and methods

The analysis was performed on 67 patients with multisegmental degenerative changes in the cervical spine, who had a clear clinical picture of cervical myelopathy. Patients were operated on at the Neurosurgical clinic in Niš, in the period from 2010 to 2016. Thirty-three patients were operated on at two levels, 25 patients on three levels and 9 patients on four levels.

The clinical picture was divided into four stages:

- The first stage - numbness in the neck, intermittent pain along the neck and arms, recurrence of ailments
- Second stage - long-lasting pain in the neck and along the arms, headaches, dizziness, buzzing in the ears, instability while walking, nonspecific vegetative complaints in the form of tightness in the chest, heart palpitations, changes in vision
- Third stage - pain along the arms with the occurrence of hypotrophy of the respective muscle group, permanent discomfort regardless of activity, position or time of day, feeling of weakness in legs and walking difficulty
- Fourth stage - the development of severe motor disorders of monoparesis, hemiparesis, triparesis or quadriparesis type

Surgery was performed on 23 patients with clinical stage II, 23 patients with clinical stage III, and 21 patients with clinical stage IV.

All patients were treated by applying multisegmental surgical approach. Complete release of intravertebral disc space was performed with the release of lateral recesses, resection of the posterior longitudinal ligament and removal of marginal osteophytes from the spinal canal. At each level, distraction of intervertebral disc space, insertion of titanium cage (cagea) and correction of intervertebral space height with consequent correction of kyphosis in that segment of the spinal cord was performed. Afterwards, the front multisegmental stabilization with a titanium plate was obligatory.

The follow-up of patients was performed by monitoring the clinical and neurological status. The numerical index of the motor strength of the most affected group of muscles was monitored preoperatively and postoperatively.

The British Medical Research scale was used to grade muscle strength:

- 1 - A noticeable or palpatory contraction, without the ability to perform movement
- 2 - Possible movement under the condition of eliminating the earth's weight
- 3 - Possibility of movement versus earth's weight
- 4 - The ability to move with less resistance (light partial paralysis)
- 5 - Normal muscle strength

Immediately after surgery, a clinical and radiological evaluation was performed. After 6 months, EMG and X-ray findings were obtained. MSCT or MRI findings were obtained after 6 to 8 months of surgery.

Results and Discussion

Of the 67 patients, 41 (61%) were women, and 26 (39%) were men. The average age of patients ranged 52.6 ± 11.2 in women, and 56.4 ± 9.2 in men, which stands in correlation with the studies where patients were treated with anterior multisegmental approach (10, 11).

All the patients recovered to a higher or lower extent, and in all of them the progression of neurological deficit stopped. In the patients' preoperative status, a statistically significantly higher representation of the motor index of 1 to 3 was observed, compared to the postoperative status of patients with a significantly higher representation of the motor index of 3 to 5 ($p < 0.05$). In 7 (10.4%) patients, there were certain swallowing problems in the postoperative period of 3 to 5 months, which is consistent with the results of other studies, ranging from 1 to 79% (12, 13).

Two patients underwent revision after two months of surgery due to the intrusion of titanium cages into the corpuses and destabilization of the front fixation plate (2.9%). Three patients underwent a repeat surgery due to the development of symptomatology to a level above surgical treatment (4.5%), (8, 14), which is correlated with other studies (in two patients, the surgical approach was expanded from two to three levels and from three to four levels).

It was observed that despite applying the multisegmental approach, in only 13 (19.4%) patients there were some problems in terms of reduced head rotation, but this did not affect their normal life activities. Reduced mobility of the neck is part of the multisegmental approach in resolving cervical myelopathy. The finding is in correlation with other studies (15, 16).

All the patients had minimal blood loss. The duration of surgery was from 2.5 - 4 hours. There was no postoperative deterioration of the neurological deficit in any of the cases. The patients were verticalized immediately after 24 hours. The results are in correlation with the world studies (17, 18)

The finding obtained at 6 months of surgery by monitoring the motor strength of the most affected muscle group indicates a significant improvement with respect to all the levels of preoperative motor weakness (Table 1, 2). Postoperative monitoring of X-ray and MRI findings shows a fully corrected neck kyphosis and decompression of the spinal canal and nerve elements in all of the patients (Figure 1, 2, 3).

Conclusion

The anterior surgical approach to multisegmental degenerative processes in the cervical spine allows for complete removal of the pathological substrate directly responsible for the development of clinical picture and neurological deficits In addition, after removal of degenerately altered disc herniation, osteophytes and stenosis of the lateral recesses, the

Table 1. Operative treatment levels in patients with cervical myelopathy
(localization and degree of neurological demage of the affected muscule group)

NEUROLOGICAL DAMAGE			0 - 1	1 - 2	2 - 3	3 - 4	4 - 5
C3 / C4, C4 / C5	8		/	1	2	3	2
C4 / C5, C5 / C6	14	33 (49,3 %)	1	1	3	7	2
C5 / C6, C6 / C7	11		1	3	4	2	1
C3/C4, C4/C5 C5/C6	8	25 (37,3 %)	1	1	4	2	/
C4/C5, C5/C6 C6/C7	17		2	4	8	3	/
C3/C4, C4/C5, C5/C6, C6/C7	9	9 (13,4 %)	3	3	2	1	/
	67 (100 %)		8 (12 %)	13 (19,5%)	23 (34%)	18 (27%)	5 (7,5%)

Table 2. Degree of demage of the most affected muscle group six months after operation

NEUROLOGICAL DAMAGE			0 - 1	1 - 2	2 - 3	3 - 4	4 - 5
C3 / C4, C4 / C5	8		/	/	2	2	4
C4 / C5, C5 / C6	14	33 (49,3 %)	/	/	1	8	5
C5 / C6, C6 / C7	11		/	1	4	4	2
C3/C4, C4/C5 C5/C6	8	25 (37,3 %)	1	/	2	4	1
C4/C5, C5/C6 C6/C7	17		1	2	4	6	4
C3/C4, C4/C5, C5/C6, C6/C7	9	5 (13,4 %)	1	2	3	3	/
	67 (100 %)		3 (4,5 %)	5 (7,5 %)	16 (24 %)	27 (40 %)	16 (24 %)

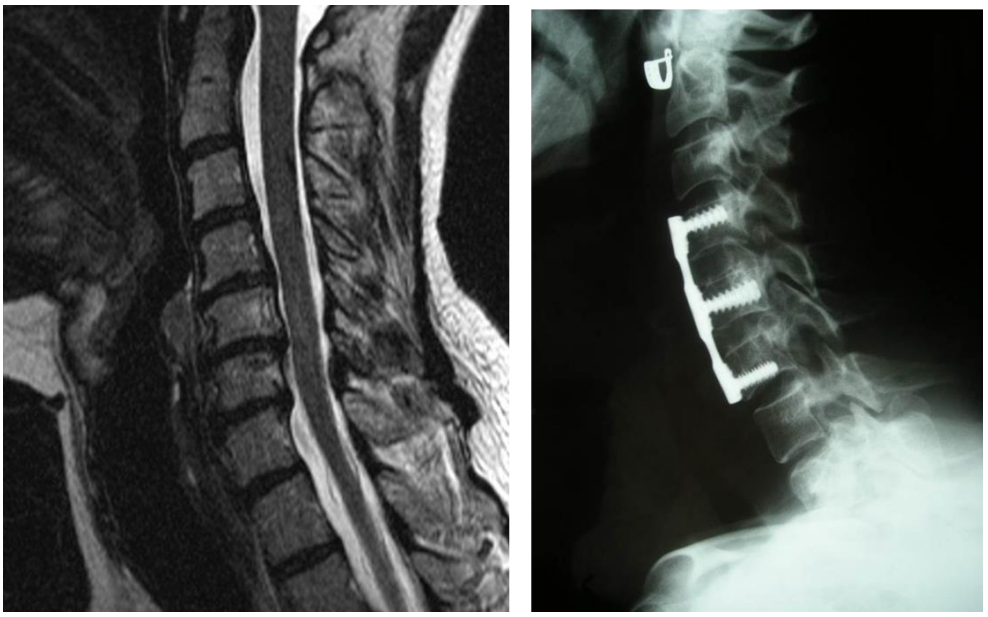

Figure 1. Treatment of two levels / corrected kyphosis

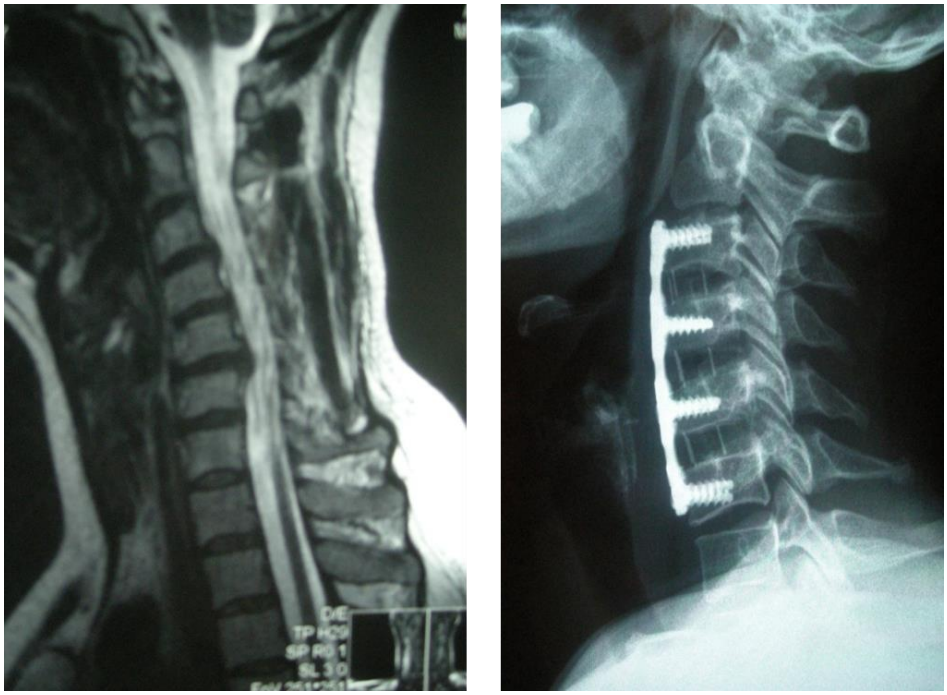

Figure 2. Treated three levels / corrected kyphosis

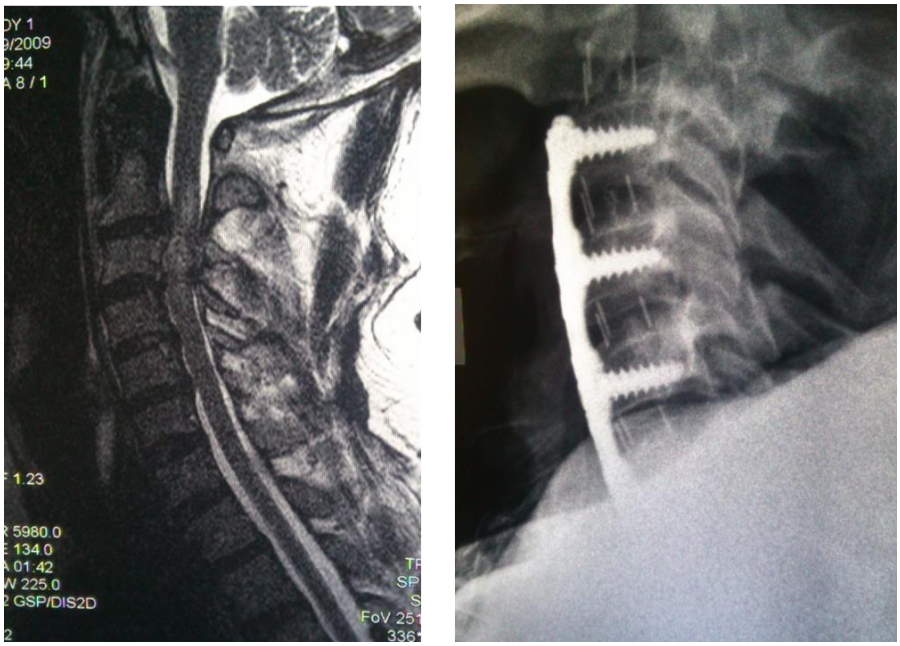

Figure 3. Treated four levels

physiological height of the intervertebral disc space is restored at all affected levels, with complete correction of the present kyphosis in this segment of the cervical spine. The importance of such surgical approach is indicated by other studies as well (11, 19, 20).

After correction of the intervertebral disc space height, we always applied the anterior plate fixation of all affected levels, which in none of the cases resulted in the development of postoperative kyphosis, as opposed to the surgical technique where only intercorporal fusion was performed without the anterior fixation (21).

The indication for surgical treatment can be clearly set out in the following cases:

• The presence of stenosis of the spinal canal with stenosis of the lateral recesses, with clinical signs of intermittent cervicobrachialgia, and resista-

nce to medication therapy, is an indication for surgical treatment, as a prevention of the development of a complete clinical picture of cervical myelopathy.

• Pain in the neck with cervicobrachialgia and with clear MRI signs of multisegmental anterior compressions and signs of cervical myelopathy, are a clear indication for surgical treatment.

• Neurological deficit of monoparesis, triparesis or quadriparesis type, with clear MRI findings of degenerative multisegmental stenoses in the cervical spine represent an absolute indication for surgical treatment

The anterior multisegmental approach to degenerative processes has an advantage over posterior surgical approach, because complete decompression of the neurovascular elements is achieved with the correction of the position of the cervical spine and restoration of mechanical stability (10, 19, 22, 23).

By using posterior decompression and laminectomy, mechanical stability in the cervical spine can be exacerbated, which is why the posterior stabilization should often be done. In addition, it does not lead to the expected recovery of neurological deficits (17, 24, 25).

Postoperative results largely depend on the stage of disease prior to performing surgery. The greater the degree of clinical and neurological damage is, the slower and more difficult the recovery will be.

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PREDNJI OPERATIVNI PRISTUP U REŠAVANJU CERVIKALNE MIJELOPATIJE KAO POSLEDICE MULTISEGMENTOG DEGENERATIVNOG PROCESA

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Progresivna degenerativna oboljenja vratnog dela kičme su sve češći uzrok razvoja cervikalne mijelopatije. Klinička slika je postepena sa dugovremenim razvojem.

Analizi je podvrgnuto 67 operisanih bolesnika sa polisegmentnim degenerativnim procesima u vratnom delu kičme, koji su imali kliničke i neurološke znake za cervikalnu mijelopatiju. Na dva nivoa bila su operisana 33 bolesnika, 25 na tri nivoa i 9 na četiri nivoa. Bolesnici su operativno lečeni u periodu od 2010. do 2016. Svi bolesnici su tretirani prednjim multisegmentnim operativnim pristupom. Pre i post operativo praćena je mišična snaga, kontrolni RTG vrata, a kontrolni IMR nakon 6 meseci.

Svi bolesnici su imali veći ili mani oporavak uz zaustavljanje progresije neuroloških ispada. Kod 7 bolesnika zapaženi su neki vidovi problema sa gutanjem. Kod dva bolesnika je urađena revizija zbog upadanja titanijumskog grefona u korpuse i prednje destabilizacije. Ponovnoj operaciji podvrgnuta su tri bolesnika nakon godinu dana, sa proširenjem segmentog pristupa sa dva na tri i sa tri na četiri nivoa. Kod 13 bolesnika registrovani su problemi sa obimom rotacije vrata, ali to nije uticalo na njihove normalne životne aktivnosti. Nalaz praćenja motorne snage najugroženije grupe mišića, nakon 6 meseci, ukazuje na postizanje značajnog poboljšanja u odnosu na sve nivoe preoperativne motorne slabosti.

Polisegmentni vratni degenerativni procesi najčešće dovode do razvoja kifoze i gubitka normalne loroze u vratnom delu kičme. Prisustvo hroničnog bolnog sindroma i razvoja neuroloških ispada u korelaciji sa NMR nalazom predstavljanju apsolutnu indikaciju za operativno lečenje. Prednjim operativnim pristupom se uklanjaju uzroci kompresije na neurovaskularne elemente, ali se pored toga vrši i korekcija visine intervertebralnih prostora i korekcija kifoze i izgubljene lordoze, što je u osnovi mehaničke stabilnosti kičmenog stuba, a samim tim i neurološke stabilnosti.

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Ključne reči: *cervikalna mijelopatija, vratna disk hernijacija, predni pristup vratnoj kičmi*

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EXPRESSION OF EPIGENETIC SILENCER EZH2 IN EARLY INVASIVE PT1 UROTHELIAL BLADDER CANCER

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Urothelial bladder cancer is the most common malignancy of the urinary tract. Early invasive bladder cancer (stage pT1) is a tumor that has invaded subepithelial connective tissue, without spread to bladder muscle, and represents a major challenge for diagnosis and therapy. EZH2 transcriptional repressor has a crucial role in oncogenesis of the bladder. The aim of this research was to investigate the expression profile of EZH2 in pT1 bladder cancer, to analyze the correlation with clinicopathological factors, and to assess the possible prognostic significance of EZH2 expression. In that purpose 279 tumor samples embedded in tissue microarrays were analyzed immunohistochemically to EZH2 expression. High EZH2 nuclear expression was observed in 44.5% of the tumors. High EZH2 expression was strongly associated with high histologic grade ($p < 0.001$). In addition, EZH2 significantly correlated to male gender, and the occurrence of carcinoma *in situ* in the adjacent urothelium ($p = 0.019$, and $p = 0.026$, respectively), while divergent differentiation and disease recurrence showed no significant association with EZH2 staining. High EZH2 expression strongly correlated with cancer specific death ($p = 0.010$). Kaplan-Meier survival analysis demonstrated that high EZH2 expression predicted worse survival of the patients ($p < 0.001$). Impact of EZH2 expression to recurrence free survival was not significant. High EZH2 expression in early invasive urothelial bladder cancer indicates aggressive behavior of the tumor and poor prognosis. EZH2 has promising roles in urothelial bladder cancer as a complementary diagnostic tool in selection of the patients that require closer clinical attention, and as a potential target for anticancer therapy.

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Key words: *urothelial bladder cancer, EZH2, immunohistochemistry, prognosis*

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Introduction

Urothelial bladder cancer (UBC) is the most common malignancy of the urinary tract, and its diagnostics and clinical management represents a major burden for healthcare systems (1). Non-muscle invasive carcinomas account for about 75% of bladder cancer. Early invasive UBC, stage pT1, is a tumor that has invaded subepithelial connective tissue (lamina propria), without evidence of the spread to muscle of the bladder wall. The accurate diagnosis of early invasive UBC is challenging, and is associated with numerous impediments including cautery artefacts, superficial, insufficient or poorly oriented transurethral resection specimens, tangential sectioning, obscuring inflammatory infiltrate (2). UBC pT1 cancer is of particular importance since it comprises a heterogeneous group of tumors in term of patients' prognosis and treatment outcome. High grade early invasive UBC is considered an aggressive and potentially lethal disease, and require more intensive clinical follow up and management (3, 4).

Recent research has highlighted the significance of epigenetic alterations in mutational landscape of bladder cancer (5, 6). It has been found that mutations in chromatin regulatory genes are more frequent in UBC than in any other solid cancer. Via their influence on expression of various transcription factors and signaling molecules, the alterations in epigenetic pathways have profound implications to fundamental features of cancer biology, including tumor

growth promotion, invasiveness and capacity to metastasize. EZH2, enhancer of zeste homolog 2, is one of the most important and most investigated molecules involved in epigenetic control (7). EZH2 acts as enzymatic subunit of Polycomb repressive complex, responsible for silencing of genetic transcription through histone methylation. EZH2 has been found to be overexpressed in various cancers, including UBC (8, 9). However, the prognostic significance of altered EZH2 in UBC has not been unequivocally established.

The aim of this research was to investigate the immunohistochemical expression profile of EZH2 in early invasive pT1 urothelial bladder cancer of low and high histologic grade, and to analyze the correlation with clinicopathological factors, as well as to assess the possible significance of EZH2 expression for disease prognosis and patients' survival.

Material and methods

Patients and tissue samples

Present study comprised tissue samples of 279 patients with urothelial bladder cancer who underwent transurethral resection of bladder tumor in Clinic of urology, Clinical center Niš, Serbia (2007 - 2012). All cases were diagnosed at the Institute of Pathology, Clinical center Nis, Serbia. The average patients' age was 66.5±9.8 years, with the predominance of male patients compared to women (76% vs. 24%). Patients' clinical history and survival data including survival time, disease-free survival, and recurrence were available for all patients included in the research. The median follow-up was 60 months. Cancer specific death was defined as death caused by bladder cancer, excluding the mortality caused by other neoplasms and non-neoplastic disease (the majority of death during the follow-up period was caused by ischaemic heart disease).

Pathohistologic analysis

The diagnosis of urothelial bladder cancer, pathologic stage and histologic grade were assessed on formalin fixed paraffin embedded tissue samples, processed by standard techniques, and stained with hematoxylin and eosin. For the purposes of immunohistochemical analysis, tissue microarrays were constructed, using manual tissue arrayer. Each tumor was represented in the composite microarray block with two cores of 2mm diameter, carefully selected from the designated area of invasive portion of tumor. The pathohistologic analysis was performed using a light microscope (Olympus BX43, Olympus Corporation, Tokyo, Japan) by two independent pathologists.

Immunohistochemical analysis

Three micrometer thick sections of tissue microarray blocks were first deparaffinized through a series of xylene, and rehydrated in a series of alcohol. Antigen retrieval pretreatment was carried out in a citrate buffer (pH 6), by heating for 20 minutes in a microwave oven at 800W. After thorough washing in phosphate buffered saline (PBS, pH 7.4), endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide solution in methanol for 20 minutes. The slides were incubated with the primary antibody in a water bath at room temperature for 1 hour. The primary antibody used in the study was Rabbit monoclonal antibody to EZH2 (D2C9), Cell Signaling technology, Mariland, United States, at dilution 1:50. The detection of positive immunoreaction was achieved using DAKO EnVision kit (EnVision® + Dual Link System-HRP (DAB +), DakoCytomation), with one hour slide incubation at room temperature. Visualization of the antigen-antibody reaction was performed with chromogene diamino-benzidine-tetrahydrochloride (DAB), which marked the sites of reaction with brown color precipitate. Finally, the sections were rinsed with PBS, counterstained with Mayer's Hematoxylin, dehydrated and mounted. Negative controls were carried out by omitting the primary antibody.

Scoring of immunohistochemical staining. EZH2 nuclear staining was assessed using a semiquntitative combined intensity and percentage / area scoring method, described elsewhere (10). In brief, tumor score values were obtained as sum of products (intensity score x percentage of tumor area) for each tissue microarray spot. Based on the median score value, all tumors were dichotomized in groups with low or high Ezh2 immunohistochemical expression.

Statistical analysis

All data were analyzed using statistical software for data processing SPSS version 20.0. Continuous variables were presented as the mean ± standard deviation. The frequencies of categorical variables were tested by using $\chi 2$ test with Yates's correction. Univariate and multivariate analysis of clinicopathologic variables was performed using a Cox regression analysis. $P \leq 0.05$ values were considered statistically significant.

Results

EZH2 expression in urothelial bladder cancer displayed exclusively nuclear staining pattern (Figure 1). Cancer cell nuclei showed positivity to Ezh2 in a form of golden to dark brown pigmentation. Significant EZH2 stain was not observed in nonneoplastic urothelium of the adjacent normal mucosa, where only small fraction of the predominantly basal nuclei showed EZH2 expression with low to intermediate intensity. High EZH2 immunohistochemical expression was observed in 44.5% of the investigated early invasive UBC, where the majority of tumors with high EZH2 were high grade carcinomas (Graph 1.).

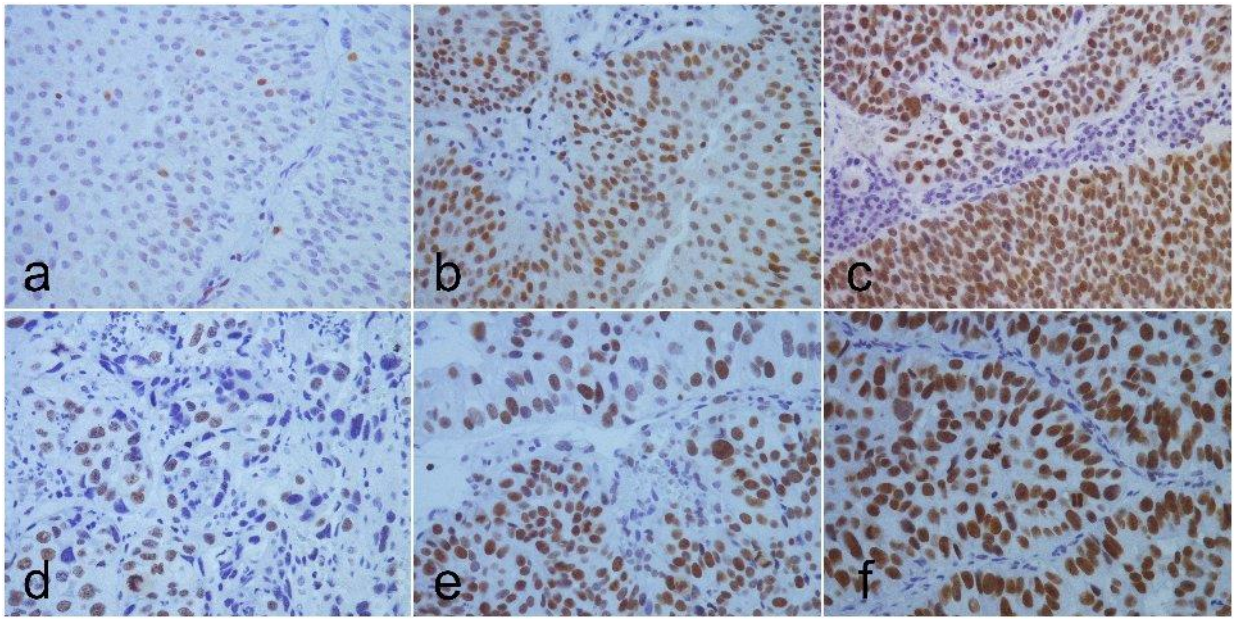
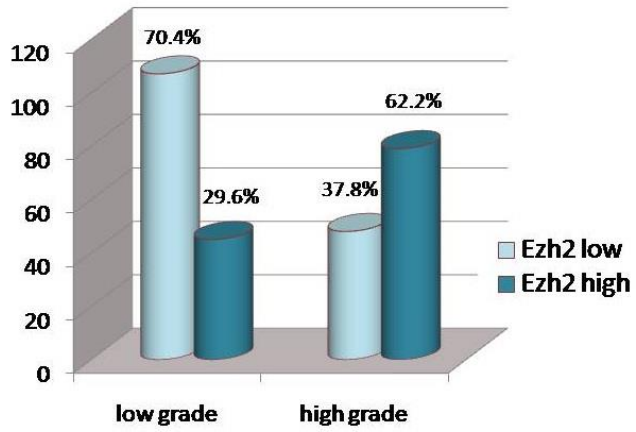


Figure 1. Immunohistochemical expression of EZH2 in low grade (a, b, c), and high grade pT1 urothelial bladder carcinoma (d, e, f); Low EZH2 expression with scattered brown stained tumor nuclei (a, d); High EZH2 expression with nuclear staining of intermediate to strong intensity in majority of tumor cells (b, e); High EZH2 expression with intense diffuse nuclear staining (c, f). Original magnification x400.



Graph 1. EZH2 immunohistochemical expression in early invasive urothelial bladder cancer of low grade (N = 152) and high grade pT1 tumors (N = 127).

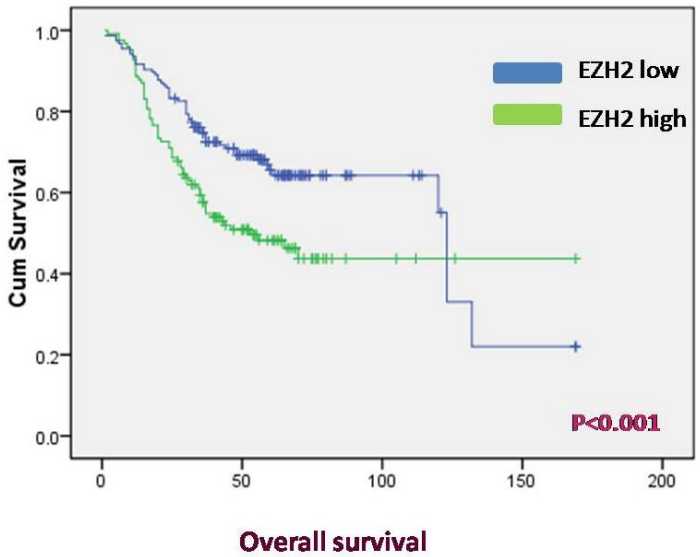
High histologic grade of the tumor correlated with nuclear EZH2 overexpression with high statistical significance ($p < 0.001$) (Table 1.). High EZH2 significantly associated to male gender, and the occurrence of carcinoma in situ in the urothelium surrounding the superficially invasive tumor ($p = 0.019$, and $p = 0.026$, respectively). Tumors with divergent differentiation (squamous, glandular, micropapillary, microcystic) demonstrated no significant variability in EZH2 staining.

High EZH2 expression strongly correlated with cancer specific death ($p = 0.010$). Almost 60% of the patients who died from direct consequences of disease progression and dissemination showed strong EZH2 positivity. Conversely, EZH2 was not associated with tumor recurrence.

Kaplan-Meier survival analysis showed that high EZH2 expression in early invasive UBC is strongly associated with worse survival of the patients and poor clinical outcome ($p < 0.001$) (Graph 2.). Impact of EZH2 expression to recurrence free survival was not statistically significant ($p > 0.05$).

Table 1. Association of EZH2 expression with clinicopathologic features of early invasive pT1 bladder cancer

Characteristics	Ezh2	Low	High	*p value*
Total (n (%))	279 (100)	155 (55.5)	124 (44.5)	
Gender				
Female	67 (24.0)	45 (67.2)	22 (32.8)	***0.019*** *
Male	212 (76.0)	110 (51.9)	102 (48.1)	
Tumor grade				
Low	152 (54.4)	107 (70.4)	45 (29.6)	***0.000****
High	127 (45.6)	48 (37.8)	79 (62.2)	
Carcinoma in situ				
Yes	19 (6.8)	6 (31.6)	13 (68.4)	***0.026****
No	260 (93.2)	149 (57.3)	111 (42.7)	
Divergent differentiation				
Negative	247 (88.5)	138 (55.9)	109 (44.1)	*0.456*
Positive	32 (11.5)	17 (53.1)	15 (46.9)	
Recurrence				
Yes	114 (40.9)	63 (55.3)	51 (44.7)	*0.516*
No	165 (59.1)	92 (55.8)	73 (44.2)	
Cancer specific death				
Yes	75 (26.9)	31 (41.3)	44 (58.7)	***0.010****
Other	42 (15.1)	23 (54.8)	19 (45.2)	
Live	162 (58.0)	101 (62.3)	61 (37.7)	



Graph 2. Kaplan Meier survival curves showing overall survival in 279 patients with pT1 urothelial bladder cancer with low and high Ezh2 expression.

In multivariate Cox regression analysis that tested the influence of clinicopathologic parameters on overall patients' survival, only histologic grade was statistically significant determinant ($p < 0.001$), while the predictive value of EZH2 expression was not established as statistically significant.

Discussion

In bladder cancer genomic landscape mutations in chromatin remodeling and histone modifying genes are frequent (5). EZH2, histone methyltransferase subunit of a Polycomb repressor complex 2, is a prominent target of oncogenetic alteration. Increased EZH2 activity leads to epigenetic silencing of numerous genes, including tumor suppressors, thus contributing to neoplastic phenotype (6, 7). E-cadherin gene (CDH1) is one of the critical EZH2 targets (11). Its downregulation is crucial for epithelial-mesenchymal transition and metastasis. This association of increased EZH2 and inhibition of E-cadherin expression has been confirmed in urothelial bladder

cancer (12). EZH2 exerts its function through canonical pathway of H3K27 methylation, however, emerging evidence indicate that EZH2 methylates non-histone substrates, and may also have functions in carcinogenesis that are methylase independent (13).

EZH2 plays a major role in oncogenesis of the bladder, and has been found to be associated with aggressive disease (8, 10, 14). Increased levels of both EZH2 mRNA and protein product were detected in cancerous tissue of transitional cell carcinoma compared to adjacent nonneoplastic bladder mucosa (8). Moreover, this increase was significantly higher in high grade lesions. Similarly, our study showed that in low grade early invasive bladder cancer high EZH2 expression is present in less than one third of the cases, while high grade tumors exhibited EZH2 overexpression in more than 60% of the cases. In addition, prevalent finding of EZH2 expression in high grade lesions was homogeneous and diffuse nuclear staining with strong intensity, while the majority of low grade superficially invasive tumors that were classified as high EZH2 expressors showed heterogeneous stain, with areas of low to intermediate nuclear signal intensity. EZH2 stain was more intense in invasive tumor areas of deepest infiltration to lamina propria, than in superficial, often papillary segments.

The study that analyzed EZH2 in a large cohort of cystectomy specimens indicated that EZH2 expression is more frequent in invasive carcinoma than noninvasive papillary lesions (15). In addition, the authors concluded that high EZH2 expression was most consistent with carcinoma in situ, implying the best diagnostic utility of EZH2 in diagnosis of flat urothelial carcinoma in situ. We found that the majority of analyzed cancers associated with the finding of carcinoma in situ in adjacent mucosa in transurethral resection specimens aligned in the group with high EZH2 expression, and this association was statistically significant. This may support the notion that deregulation of EZH2 expression is an early event in bladder carcinogenesis, thus invasive tumor shares the same alteration of epigenetic factor with in situ precursor lesion.

One of the hallmarks of non muscle invasive UBC is a relatively high recurrence rate, often associated with tumor progression (1, 2). About 40% of the patients included in present study developed one or more recurrent bladder tumor during a follow-up period. However, EZH2 expression was not associated with recurrence of the disease, and provided no significant prognostic information in analysis of recurrence-free survival. Recent study described a model for high grade superficial urothelial carcinoma, where Rb-E2F-Ezh2 axis disruption provided a genetic base for tumor development (9). This study verified significant increase of EZH2 expression in tumor samples showing progression in recurrence. Moreover, increased EZH2 activity that mediates the increased progression risk of non muscle invasive UBC can be precluded by increased PIK3CA expression that acts as functional opponent to EZH2 (16). This implies that high EZH2 overexpression may not indicate relapse of the superficially invasive tumor, but its finding indicates more aggressive phenotype of the recurrent neoplasm.

Among all forms of UBC, early invasive pT1 bladder cancer, especially high grade non-muscle invasive carcinoma, is the most significant problem for clinical treatment. Therefore, numerous approaches to better stratify patients that would benefit from early radical cystectomy have been made (3, 4, 17).

The use of immunohistochemical biomarkers is widely available and reliable method for analysis and classification of bladder cancer to prognostic groups. The prognostic significance of EZH2 in non muscle invasive bladder cancer yielded discrepant findings (15, 18). Our results indicate that high EZH2 expression in pT1 UBC is a strong predictor of shorter overall survival and is significantly associated to cancer specific death. Although multivariate analysis of a significant model failed to establish EZH2 expression as the independent predictor of poor patients' outcome, further studies are warranted to clarify the prognostic potential of EZH2 expression in urothelial neoplasms of the bladder.

During the recent years the inhibition of EZH2 expression has been recognized as an exciting novel therapeutic approach in cancer treatment (19 – 21). Inhibitors developed against EZH2 achieve their goal through direct or indirect mechanisms. Anticancer therapies targeting EZH2, now well established oncogene in many malignancies, have already imparted promising results in clinical trials in treatment of solid tumors and lymphoma. However, studies of EZH2 inhibitors in bladder cancer treatment are scarce, and, in spite of the profound understanding of EZH2 roles in bladder carcinogenesis, it will require a lot of work and solid evidence of efficacy to fully establish the clinical settings of using these therapeutics in management of urothelial bladder cancer.

Conclusion

Urothelial bladder cancer is one of the most common cancers worldwide and a major healthcare issue due to expensive process of diagnostics and clinical management. Early invasive bladder cancer is challenging to diagnose and very difficult to define in terms of behavior and evolution prediction. High EZH2 expression is found in one third of low grade early invasive UBC, while in high grade pT1 lesions it is twice as often. High EZH2 expression in early invasive urothelial bladder cancer indicates aggressive behavior of the tumor and poor prognosis. EZH2 has promising roles as a complementary diagnostic tool in selection of the patients that require closer clinical attention and second transurethral resection, and as a potential target for anticancer therapy.

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EKSPRESIJA EPIGENETSKOG PRIGUŠIVAČA EZH2 U RANO INVAZIVNOM PT1 UROTELNOM KARCINOMU MOKRAĆNE BEŠIKE

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Urotelni karcinom mokraćne bešike je najčešća maligna neoplazma urinarnog trakta. Rano invazivni karcinom bešike (stadijum pT1) je tumor u kojem je prisutna invazija subepitelnog vezivnog tkiva, bez zahvatanja mišićnog sloja. Predstavlja veliki izazov za dijagnozu i lečenje. EZH2 transkripcioni represor igra ključnu ulogu u onkogenezi mokraćne bešike. Cilj ovog istraživanja bio je da ispita profil ekspresije EZH2 u pT1 karcinomu bešike, analizira korelaciju ekspresije EZH2 sa kliničko-patološkim faktorima i da proceni njen prognostički značaj. U tom cilju je imunohistohemijski analizirano 279 tumora ukalupljenih u tkivne mikroareje na ekspresiju EZH2. Visoka jedarna ekspresija EZH2 zabeležena je u 44,5% tumora. Visoka EZH2 ekspresija bila je udružena sa visokim histološkim gradusom ($p < 0,001$). Pored toga, EZH2 značajno je korelirao sa muškim polom i pojavom karcinoma in situ u okolnom urotelu ($p = 0,019$ i $p = 0,026$), dok divergentna diferencijacija tumora i pojava recidiva bolesti nisu pokazale značajnu udruženost sa ekspresijom EZH2. Visoka EZH2 ekspresija bila je snažno povezana sa specifičnim mortalitetom uzrokovanim karcinomom ($p = 0,010$). Kaplan-Majer analiza preživljavanja pokazala je da visoka ekspresija EZH2 predviđa lošije preživljavanje obolelih ($p < 0,001$). Uticaj EZH2 na vreme bez razvoja relapsa tumora nije se pokazao statistički značajnim. Visoka ekspresija EZH2 u rano invazivnom karcinomu mokraćne bešike ukazuje na agresivno ponašanje tumora i lošiju prognozu. EZH2 ima potencijalno značajne uloge kao komplementarno sredstvo u dijagnostici za selekciju bolesnika koji zahtevaju intenzivniju pažnju kliničara i kao potencijalna meta antikancerogene terapije.

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Ključne reči: *urotelni karcinom bešike, EZH2, imunohistohemija, prognoza*

Case report

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PANCREATIC METASTASIS FROM RENAL CELL CARCINOMA: A CASE REPORT

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Metastatic pancreatic cancer is rare and makes 2% to 5% of all malignant tumors of this gland. Predominantly it is a metastatic renal cell carcinoma (RCC - renal cell carcinoma), which shows expressed affinity to the pancreas, which is often the only place of its expansion, typically several years to several decades after nephrectomy. The average time of detection of metastases is 7 years (described is the case after 32.7 years). As multifocal lesions they occur in 20-45% of cases when their treatment will depend on the resectability of changes, which is possible in about 60% of patients. We present a case of 69-year-old female patient, in whom solitary changes in the body of the pancreas were detected 3.5 years after nephrectomy for RCC. At the time of examination, the patient had no symptoms, a change was detected by control computed tomography (CT) of the abdomen. After distal splenopancreatectomy metastasis kidney cancer was confirmed. Extensive and regular follow up of the patient after nephrectomy for RCC was necessary and imposed by the unpredictable nature of this tumor. Despite the existing radio-biological therapy, surgery occupies an important place in the treatment of these metastases, the radical surgical approach in the case of resectable metastases offers the chance for years of survival.

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Key words: *pancreatic metastasis, renal carcinoma, pancreatic resection*

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Introduction

Metastatic pancreatic cancer is rare and makes 2% to 5% of all malignity tumors of this gland (1-19). The most common pancreatic metastases are RCC, lung carcinoma, lobular carcinoma of the breast, adenocarcinomas of the colon, melanoma, leiomyosarcoma (2-7, 13-19). However, pancreas is from renal cell carcinoma elective and frequently only site for metastases, a few years to several decades after nephrectomy (20-22, 26). Expressed affinity of malignancy cells to pancreatic parenchyma is probably effect of the character of the tumor, while the dissemination mechanism is not yet fully understood (23).

Case report

We report a case of 69-year-old female patient, who had 3.5 years previously undergone a radical nephrectomy for RCC, proven histopathology, the presence of typical well-differentiated clear cells without regional lymphadenopathy or distant metastases. Regular follow-up of patient for 3 years does not indicate a recurrence of the disease. After 3.5 years of nephrectomy abdominal ultrasonography, then computed tomography (CT), revealed hypervascular tumor in the body of the pancreas (Figure 1).

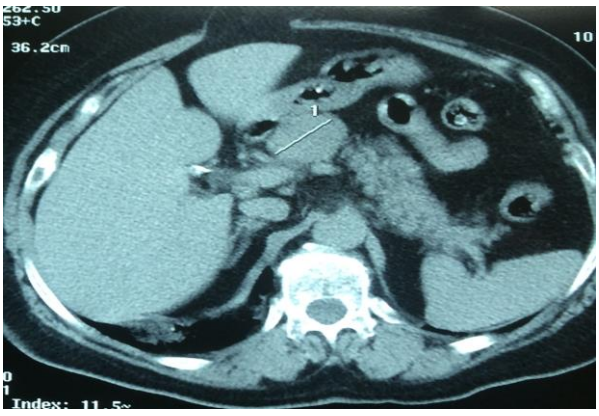

Figure 1. Computerized tomography of the abdomen with marked metastatic tumor of the pancreas

At the time of medical examination the patient had no symptoms, laboratory findings were with normal limits, CA 19-9 were regular. Distal pancreatectomy with splenectomy was performed (Figure 2). Histopathology confirmed metastatic RCC (Figure 3).

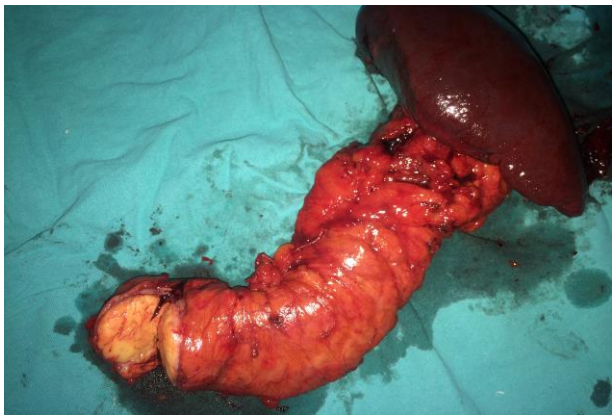

Figure 2. Metastatic tumor, macroscopically, after distal spleno-pancreatectomy

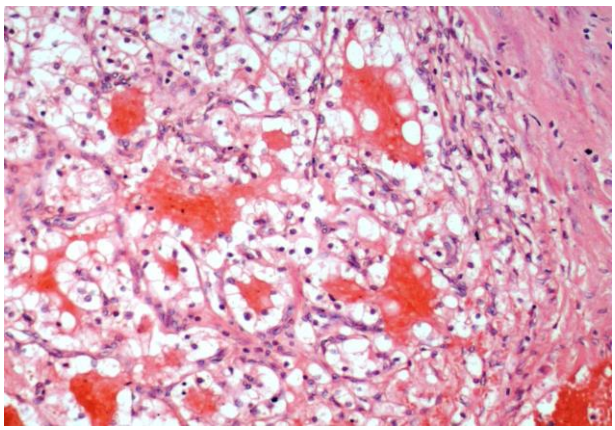

Figure 3. Histology of a renal carcinoma metastasis: clear cell type

The postoperative course was normal; the patient was discharged on 9th postoperative day. Twelve months after the second surgery, the patient was well, with no signs of disease recurrence.

Discussion

The pancreas represents elective place metastasis of RCC, which described numerous studies (1-11). It typically occurs several years after nephrectomy in the seventh decade of life. The average time of detection of metastases is 7 years, but the case is described after 32.7 years (3, 4, 9, 11), usually during a routine examination of asymptomatic patients, as is the case in 50% of patients (5, 6, 12, 13). In other cases the symptoms are described as pain, weight loss, gastrointestinal bleeding, icterus (5, 6, 12-14).

The mechanism of spread of the disease and the connection between RCC and pancreatic cancer are still not fully understood, and the affinity of metastatic RCC to the parenchyma of pancreas explains the nature of the tumor, but not their anatomical proximity. The usual routes of malignant processes cannot explain certain described cases of metachronous metastases on previously resected pancreas because of the same disease, or secondary multifocal lesions in the pancreas, along with their absence in other organs (3, 15).

Multifocal lesions of pancreatic metastasis have been reported to be in the range 20% to 45% which should be considered when selecting therapy (4, 15, 16-18).

The diagnosis usually relies on computed tomography (CT) or magnetic resonance (MR), when it is difficult to make the distinction of metastases from primary carcinoma of the pancreas. The differential diagnosis of metastases should be differentiated from primary pancreatic neoplastic process, focal infiltration of lymphoma and pancreatitis. On ultrasound the metastases were present as hypoechoic, whereas CT presentations usually showed appropriately limited hypervascularized lesions without infiltration of the retropancreatic space and adipose tissue which is commonly seen with primary pancreatic cancer and lymphoma.

In addition to those listed, PET scan and echo endoscopy may also be considered useful procedures. In nephrectomized patients the diagnosis is easily established due to RCC. In some unclear situations percutaneous biopsy of lesion controled by CT may be perfomed (3, 13, 19, 20).

Resection of the pancreas (a cephalic duodenopancreatectomy sec. Whipple or distal pancreatectomy), if possible, is the most effective treatment of solitary metastatic of RCC. Total pancreatectomy with the multiple lesions is indicated by some authors (21-23). Radical lymphadenectomy is unnecessary (11). A number of studies (with 5 or more patients) indicate good results of surgical treatment: the 5-year survival was present, depending on the series, in 30% to over 80% of patients (2-6, 15, 17, 24-34). Reddy et al. (2008) reported that metastatic size greater than 4 cm and perineural infiltration were related with a significantly lower percentage of survival (25), while Motzer et al. (2004) reported that the factors of poor prognosis after resection therapy was interval between nephrectomy and metastases < 12 months, previous radiotherapy, ≥ 2 metastases (35).

In the treatment of primary RCC and its metastases, chemotherapy has proved to be ineffective. There are a number of proposed protocols that include biological (molecular) therapy such as sunitinib, sorafenib, bevacizumab, everolimus, temsirolimus. Highly toxic interferon alpha and interleukin-2 is used as second-line therapy (36-39).

Radiation therapy is an alternative treatment of inoperable lesions, as well as in high-risk patients, alone or in combination with any of the above-mentioned biological therapy or surgery. In the literature there is only one study which publishes the results of

RT applied in 4 patients with multiple metastatic RCC in the pancreas.

Iinterferon alpha was administered combined with RT, where the stabilization of the disease was achieved in 3 patients (average 34 months), while in one patient partial response was achieved to the combined therapy (40).

Some authors suggest the possibility of treatment of multiple metastatic RCC in pancreas with radiofrequency ablation (RFA). Associated with a high percentage of significant complications such as necrotizing pancreatitis, bleeding, perforation of the duodenum, this type of therapy is reserved for individual cases in highly specialized centers (41-43).

Conclusion

Extensive and regular follow up of the patients after nephrectomy for RCC is necessary and imposed by the unpredictable nature of this tumor for early detection of pancreatic metastases, often in asymptomatic patients.

The radical surgical approach in case of resectable changes offers the chance for years of survival. The best results are achieved in patients with good general condition, with long disease-free period after nephrectomy, when pancreatic resection is performed in high-volume centers due to solitary metastasis up to 4 cm.

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PANKREASNE METASTAZE RENALNOG KARCINOMA: PRIKAZ SLUČAJA

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Metastatski tumor pankreasa je redak i čini 2% do 5% svih malignih tumora ove žlezde. Predominantno se radi o metastazama renalnog karcinoma (RCC - renal cell carcinoma), koje pokazuju izraženi afinitet prema tkivu pankreasa, koji je često jedino mesto njegovog širenja, tipično nekoliko godina, do nekoliko decenija nakon nefrektomije. Prosečno vreme detekcije metastaza je period od sedam godina (opisan je i slučaj nakon 32,7 god). Kao multifokalne se javljaju u 20-45% slučajeva, kada će njihov tretman zavisiti od resektabilnosti promena, što je moguće kod 60% bolesnika. U ovom radu opisan je slučaj 69-godišnje bolesnice, kod koje je detektovana solitarna promena u telu pankreasa, 3,5 godine nakon nefrektomije zbog RCC. U momentu pregleda, bolesnica je bez ikakvih simptoma, a promena je otkrivena kontrolnom kompjuterizovanom tomografijom (CT) abdomena. Nakon distalne splenopankreatektomije, potvrđeno je da se radi o metastastazi karcinoma bubrega. Dugogodišnje i redovno praćenje bolesnika nakon nefrektomije zbog RCC je obavezno i nametnuto nepredvidljivom prirodom ovog tumora. Uprkos postojećoj radio-biološkoj terapiji, hirurgija zauzima važno mesto u lečenju ovih metastaza, gde radikalni hirurški pristup u slučaju resektabilnih promena nudi šansu za višegodišnje preživljavanje.

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Ključne reči: *pankreasne metastaze, renalni karcinom, resekcija pankreasa*

Review article

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SIGNIFICANCE OF MEDICAL LAW IN MEDICAL SCHOOL CURRICULUM

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Incorporating the medical law as a part of medical school curriculum will enable medical students to be fitted and aspired for a lifetime of good practice and learning not just medical skills although improving medical care system in general. Due to acquired knowledge student will be aware and will understand legal, ethical and professional responsibilities that medical care system requires of them.

Acquired knowledge will enable students to develop critical thinking about ethical, legal and professional issues. In that matter the students will be able to respond delicate situations that they will be confronted with. It will make influence in minimizing or eliminated some legal or ethical uncertainty in order to help them to preserve their professional integrity. Also acquired knowledge will enable future doctors to make decisions that are ethically, legally and professionally justifiable, they will be able to respond appropriately to any challenges in medical practice also novelties and results in science and society changes. Thus the knowledge, behavior, skills and attitudes will be integrated into medical practice and medical care system.

It has been said a doctor who knows nothing of law, and a lawyer who knows nothing of medicine, is deficient in essential requisites of their respective, professions.

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Key words: *Medical law, Medical school curriculum*

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Introduction

Medical law is a branch of law which will ensure the quality and rights of all participants in medical service system. Medical law covers an area of regulations relating to the medical operations, the industry operators, the procedures involved in the medical operations, characteristics of medical experts performing medical operations, as well as the relations that occurred while performing medical activities. The medical law also covers regulative of drugs and other medical devices (1, 2). The studying of medical law and bioethics are recognized as claimed for the medical professionals in every day clinical practice.

Studying the basis of medical law by medical students allows them to be aware of basic elements of medical law, establishing the basis for the future good medical practice, developing the adequate relationship with patients, exploring the essential needs of patients and medical care system, develops awareness and understanding of ethical, legal and professional responsibilities required of them as students and future doctors, to reflect adequately resolving ethical, legal or professional issue (1, 2). Adopting the knowledge received during studies future doctors will be able to aim high standards of medical practice, insuring that their professional engagement is not only practicing medical techniques but also providing the patients the full care involving respect of their human rights, also following guidelines of good medical practice.

The standards in medical school curriculums must go beyond traditional basic science and clinical subjects. Developing medicine, medical practice and medical law beside them created the need to translate physicians' technical skills in medicine into effective research and medical care for patients (3).

Core Content of Medical law

The Core Content of Medical law must be determinate as framework of teaching medical law. Students must adopt the basis of medical law which will improve their not just medical skills to become good doctors. Recognizing the foundations of medical law medical students will adopt and apply understanding the framework of good medical prac tice and duties that are required. Medical students, as

future doctors, will explore and underscore the key importance of good medical practice, health benefits for individual patients and health care system, minimizing malpractice and respecting the patients' autonomy and will, resolving the legal issue and improves reflective and critical thinking of the core content issue (4).

Foundations of Medical law

Gaining knowledge, skills, behaviors, and aptitudes during medical courses, medical students will develop appropriate attitude to essential ethical and legal issues, methods of ethical reasoning that informed decisions in everyday practice, to establish the framework of medical practice according legal principles and be aware of their duties (2-4).

Mastering these basic elements of medical law will enable medical students to adopt principles that will be incorporated in their practice. Thus, students will master basics of good medical practice, to understand and be aware of their role in medical care system, duties and responsibilities, relations to the patients and patients' health, the importance of confidentiality, good communication, respect of patients' autonomy, honesty, right of patients to be informed of every procedure and its consequences, patients' right to choose if they want to be treated or not to, to provide second opinion and adhere legal responsibilities to protect patients' rights.

Medical law education is often based on the rules, but the recommendation are that medical law focuses also on fundamental of legal reasoning, legal skills and behaviors (4).

As future doctors, students should be able to develop appropriate relationship between everyone included in medical care system and will be able to respond efficiently to any legal issue, errors made during procedures. It is important for students to be able to make difference between moral, legal and human right and how these rights can involve their practice (4).

Aims of teaching medical law

Incorporating the Medical law as a part of Medical school curriculum will enable medical students to be fitted and aspired for a lifetime of good practice and learning not just medical skills although improving medical care system in general. Due to acquired knowledge a student will be aware and will understand legal, ethical and professional responsibilities that medical care system requires of them.

Understanding some core rules, principles and concepts is important part of medical training (4).

Some Medical schools in their Medical law curriculum focus on future physicians as expert witnesses, other Medical schools lay focus on avoiding lawsuits as defensive medical technique. These two approaches are physicians centered. Medical schools focused on patients centered courses teach medical law more as analyses of ethical questions faced to the physicians, the way to work through boundaries and questions are set by law. These approaches are not exclusive one to another and many Medical schools embody them in one unique approach (5).

Acquired knowledge will enable students to develop critical thinking about ethical, legal and professional issues. In that matter the students will be able to respond to delicate situations that they will be confronted with. It will make influence in minimizing or eliminated some legal or ethical uncertainty in order to help them to preserve their professional integrity. Also the acquired knowledge will enable future doctors to make decisions that are ethically, legally and professionally justifiable, they will be able to respond appropriately to any challenges in medical practice also novelties and results in science and society changes. Thus the knowledge, behavior, skills and attitudes will be integrated into medical practice and medical care system (5).

Medical schools in order to shape their coursework of Medical law must get answers of the questions:

◇ What should medical students learn about the law?
◇ When should they learn about the law?
◇ How should they learn about the law?
◇ From whom should they learn about the law?

◇ *The What*

Medical schools generally teach medical jurisprudence (impact of law to the medicine) and medical forensics (impact of medicine to the law).

◇ *The When*

The standing point of most authors in reviewed literature is that Medical law should be taught during pre-clinical and clinical years, horizontally (across one year) and vertically (through all years of studies).

◇ *The How*

Most Medical schools clumps law and ethics, some of them shaped their courses as a mixture of lecture and group discussions. There are some initiatives to provide trainings among medical and law students as kind of interdisciplinary practice.

◇ *The Who*

The lawyers should be included into Medical law teaching courses or to be a part of team teaching model (that is, adds a J.D. to an M.D. or Ph.D.).

Most of Medical schools involve lawyers into Medical law teaching courses, with faculty appointments ranging from full-time to adjunct, many of them are in legal practice. These kinds of appointments are characteristic to legal systems that allow this kind of arrangements to lawyers.

It is important that physicians encounter law be positive and productive not only for physician's positions, but for the welfare of patients whom medical profession exists to serve, as it is mentioned in paper by Campbell, 2012. (4).

Medical school curriculum and clinical implications

Teaching medical law and integrating medical law into medical schools curriculum enables medical students to acquire knowledge of basic principles of medical care system.

As a part of medical care system every future doctor must be aware of basic priciples of medical law, that are regulated by Act of Health care ("Gazette of RS", no. 107/2005, 72/2009 - other Act, 88/2010, 99/2010, 57/2011, 119/2012, 45/2013 – other Act 93/2014, 96/2015 and 106/2015) (6).

Health care regulated by Act of Health care ("Gazette of RS", no. 107/2005, 72/2009 - other Act, 88/2010, 99/2010, 57/2011, 119/2012, 45/2013 – other Act 93/2014, 96/2015 and 106/2015) is organized and comprehensive activity of the society with the aim to achieve the highest possible level of preservation of health of citizens and families. Health care includes the implementation of measures for the preservation and improvement of citizens' health, prevention, suppression and early detection of diseases, injuries and other health disorders and timely and efficient treatment and rehabilitation (7).

A student must acquire knowledge of basic principles of health care system. These principles are:

▪ The principle of access to health care

The principle of access to health care is delivered by providing adequate health care to citizens of the Republic of Serbia, which is physically, geographically and economically accessible, and culturally acceptable, especially health care at the primary level (8).

▪ The principle of equity of health care

The principle of equity of health care achieved by prohibiting discrimination providing health care on the basis of race, sex, age, national origin, social background, religion, political or other opinion, property status, culture, language, type of illness, mental or physical disability (9).

▪ The principle of comprehensive health care

The principle comprehensive health care is achieved by the inclusion of all citizens into health care system by implementing integrated measures and procedures of health care that include health promotion, disease prevention at all levels, early diagnosis, treatment and rehabilitation (10).

▪ The principle of continuity of health care

The principle of continuity of health care is achieved by organization of health care as a whole that has to be functionally linked and coordinated on every levels of health care system, from primary through secondary to tertiary level of health care and that provides continuous health care to the citizens at any age (11).

▪ The principle of continuous improvement of the quality of health care

The principle of continuous improvement of the quality of health care is achieved through the measures and activities that are in line with the modern achievements in medical science and medical practice, that increases the possibility of a favorable outcome and reduces risks and other adverse effects on health and the health status of individuals and the community as a whole (12).

▪ The principle of efficiency of health care

The principle of efficiency of health care presents achieving the best possible results related to available financial resources and achieving the highest level of health protection with the lowest consumption of resources (13).

Besides knowing basic health care principles, medical students must understand that providing health care has been accomplished with respect of the highest possible standards of human rights and values, and the right to physical and psychological integrity and security of personality, as well as the appreciation of patients' moral, cultural, religious and philosophical beliefs (14).

International law principles are integral part of Serbian legal system. Republic of Serbia has ratified European Convention on Human Rights and Fundamental Freedoms 1950. Ratification of Convention obliged Republic of Serbia to implement the values of the Convention in Serbian legal system, beginning from Constitution as the highest legal Act to lower legal Acts (15).

There are several Articles at Convention that are relevant to medical law, such as Article 2 (right to life), Article 3 (right not to be subject to inhuman and degrading treatment and torture), Article 5 (right to liberty and security), Article 8 (right to privacy and family life) and Article 12 (right to marry and found a family). Many of these rights are not absolute and are subject to exceptions. Article 8, for example, envisages exceptions to the right to privacy (including confidentiality) in the interests of national security, public safety or the country's economic well-being, for the prevention of disorder or crime, the protection of health or morals, and the protection of the rights and freedoms of others (16) (Table 1).

Global health law has been defined as the legal norms, processes, and institutions that are designed primarily to attain the highest possible standard of physical and mental health for the world's population (17). Also there are limitations of global health that must be considered, as it is specified in Table 2.

Table 1. Significance of Medical law in Medical school's curriculum

Core Content of Medical law	**D**eterminates as framework of teaching medical law. **S**tudents adopting the basis of medical law which will improve their not just medical skills to become good doctors.
Foundations of Medical law	**G**aining knowledge, skills, behaviors, and aptitudes during medical courses, medical students will develop appropriate attitude to essential ethical and legal issues, methods of ethical reasoning that informed decisions in everyday practice, to establish the framework of medical practice according legal principles and be aware of their duties.
Aims of teaching medical law	It is important that physicians encounter law be positive and productive not only for physicians positions, but for the welfare of patients whom medical profession exists to serve.
Medical school curriculum and clinical implications	**E**nables medical students to acquire knowledge of basic principles of medical care system.

Table 2. Limitations of Global Health Law (modified by Gostin and Sridhar, 2014)

Limitation	Description
National sovereignty	Countries are reluctant to forgo self-governance or cede authority to international actors.
Rise of nongovernmental actors	Businesses, foundations, and civil-society groups have major effects on health but are hard to govern at the international level.
Divergent interests of emerging economies and high-income countries	High-income countries defend trade liberalization (e.g., intellectual property), whereas low- and middle-income countries focus on health justice (e.g., access to medicines and fair allocation of scientific benefits).
Funding earmarked by private donors for specific sectors, diseases, or regions through multilateral agencies ("multibi" financing)	Countries route assistance through the WHO and other multilateral agencies but hold tight control over its use, limiting WHO control of its resources and ability to set priorities and diminishing the perceived independence of the WHO
Funding for capacity building	Global health law rarely requires high-income countries to build capacities in lower-income countries to fulfill international obligations.
Compliance and incentives	WHO norms (whether soft or hard) rarely contain effective methods for holding countries and stakeholders accountable
Adjudication and enforcement of norms	The WHO lacks power to adjudicate most disputes and enforce norms.

Conclusion

Integration of medical law into Medical school curriculum will contribute to the knowledge of basic principles of health care system by undergraduate students. Acquiring knowledge of these principles will lay the foundation of good medical practice. Adopting the essential values, students, as future doctors, will provide health care with the best possible standards of human rights, providing the best possible medical treatments to all citizens. Finally, it could be cited, a doctor who knows nothing of law, and a lawyer who knows nothing of medicine, is deficient in essential requisites of their respective, professions (18).

In modern law, medical paternalism no longer rules' as it was summed previously (3-5). Modern medical law requires physicians seeking consent to provide sufficient information to enable patients to agree to proposed treatment with appropriate know-

ledge of risks, adverse effects and possible alter-natives. Central to this is the importance of good communication, as it was specified in the paper by Harpwood, 2016 (16).

Future doctors during their medical studies, trainings and residency, develop anti-patient bias. The importance of teaching medical law is to bring up to medical students the foundations of medical law, procedures, regulations, doctors and patients' rights, and, in that way, develop the relationship between doctors and their patients. Thus, it will contribute to develop the patients-centered system.

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ZNAČAJ IZUČAVANJA PREDMETA MEDICINSKO PRAVO NA MEDICINSKIM FAKULTETIMA

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Rad se bavi pitanjem značaja uvođenja predmeta Medicinsko pravo u programe medicinskih fakulteta. Izučavanje medicinskog prava omogućilo bi studentima medicine osposobljenost da prepoznaju pravne, etičke i profesionalne obaveze koje zdravstveni sistem zahteva, što doprinosi unapređenju dobre medicinske prakse i celokupnog sistema zdravstvene zaštite.

Sticanje znanja iz oblasti Medicinskog prava omogućilo bi studentima da razviju kritičan odnos prema etičkim, pravnim i profesionalnim izazovima, i rešavanju delikatnih situacija u praksi. Ovako stečena znanja svela bi na minimum ili u potpunosti eliminisala pravne ili etičke nedoumice, a sve u cilju očuvanja profesionalnog integriteta lekarske profesije. Odluke budućih lekara bile bi u skladu sa etičkim, pravnim i profesionalnim standardima, što bi im omogućilo da na adekvatan način odgovore izazovima u praksi. Stečena znanja, veštine, primeren odnos prema pravima i obavezama postali bi deo medicinske prakse i zdravstvenog sistema.

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Ključne reči: *Medicinsko pravo, program medicinskih fakulteta*

Review article

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NITRATES AND NITRITES SIGNIFICANCE IN THE DEVELOPMENT OF ALCOHOLIC LIVER DISEASE

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Alcoholism is one of the most common addictions affecting health and the immune system in people worldwide. Chronic alcohol consumption over a prolonged period of time causes metabolic liver injury, along with arginine metabolism and nitric oxide (NO) synthase disorders. Ethanol intoxication under cumulative nitrooxidative and nitrosative stress conditions, as well as in inflammation, stimulates the production of NO anion (NO^-) and superoxide anion (O_2-), i.e. peroxynitrite formation in hepatocytes and endothelium. Mitochondrial dysfunction and disorders of adenosine triphosphate (ATP) molecules synthesis in hepatocytes cause disorders of intra - and extracellular antioxidants synthesis (glutathione and superoxide dismutase) and neutralization of toxic nitrates and nitrites. Peroxynitrites damage cell membranes lipoproteins, as well as the membrane enzyme systems and the mitochondrial matrix. They also damage the enzymes of ethanol and arginine metabolism in cytosol, and nucleic acid repair enzymes in hepatocytes. In the development of alcoholic liver disease (ALD), peroxynitrites cause reversible injuries of the structure and function of hepatocytes that proceed irreversibly, and vascular sinus endothelial damage, mediated by the mechanisms of apoptosis and necrosis.

Considering the fact that 3.3 million people die of ALD and its complications annually, the measures should be taken and aimed at reducing the onset, development, and progression of ALD. The priority is timely ALD diagnosis, as well as the severity of alcoholic liver damage. The studies have shown that the values of peroxynitrite elevation correlate with the severity of liver injury. It can be concluded that timely determination of peroxynitrite values followed by suitable antioxidant therapies may slow down the processes of hepatocyte apoptosis and necrosis, as well as the course of ALD.

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Key words: *alcohol, nitrates, nitrites, liver damage*

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Introduction

Alcoholic liver disease is the third most common cause of death, after cardiovascular diseases and malignancies. According to the World Health Organization epidemiological data, the incidence of alcoholics in general population is about 5%, and in adult males it is about 10%. Excessive alcohol consumption over a longer period of time results in chronic liver damage.

Chronic alcohol consumption leads to liver damage. Alcohol-related liver diseases include alcoholic fatty liver disease, alcoholic hepatitis, and alcoholic cirrhosis. Alcoholic fatty liver disease and alcoholic hepatitis are reversible, but alcoholic cirrhosis is a non-reversible disease. Hepatocellular carcinoma is a severe complication of late-stage chronic liver disease. The severity of liver damage depends on the amount, concentration and duration of alcohol consumption (1). Alcohol toxicity is related to alcohol concentration. Weekly alcohol limit is cut to 14 units for women and men are advised not to take more than 21 units per week. One unit of alcohol is equal to a small glass of strong spirits, one glass of wine, or a half-pint glass of beer. Drinking more alcohol than the recommended safe limits for at least five years most certainly results in alcoholic liver disease. Liver injuries depend on factors that include genetic predisposition, nutritional status, gender, ethnicity, and social status. According to the World Health Organization data, 3.3 million of deaths resulted from harmful use of alcohol every year (2,3).

Alcohol primarily shows hepatotoxic effects. Acetaldehyde and reactive species play an important role in alcohol hepatotoxicity by the following biological mechanisms: the process of lipid peroxidation, protein covalent binding, activation of immunological mechanism, endotoxemia effects (4).

In oxidative stress, endotoxins released by gram-negative bacteria activate macrophages of the liver and blood. Macrophages inflammatory cytokines induce the expression of inducible nitric oxide synthase (iNOS). The peroxynitrite anion and hydroxyl radicals modify proteins, lipids, carbohydrates, and nucleic acid in cells by the process of nitrooxidative and nitrosative stress. Modification of these biomolecules results in their structural and functional changes, contributing to tissue and vascular endothelial injury. Irreversible hepatocyte damage and endothelial dysfunction cause hemodynamic disorders and the development of portal hypertension (5).

The mechanisms underlying the toxic effects of alcohol have not been fully understood yet. However, data collected from experimental and clinical studies show toxic effects of ethanol and its metabolites, as well as nitrates and nitrites toxicity, reflected by cumulative oxidative stress and antioxidant defense system exhaustion (6, 7).

Alcohol and oxidative stress in hepatocytes

Alcohol (ethanol) metabolism occurs in the liver by three enzymatic pathways: alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS) and catalysis (Figure 1).

The primary metabolic pathway of oxidative metabolism of ethanol is in cytosol by the cytosolic enzyme ADH. As the ethanol is oxidized, acetaldehyde is produced. The coenzyme nicotinamide adenine dinucleotide (NAD) is reduced and becomes nicotinamide-adenine-dinucleotide-hydrogen(NADH). Acetaldehyde enters the mitochondria where it is oxidized to acetate by acetaldehyde dehydrogenase (ALDH). The acetate is then involved in the Krebs cycle and broken down into water, carbon dioxide and acetyl CoA. Chronic alcohol consumption enhanced acetaldehyde production, as well as accumulation of reduced NAD (NADH) in the cytosol and mitochondria (8, 9).

Continuous alcohol intake induces microsomal ethanol oxidation in microsomes by the activity of the MEOS. In ethanol intoxication, the role of the MEOS in ethanol metabolism is much greater. The microsomal ethanol oxidizing system involves nicotinamide-adenine-dinucleotide-hydrogen-phosphate (NADPH) oxidase, cytochrome oxidase, and cytochrome P450-dependent enzyme. The ethanol inducible cytochrome P-4502E1 isoenzyme is responsible for ethanol metabolism. In ethanol intoxication, MEOS takes a role in ethanol metabolism. Stimulated activity of cytochrome P-4502E1 is a major pathway of oxidative stress in hepatocytes, producing great amounts of free radicals. Free radicals produced by microsomal NADH oxidase also include: hydroxyl radicals, hydroxyl ethyl radicals, superoxide radicals, and hydrogen peroxides (10). Significant amounts of superoxide radicals and hydrogen peroxide are produced in further metabolism of acetaldehyde by the activity of xanthine oxidase that is stimulated by reduced NAD and elevated NADH concentrations during the metabolism of ethanol into acetaldehyde. Acetaldehyde is highly reactive, it binds to cellular membrane lipoproteins, resulting in alteration of membrane fluidity. It causes structural and functional impairment of lipoproteins, as well as enzyme inactivation. Xanthine oxidase causes activation of carcinogenic substances (11).

The third pathway of ethanol oxidation occurs in peroxisomes by the activity of catalase and the presence of hydrogen peroxide. Oxidation of ethanol also produces acetaldehyde.

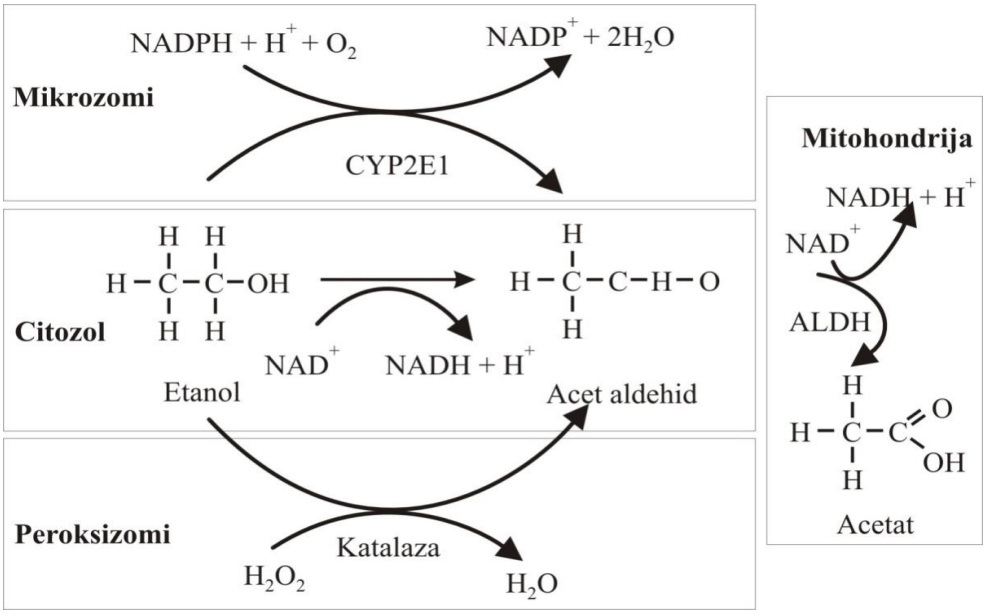

Figure 1. Ethanol metabolism

Ethanol metabolism as a source of reactive species

Ethanol metabolism is a significant source of reactive species generation. Reactive species are produced by ethanol and acetaldehyde metabolism during the following processes: microsomal induction, purine metabolism, stimulation of iron mobilization, increased concentrations of reduced NAD, and during the processes of neutrophils and macrophages stimulation. Reactive species are produced during ethanol metabolism, in enzyme reactions catalyzed by ADH, ALDH, microsomal cytochrome P-4502E1 oxidase, microsomal NADPH oxidase and microsomal xanthine oxidase (12, 13). Microsomes and mitochondria are the major source of reactive species production. The activity of the enzyme cytochrome P-4502E1 generates permanent free radicals and acetaldehyde. Microsomes and mitochondria have a primary role in ethanol oxidation. Hydroxyethyl radicals are generated by liver microsomes, involving cytochrome P-4502E1 induction. Alcohol induces production of superoxide radicals and hydrogen peroxide in mitochondria. Hydrogen atom abstraction from ethanol leads to production of hydroxyethyl radicals out of hydroxyl radicals:

$$CH_3CH_2OH + OH^- \rightarrow CH_3C^-HOH + H_2O$$

Hydroxyl ethyl radicals may react with oxygen and form peroxy radicals. Peroxy radicals reaction with molecular oxygen enables production of superoxide anion radicals:

$$CH_3C^-HOH + O_2 \rightarrow CH_3C(OO^-)HOH$$
$$CH_3C(OO^-)HOH \rightarrow CH_3CHO + O_2^- + H^+$$

Peroxynitrite radicals are formed in vivo from the reaction of nitric oxide and superoxide anion radical (14, 15).

Nitric oxide

Nitric oxide is an intercellular signaling molecule synthesized in blood vessels endothelial cells, macrophages, and other cells. It participates in regulation of a variety of physiological functions in the body, such as: regulation of vascular tonus, platelet aggregation, leukocytes adhesion, smooth muscle cells proliferation, apoptosis, and neurotransmission. The rate of nitric oxide diffusion through the cell membrane is rapid in almost all organs. Thus, it can control biological functions in the body. Nitric oxide is a highly reactive molecule. Biological reactivity of NO molecules is based on guanylyl cyclase, transformation into peroxynitrite, and interaction with thiol groups (16).

Nitric oxide synthesizes from L-arginine by the activity of NOS. This enzyme catalyzes the NADPH – dependent oxidation of L-arginine to NO, citrulline, and NADP (Figure 2). There are three NOS isoforms: the neuronal NOS (nNOSor NOS-1), inducible or inflammatory NOS (iNO Sor NOS-2), and endothelial NOS (eNOSor NOS-3). Endothelial and neuronal NOS isoforms are constitutive, Ca^{2+} dependent forms, while iNOS is Ca^{2+}independent NOS isoform. Functional eNOS has been identified in the liver sinusoidal endothelial cells and may contribute to local perfusion and portal pressure (17).

The activity of constitutive isoforms produces short-lived NO molecules. Nitric oxide is an important signaling molecule. There are many sources of NO production in the body. Nitric oxide is a primary mediator of liver cells injury; it also takes part in a potential protective mechanism against stimulants that cause cellular damage. The activity of iNOS produces large amounts of NO molecules for an extended period of time. The expression of iNOS is induced by cytokines and microbial products (18).

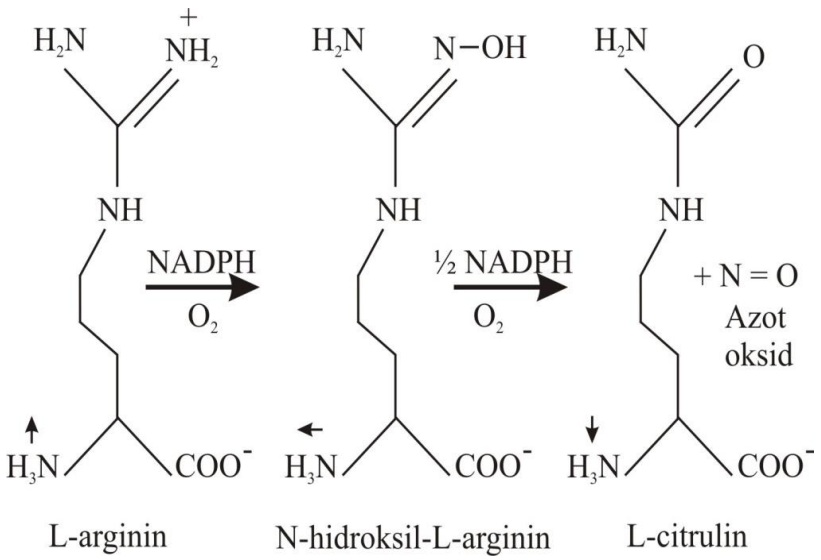

Figure 2. NO synthesis

Nitric oxide may have cytotoxic effects. Factors contributing NO cytotoxicity include localization and amount of NO generation, as well as the presence of oxidative stress, the amount of ROS, specific localizations of ROS (type of cells, intra - or extracellular localization). Peroxynitrite toxicity is manifested by interaction with biomolecules (19).

Nitric oxide and its metabolites may damage repair mechanism for nucleic acids. Peroxinitrites cause nucleic acids damage by activating inflammation and by the nuclear enzyme PARP mediation. Cell death occurs by necrosis and apoptosis (20). In the living organisms, the aggressive behavior of peryxonitrites is an important mechanism for the initiation and progression of a large number of acute and chronic diseases. Major NO cellular effects are illustrated in Figure 3.

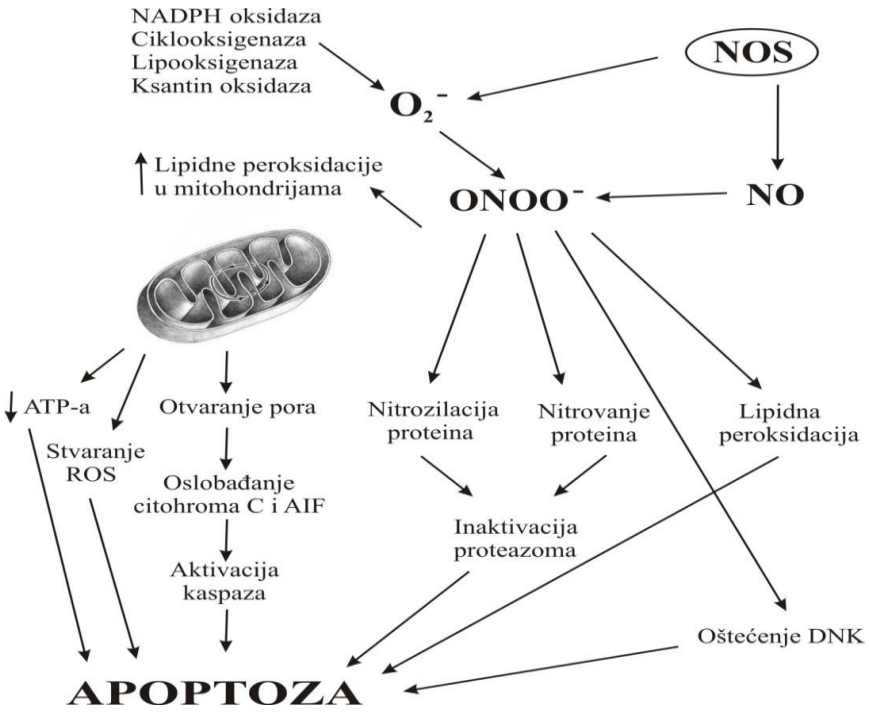

Figure 3. Major NO cellular effects

In the conditions of oxidative stress in liver damage, specific vasodilatory activity of eNOS is modulated or decreased. It leads to the development of increased intrahepatic vascular resistance. Thus, impaired microcirculatory perfusion occurs, as well as unequal necrosis in liver tissue as a consequence (21).

Nitric oxide is removed by rapid diffusion in the form of integrated complexes with erythrocytes. It is converted into nitrate by the reaction between NO and erythrocyte oxyhemoglobin. High concentrations of O_{2-} are quickly removed by deionization, by various superoxide dismutase (SOD) isoenzymes activities. In the living organisms, the aggressive behavior of peryxonitrites is an important mechanism for the initiation and progression of a large number of acute and chronic diseases.

Nitric oxide has a role in regulating ET-B endothelin receptor synthesis. In the conditions of ischemia-reperfusion and inflammation with endotoxemia, the roles of endothelin and its ET-B receptors change. Under the conditions of inflammatory stress, endotoxemia, ischemia and reperfusion in ALD, endothelin receptor gene expression is elevated, as well as ET-B receptors density, and endothelin level in the liver tissue (22, 23).

Peroxinitrites and mitochondria

Mitochondria are involved in hepatocytes vital processes, including energy production, calcium homeostasis and the control of biosynthetic pathways. Mitochondria also have an important role in the mechanism of cell death. Under the conditions of high NO production, peryxonitrites cause the impairment of mitochondrial function. Peroxynitrites react with mitochondrial components, thus having effects on every function of these organelles. Peroxynitrites enter the mitochondria from the extra mitochondrial space, or they may directly be produced in mitochondria. Mitochondria can produce NO by the activity of Ca^{2+} dependent mitochondrial NOS (mtNOS) (24). At the level of mitochondrial membranes, electron leakage from the respiratory chain causes pa-

rtial reduction of molecular oxygen to superoxide anion radical (O_2-). The role of NO in mitochondria is to regulate oxygen consumption by reversible inhibition of cytochrome C oxidase (complex IV of electron transport chain), by competing with oxygen, and by binding to binuclear site. During inflammation, reperfusion-ischemia in high NO production, electron transfer at the level of cytochrome C oxidase dysfunction is stimulated. Electron leakage from the respiratory chain occurs, as well as increased production of O_2- within the mitochondrial matrix and peroxynitrite (25, 26).

Peroxynitrites have a short half-life (10-20 ms). Peroxynitrite anion is highly diffusible. The injuries mediated by peroxynitrite are explained by its unique chemistry, including direct oxidation, nitrosative stress, as well as nitration reaction. Intensive processes of nitration and protein nitration lead to protein function modification. Peroxynitrites may directly oxidase low molecular weight thiols as well, such as GSH thiols (27).

Toxicity of peroxynitrites in mitochondria may result from direct oxidative reactions and ROS – mediated damage. Carbon dioxide (CO_2) is produced in mitochondria during decarboxylation in tri-carboxylic acid cycle. Carbon dioxide reacts with peroxynitrites. Direct reaction between peroxynitrites and CO_2 causes production of unstable nitrosoperoxycarbonates-that which quickly decompose into highly reactive radicals, carbonate radicals and NO radicals (26).

Peroxynitrites inhibit most components of the electron transport chain. Inhibition by peroxynitrites is achieved by different mechanisms: cysteine oxidation mechanism, nitration, tyrosine nitration, and iron-sulfur centers damage (27). Contrary to this, cytochrome C oxidase (complex IV), that is easily inhibited by NO, is resistant to peroxynitrites. Cytochrome C oxidase in its reduced form may act as an endogenous reduction catalyst of peroxynitrites into nitrites by its two electrons (28). The other target of peroxynitrites is cytochrome C that significantly decreases its redox properties by nitration. Nitration of cytochrome C increases its peroxidase activity that results in hydrogen peroxide production and exacerbation of oxidative damage of mitochondrial proteins (29).

Peroxynitrites additionally damage energy metabolism by the inhibition of aconitase enzyme, which inhibits tricarboxylic acid in mitochondria. Aconitase enzymes are found in mitochondrial matrix. Peroxynitrites inhibit aconitase enzymes via oxidative disruption of the 4Fe-4S center of the enzyme (30). Peroxynitrites also disturb energy metabolism by the inhibition of mitochondrial creatinine kinase that are present in the mitochondrial intermembrane space (31).

The reaction of peroxynitrite production is 3-fold faster than the reaction of SOD dismutation of O_2- to hydrogen peroxide. A 10-fold increase in O_2- and NO production causes 100-fold increased formation of peroxynitrites. However, under inflammatory conditions, O_2- and NO can be extremely increased by 1.000-fold, resulting in the increased formation of peroxynitrite by 1.000.000-fold. Superoxide dismutase is found in mitochondria, cytoplasm, and extracellular space. Superoxide dismutase is found in mitochondria, cytoplasm, and extracellular space. Decreased SOD activity, cycloxigenase, cyclooxigenase, xanthine oxidase accumulation of O_2- in cirrhotic liver, decrease of NO, increase of peroxynitrites in sinusoidal endothelial cells, increase of vascular resistance and the development of portal hypertension show that AOS therapy is significant in preventing hemodynamic disbalance in chronic alcoholic liver disease, ALD (32).

Peroxynitrites and apoptosis

Apoptosis is programmed cell death. Hepatocyte apoptosis is a typical consequence of increased peroxynitrite levels. Apoptosis is characterized by morphological changes, such as nuclear and cytoplasmic membrane condensation. The process of hepatocyte apoptosis results in the formation of small apoptotic bodies. Apoptotic bodies are membrane - bound particles that are quickly degraded by the phagocytes. Apoptosis is characterized by proteolytic cysteine protease activation, which is known as caspase. Caspase activity requires maintained ATP level. There are a few mechanisms that explain activation of programmed apoptosis by peroxynitrites, and they are greatly dependent on the cell type (33).

Apoptosis is activated by either death receptor activation (outer route), or by mitochondrial outer membrane permeabilization (inner route).

The common pathway of these two peroxynitrite-mediated apoptosis mechanisms includes outer mitochondrial membrane permeabilization. Permeabilization of the outer mitochondrial membrane enables the leakage of various proapoptotic signaling molecules that promote cellular apoptosis via caspase-dependent mechanisms, or caspase-independent mechanisms (34). Permeabilization of outer mitochondrial membrane may be activated by pore formation within the outer membrane by proapoptotic proteins (Bak, Bak1), by the process of anti-apoptotic protein inhibition, or by the phenomenon known as mitochondrial permeability transition (35).

Mitochondrial permeability transition is a pronounced characteristic of peroxynitrite-induced cell death. Mitochondrial permeability transition involves permeabilization of the inner mitochondrial membrane by a multiprotein complex, called permeability transition pores (PTP). The multiprotein complex consists of adenine nucleotide translocase, cyclophilin D, and the voltage-dependent anion channel. Formation of PTP occurs with calcium overload or by oxidative modification of critical thiol groups within nucleotide translocase. Permeability of transition mitochondrial pores causes the cessation of electrons transmission and ATP production. It occurs by the dispersion of mitochondrial membrane potential (36, 37).

In addition to direct effect on mitochondria, peroxynitrites may activate apoptotic mechanism by signal cellular modulation. A serine/threonine protein kinase has an important role, since its activation induces powerful protection mechanism to limit apoptosis in a variety of stressful conditions, including oxidative stress (38).

Peroxynitrites and necrosis

Hepatocyte necrosis occurs after cellular exposure to high concentrations of oxidants. Necrosis is associated with the exhaustion of cellular ATP capacity. Cellular ATP deficiency is a consequence of membrane disruption, the release of harmful degraded cellular substances, and the development of secondary inflammation. Many studies have shown that peroxynitrite-dependent cellular necrosis is mediated by a complex process, including DNA damage and activation of DNA repair enzyme (nuclear enzyme poly ADP-ribose polymerase PARP-1). The nuclear enzyme poly ADP-ribose polymerase enzyme system (PARP) is composed of PARP-1 and enzyme system poly ADP-ribose polymerase. The nuclear enzyme poly ADP-ribose polymerase type 1 detects and signals DNA strand breaks induced by different reactive species (hydrogen peroxide, peroxynitrites, nitrosyl anion and oxygen radicals, carbonate or hydroxyl radicals) (20).

Peroxynitrites-dependent cytotoxicity is mediated by lipid peroxidation, protein nitration and oxidation, DNA oxidative damage, activation of matrix metalloproteinase, and inactivation of a variety of enzymes in cells.

Inactivation of mitochondrial enzymes, as well as inhibition of ATP molecules synthesis, leads to mitochondrial swelling and increased permeabilization of mitochondrial membranes. Permeabilization of mitochondrial membrane causes the leakage of proapoptotic molecules, cytochrome C, and apoptosis-induced factor. In addition to harmful effects on mitochondria, peroxinitrites cause DNA breaks and induce severe oxidative damage to genomic DNA. DNA breaks are activated by nuclear enzyme PARP. Activated PARP depletes NAD to synthesize poly ADP-ribose polymerase (PAR). Mild DNA damage activates DNA repair mechanism.

In the case of moderate mitochondrial permeabilization and PARP activation, along with maintenance of cellular ATP, a cell may degrade by apoptosis. In reperfusion-ischemia, in very pronounced oxidative stress and nitrosative-induced DNA damage, cells can degrade by necrosis. Expressed mitochondrial permeabilization then occurs, as well as pronounced PARP activation, leading to massive NAD and cellular ATP exhaustion (39, 40).

In chronic, progressive liver disease there are differences in the degree of inflammation and fibrosis that originate from different nitrate and nitrite levels in blood. Recent studies have shown that an increase in NO_2 + NO_3 concentration is directly proportional to the degree of chronic liver injury. An increase in NO_2 + NO_3 concentration at the expense of peroxynitrite formation may be a causative factor for the development of cirrhosis and its complications in patients with cirrhosis (41).

In patients with cirrhosis, overproduction of nitrates and nitrites is involved in the pathogenesis of hepatic hemodynamic abnormalities development In end-stage liver cirrhosis with ascites, peroxynitrites and NO are key pathogenic factors responsible for the development of portal hypertension and its complications (42).

Conclusion

Alcohol abuse and ALD are global health problems. Considering the fact that 3.3 million people die of ALD and its complications annually, priority is given to timely diagnosis of ALD and alcoholic liver disease staging.

It can be concluded that the analysis of NO_2 + NO_3 values enhances the understanding of alcohol-induced liver disease pathogenesis, alcoholic liver disease staging, as well as monitoring of the disease progression. It can also be concluded that timely determination of toxic nitrates and nitrites values and suitable antioxidant therapy may slow down the processes of hepatocytes and vascular endothelial cells apoptosis and necrosis, i.e. the progression of ALD and hemodynamic disorder.

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NITRATI I NITRITI U RAZVOJU ALKOHOLNE BOLESTI JETRE

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Alkoholizam predstavlja jednu od vodećih bolesti zavisnosti koja štetno utiče na zdravlje i imunitet ljudi širom sveta. Hroničnim konzumiranjem alkohola u dužem vremenskom periodu nastaje poremećaj metaboličke funkcije jetre uz poremećaj metabolizma arginina i poremećaj sinteze azot oksida (NO). Pri etanolnoj intoksikaciji u uslovima kumulativnog nitrooksidativnog i nitrozivnog sresa, kao i inflamacije, nastaju NO anjon (NO^-) i super oksid anjon (O_2-), tj. peroksinitriti u hepatocitima i endotelu. Disfunkcija mitohondrija i poremećaj sinteze adenozin tri fosfata (ATP) molekula u hepatocitima dovodi do poremećaja sinteze intra - i ekstracelularnih antioksidansa (glutationa i superoksid dizmutaze) i neutralizacije toksičnih nitrita i nitrata. Peroksinitriti oštećuju lipoproteine membrana ćelija i sisteme enzima membrana i matriksa mitohondrija. Takođe, oštećuju enzime metabolizma etanola i arginina u citozolu i enzime reparacije nukleinske kiseline hepatocita. U razvoju alkoholne bolesti jetre (ABJ) peroksinitriti mehanizmima apoptoze i nekroze oštećuju najpre reverzibilno, a zatim ireverzbilno strukturu i funkciju hepatocita, kao i vaskularni endotel sinusa.

S obzirom da godišenje umire oko 3.3 miliona ljudi od ABJ i njenih komplikacija, treba raditi na smanjenju pojave i progresije ABJ. Prioritet predstavlja pravovremeno postavljanje dijagnoze ABJ, kao i dijagnoza stepena alkoholnog oštećenja jetre. Istraživanja pokazuju da vrednosti peroksinitrita rastu sa oštećenjem jetre. Može se zaključiti da pravovremeno određivanje vrednosti peroksinitrita, uz adekvatnu terapiju antioksidansima, može usporiti mehanizme apoptoze i nekroze hepatocita, kao i tok ABJ.

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Ključne reči: *alkohol, nitrati, nitriti, oštećenje jetre*

Case report

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LOBECTOMY OF THE CENTRAL LOBE AS A METHOD OF CHOICE IN THE TREATMENT OF LARGE ENDOBRONCHIAL MID-LOBES HAMARTOMA: CASE REPORT

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Pulmonary hamartoma is a rare benign tumor change in the lungs. Often, it is discovered randomly as a side finding on the chest x-ray in the form of asymptomatic, solitary, round tumor change, coin size, with "popcorn" calcifications. Although considered benign tumor with good prognosis, it differs diagnostically from carcinoid, tuberculosis, bronchogenic carcinoma, metastases and hydatid cysts. In histopathology diagnosis, the following are used: chest X-ray, computed tomography, bronchoscopy, fine needle aspiration cytology, surgical extirpation and histopathology verification. It is thought that symptomatic and large pulmonary hamartoma should be removed. Whenever possible, pulmonary hamartoma should be removed by minimally invasive-bronchoscopic procedure or by video-assisted thoracoscopic surgery. For classical surgical approach, lateral and anterolateral thoracotomy are more convenient, and lung hamartoma can be enucleated, wedge resectioned, removed by segmentectomy, lobectomy, and possibly by pulmectomy. The following case study describes the lobectomy of the central lobe through anetolateral thoracotomy as a method of choice to treat a large, symptomatic PH in the middle lobe, in a patient 21 years of age.

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Key words: *lung hamartoma, benign tumor, lobectomy of the central lobe*

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Introduction

Pulmonary hamartoma (PH) is a rare but most common benign tumor change in the lungs. It occurs in 0.025-0.32% of all lung neoplasms (1). PH accounts for 77% of all benign lung neoplasms and about 6% of all solitary lung nodules (2,3). It was first described by Albrecht in 1904 (4). It is more likely to occur in men with peak incidence in the sixth and seventh decades of life and male female ratio 2-4:1 (5). Pulmonary hamartoma is most often detected incidentally as an early finding of the chest Rtg in the form of a solitary, round, coin size tumor change (coin lesion) with "popcorn" calcifications (4). The presence of PH is usually asymptomatic. Rarely, depending on size and localization, PH can give symptoms in the form of cough, haemoptysis, chest pain, fever and signs of obstruction of the bronchial tree (6). Usually, these are solitary nodes ≤ 1-20 cm in the parenchyma of the lungs on the periphery, but they can be found both centrally and endobronchially (7). In 1.4% of cases PH was localized endobron - hially (8). Hamartomas are considered benign tumors with good prognosis and are often clinically monitored due to their slow growth (2). However, although it is traditionally considered that PH does not alter malignantly, there are some reports indicating the possibility of malignant alteration (4, 9).

Case study

Patient I.V. aged 21, was admitted to the Clinic of Lung Diseases Knez Selo due to the cough problems, high body temperature, general weakness and fatigue, pain in the muscles and joints. The symptoms started 7 days before the admission. He was treated clinically by Hemomycin tbl. when there was a slight drop in body temperature. On the chest x-ray P-A in the middle pulmonary field, an oval, soft

tissue change, diameter of about 8 cm was observed, with "popcorn" calcifications (Figure 1). The patient is a non-smoker and was not in contact with domestic animals. Immediately upon admission, a serological analysis was performed on Echin spp antibodies by an indirect method that was negative. Spirometrically, there is an easy-to-restrictive ventilatory disorder FEV1 85% (3.64 L), FVC 89% (4.49 L), FEV1%/FVC 81%. The lung multislice computed tomography (MSCT) discovered on the right, dominantly in the middle lobe, a change of 79x70x65 mm, extending from the right hilum to the pleura lateral wall, with which it is in wide contact, a highly heterodenous structure, with soft tissue attenuations and attenuations of calcium and fat, with postcontrast coloring of solid soft tissue parts, without adhesive growth; several subpleural micronodal changes were present on both sides (Figures 2 and 3). It was concluded that this was a hamartoma change (Ddg. of other etiology), and the observed subpleural micronodal changes were of non-specific characteristics. Bronchoscopically, mouthpiece for DB4 was completely frozen with a ball-like neo formation of a glossy, white, smooth surface ("membrane"), due to which transbronchial biopsy (BB) was not taken. It was concluded that the tumor change was in the middle lobe, of the neat environment and that it worked cystic and benign.

The patient was admitted to our facility for the operative treatment of the mentioned tumor change in the middle lobe. The patient underwent surgery. At first, video-assisted thoracoscopic surgery (VATS) was made to the right with the idea of hamartoma enucleation. After the introduction of the endocamera in the right pleural space, one finds an oval, white mass of about 8 cm in diameter, which engages almost the entire middle lobe. It was decided to perform the lobectomy of the central lobe. The approach was anterolateral thoracotomy through the 5th interrib space to the right. First, once again, the enucleation of the change was attempted. However, during the preparation of tumefact it was seen that the same was inseparably linked to the bronchial cartilage for the middle lobe (Figure 4). The preparation of tumor was done partly sharp, partly dull, with monopolar and bipolar instruments. Then, with the combined, front and back approach, the central lobe blood vessels were uncompressed, proximal double-bridged and cut. The middle lobe bronchus was first cut down more central from the level of the tumor growth, and then it was taken away by a bronchial splitter. A tumor with a bronchial stump and the rest of the central lobe tissue was sent to the histopathology examination (HP) (Figure 5). HP findings indicate that it is a 8x6x5 cm capsulated oval tumor, on a cross-section of multishape appearance, a brown, white-yellow color with the presence of car-tilaginous consistency and dense yellowish areas, as well as cystic formations up to 25 mm, partly filled with brown mass. HP diagnosis is hamartoma pul-monis.

Immediate postoperative procedure was neat and the wound has healed per primam. On the seventh postoperative day, the patient was released for home treatment. In a series of control x-ray examinations, the findings on the lungs are correct and correspond to the operation performed (Figure 6). Control check after six months has shown that the patient is subjectively well, there is no recurrence of tumor. The patient returned, all the way to the usual life activities.

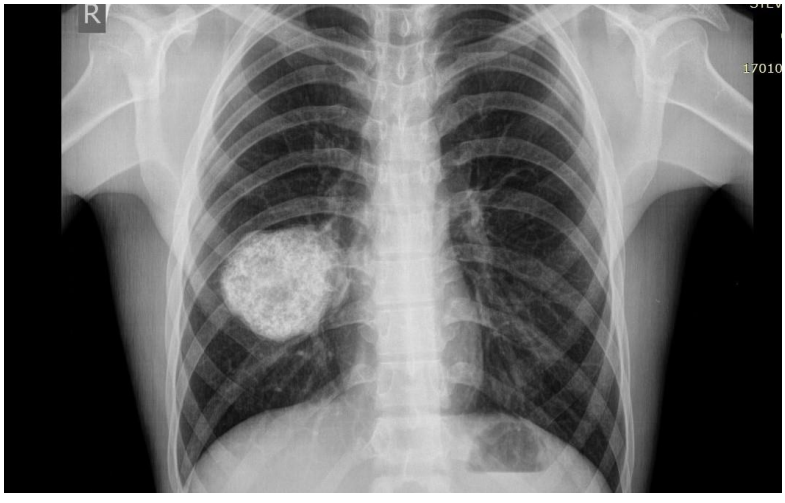

Figure 1. Rtg P-A, preoperatively, a large hamartoma in the middle lobe with "popcorn" calcifications

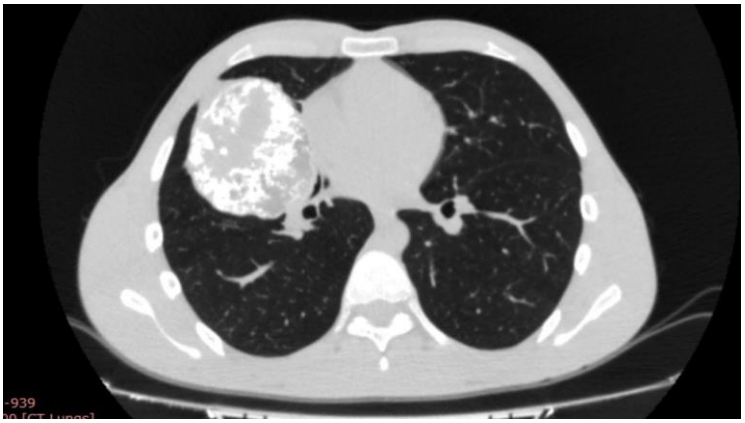

Figure 2. MSCT presentation of hamartoma in the middle lobe (axial cross-section)

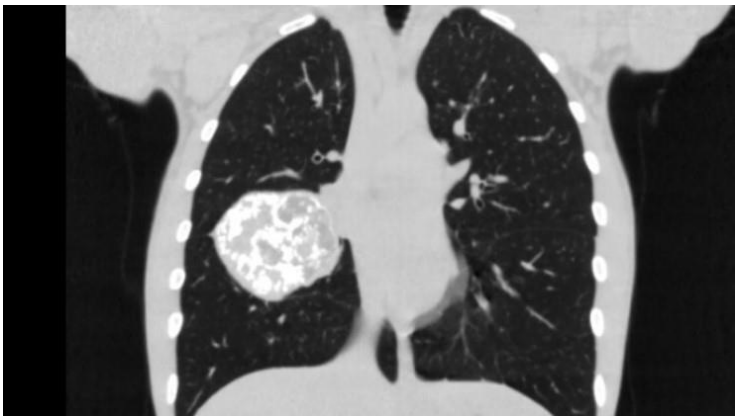

Figure 3. MSCT presentation of hamartoma in the middle lobe (coronal cross-section)

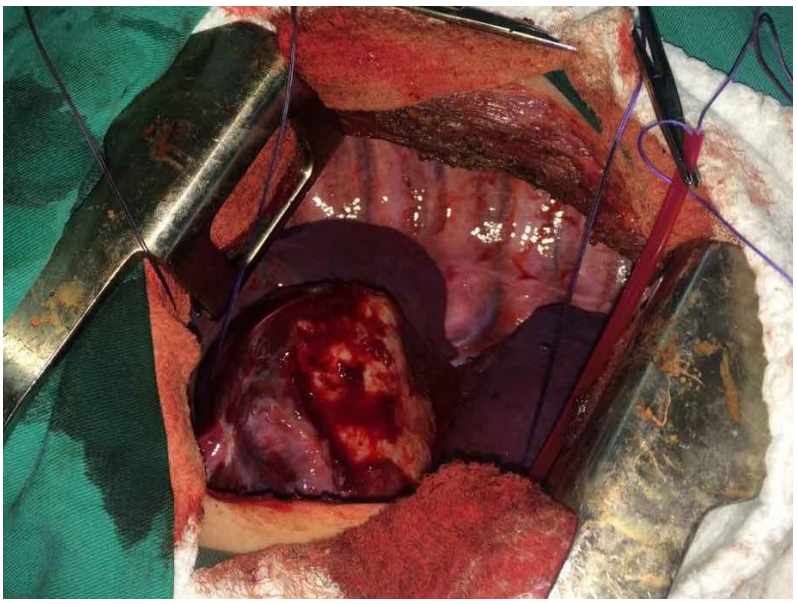

Figure 4. Intraoperative finding, a large hamartoma that engages almost the entire middle lobe

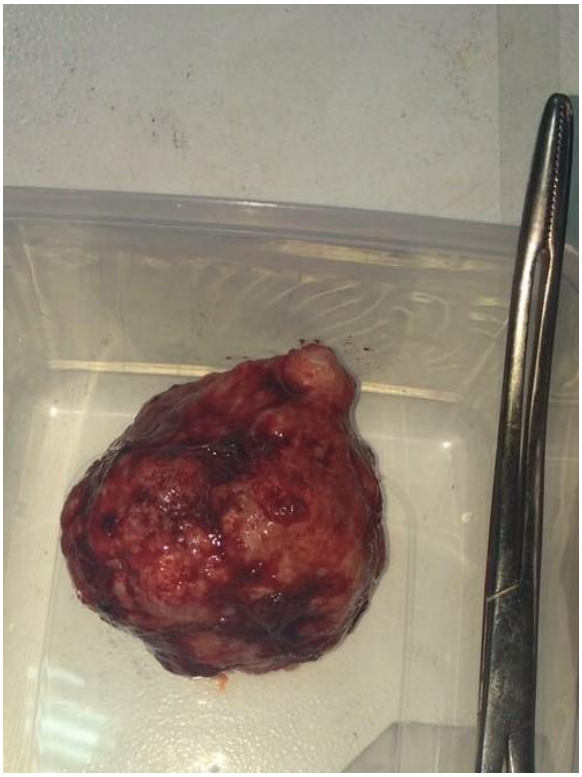

Figure 5. Enucleated hamartoma with a bronchial stump for the middle lobe

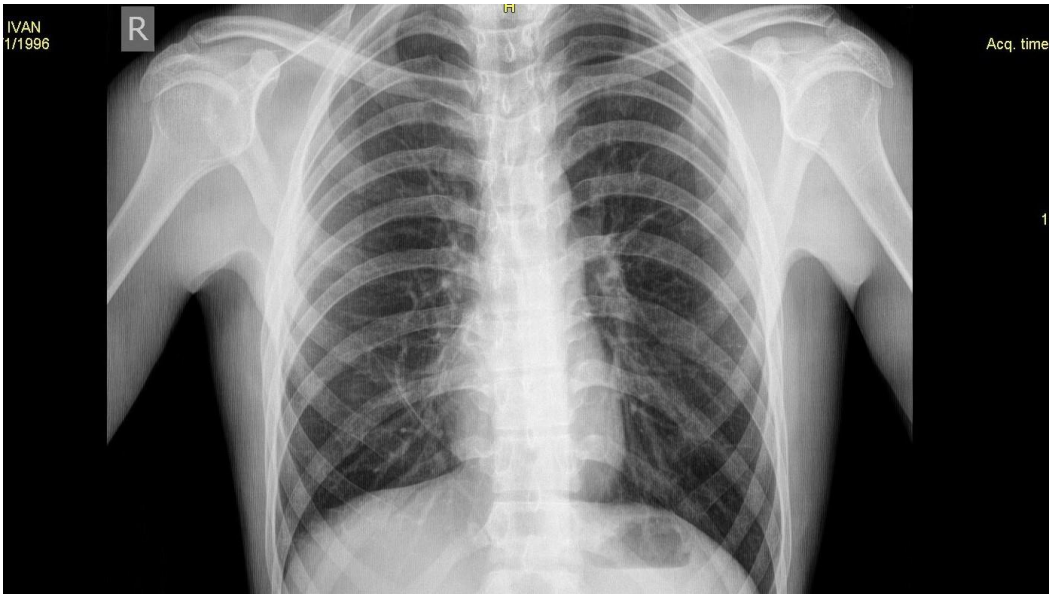

Figure 6. Rtg P-A three months after surgery, lung reexpansion is complete

Discussion

The pulmonary hamartoma is formed of embryonic remains, it is present in the fetal period, but is rarely detected before growing up (10). Although considered a benign tumor with a good prognosis, differentially diagnostically it is similar to carcinoid, tuberculosis, bronchogenic carcinoma, metastasis and hydatidic cyst (11). Hamartoma is typically of a smooth surface, a well-limited, moderately firm,

round or oval nodus of heterogeneous internal structure (2). The cytologic PH typically comprises a mixture of mesenchymal elements from fibromycose or cartilaginous connective tissue to mature cartilage and benign bronchial epithelial cells randomly alligned with-out necrosis in the background (5).

The pulmonary hamartoma usually occurs as a single nodus, but the occurrence of multiple, cystic and diffuse endotracheal hamartomas (12, 13) is possible. Multiple PHs have been described in Carney's triad: pulmonary chondromes, gastric epithelium leukemias, and functional extra-adrenal paragangliomas (11). There have been cases of malignant PH alterations that are extremely rare (4, 9, 14).

In PH diagnosis, the following are used: chest x-ray, computed tomography (CT), bronchoscopy, fine needle aspiration cytology (FNAC), surgical extirpation and HP verification. Positron-emission tomography (PET) is useful in the differentiation of malignant from benign solitary nodus in the lungs, but easy intake of 18F-FDG by PH was described, which makes it difficult to differentiate PH from the malignant nodular changes in the lungs (15).

Radiologically, the presence of "popcorn" calcification in PH is a pathognomonic radiological mark of hamartoma and is seen in 10-15% PH on the Rtg of the thorax, and according to some authors in up to 20-30% of cases (6,16).

Indications for removing PH and the way it will be removed are still controversial (16). It is believed that PH, which is symptomatic and large, should be removed (4). Surgical resection of tumefact in the lungs when diagnosis of PH is not confirmed is performed:

1) when it is difficult to distinguish between PH, metastasis, cancer, tuberculosis and other tumors of the lungs,

2) when patients with PH have respiratory problems due to endoluminal PH growth with postoperative complications,

3) when tumefact (PH) is rapidly increasing,

4) in patients who experience strong psychological pressure due to the presence of tumefact in the lungs of unknown origin and nature,

5) due to potential malignant alteration of PH (which is extremely rare, although described) (14, 16, 17).

In order to diagnose and treat the symptoms caused by PH, it can be removed bronchoscopically and surgically. It is desirable to remove PH, whenever possible, bronchoscopically (smaller, endoluminal PH) (18). Out of the surgical methods, VATS and conventional surgery are used (lateral and anterolateral thoracotomy are preferred) (17). Depending on the local finding, PH can be enucleated, wedge resectioned, removed by segmentectomy, lobectomy and, possibly, pulmectomy (in case of vast PH) (1). Hamartomas are moderately solid tumors and can be manually suppressed in the pulmonary parenchyma to the surface, after which a small incision on the visceral pleura is made, and the tumor is completely enucleated. Quite often a planned VATS procedure, due to the inability to identify intraparenchymal PH, has to be converted. In this case, it is desirable to make as small a cut on the chest (miitorocotomy) through which PH will be palpated and enucleated.

In the case presented PH was large and symptomatic. It was not possible to remove the same endobronchially. It was decided to remove PH surgically. First, VATS was made, and then the conversion into anterolateral torocotomy, with the idea to, with the smallest operative trauma per patient, remove hamartoma. Lobectomy of the central lobe was inevitable since hamartoma was large, it engaged almost the entire middle lobe and intimately grew with the bronchial tissue to the middle lobe with a break in his lumen.

Conclusion

Pulmonary hamartoma is a rare but most common benign tumor change in the lungs. Pulmonary hamartoma is most often detected incidentally as a side finding on the chest x-ray, in the form of asymptomatic, solitary, round tumor change, size of a coin, with "popcorn" calcifications. Differentially diagnostically lung hamartoma looks like carcinoid, tuberculosis, bronchogenic carcinoma, metastasis and hydatid cyst. In pulmonary hamartoma diagnostics chest x-ray, CT, bronchoscopy, FNAC, surgical extirpation and HP verification are used. Pulmonary hamartoma, which is symptomatic and large, should be removed. Whenever possible, pulmonary hamartoma should be removed minimally invasive-bronchoscopic or by VATS. For classic surgical approach, lateral and anterolateral thoracotomy are most convenient, and the lung hamartoma itself can be enucleated, wedge resected, removed by segmentectomy, lobectomy, and possibly by pulmectomy. In the presented case study, the lobectomy of the central lobe through anetolateral thoracotomy has been shown to be a method of choice in the treatment of a large, symptomatic lung hamartoma that engages the entire central lobe and has endoluminal growth in the bronchus for the middle lobe.

Disclosure: The authors declare no conflict of interest.

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Prikaz bolesnika

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LOBEKTOMIJA SREDNJEG REŽNJA KAO METODA IZBORA U LEČENJU VELIKOG ENDOBRONHIJALNOG HAMARTOMA SREDNJEG REŽNJA: PRIKAZ SLUČAJA

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Plućni hamartom je retka benigna tumorska promena u plućima. Često se otkrije slučajno, kao uzgredni nalaz na standardnom rendgenogramu grudnog koša u vidu asimptomatske, solitarne, okrugle tumorske promene veličine novčića sa „popcorn" kalcifikacijama. Iako se smatra za benigni tumor sa dobrom prognozom, diferencijalno dijagnostički liči na karcinoid, tuberkulozu, bronhogeni karcinom, metastaze i hidatidnu cistu. U dijagnostici plućnog hamartoma koriste se: standardni rendgenogram grudnog koša, kompjuterizovana tomografija, bronhoskopija, aspiraciona biopsija tankom iglom, hirurška ekstirpacija i histopatološka verifikacija. Smatra se da simptomatske i velike plućne hamartome treba ukloniti. Kad god je to moguće, plućni hamartom treba ukloniti minimalno invazivno, bronhoskopski ili video-asistiranom torakoskopskom hirurgijom. Za klasičan hirurški pristup najpodesnije su lateralna i anterolateralna torakotomija, a sam plućni hamartom se može enukleisati, klinasto resecirati, ukloniti segmentektomijom, lobektomijom i eventualno, pulmektomijom. Prikaz slučaja koji sledi opisuje lobektomiju srednjeg režnja kroz aneto-lateralnu torakotomiju kao metodu izbora u lečenju velikog, simptomatskog PH u srednjem režnju kod bolesnika starog 21 godinu.

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Ključne reči: *hamartom pluća, benigni tumor, lobektomija srednjeg režnja*

Original article

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APPARENT DIFFUSION COEFFICIENT(ADC) OF PERITUMORAL TISSUE IN DIFFERENTIATION OF BRAIN METASTASES FROM GLIOMAS

Zoran Radovanović

Peritumoral edema of high grade gliomas represents a combination of neoplastic cell infiltration and vasogenic edema, while peritumoral edema of intracranial metastases is purely vasogenic. The aim of this study was to examine whether ADC can be used as a noninvasive parameter to distinguish peritumoral brain tissue in metastases from peritumoral tissue in cerebral gliomas.

A prospective study involved 71 patients, 22 with histologically proven intracranial metastases and 49 with gliomas. All patients underwent conventional MRI and DWI up to 7 days before undergoing surgery. ADC values were obtained in three regions of interest within peritumoral brain tissue and compared with the histopathological findings.

The mean minimum ADC values in the peritumoral regions of low grade gliomas were significantly higher (< 0.001) than those of high grade gliomas. The mean minimum ADC values in the peritumoral regions of metastases were significantly higher than those in high grade gliomas. The ADC values of peritumoral brain tissue of lung carcinoma metastases (0.000947 ± 0.000043 mm^2/s), melanoma (0.000842 ± 0.000018 mm^2/s) and breast metastases (0.000783 ± 0.000048 mm^2/s) were significantly higher than the ADC values of peritumoral brain tissue of astrocytoma grade I (0.000775 ± 0.000013 mm^2/s), grade II (0.000411 ± 0.000005 mm^2/s), grade III (0.000121 ± 0.000004 mm^2/s) and glioblastoma multiforme (0.000076 ± 0.000011 mm^2/s).

The minimum ADC values of the peritumoral edema in brain metastases were significantly higher than those in gliomas. ADC values can provide additional diagnostic information for distinguishing gliomas from metastases.

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Key words: *brain imaging, brain metastases, diffusion-weighted imaging, cerebral gliomas*

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Introduction

Conventional Magnetic Resonance Imaging (MRI) often shows difficulties in the differentiation of glioblastomas from solitary brain metastases, as they both can demonstrate similar imaging characteristics and contrast-enhanced patterns. Preoperative distinction between these tumors is important for their surgical approach and therapeutic procedures, which could be completely different (1). Patients with glioblastomas are almost always treated by surgical resection, while patients with suspected brain metastases without a clinical history of systemic cancer should undergo a complex systemic staging to determine the site of primary carcinoma and evaluate other distant metastases before any surgical intervention or medical therapy (2]) Diffusion-weighted imaging (DWI) provides valuable information beyond just anatomy and structure of the brain tissue (3). Apparent diffusion coefficient (ADC) value is a quantitative parameter of DWI which reflects diffusion movements of water molecules in the brain tissue. The degree of restriction of water diffusion is correlated with tissue cellularity, medium viscosity and integrity of cell membranes (4).

The key for distinguishing gliomas from metastases can lie in detecting the changes within the peritumoral area – which is the area beyond the enhancing margin on postcontrast T1W imaging. Most of these tumors are surrounded by a T2W hyperintensity that has been named vasogenic edema (5). Local disruption of the blood-brain barrier increases capillary permeability and induces a pressure gradient from the vascular to the extracellular compartment that results in the retention of plasma fluid and protein in the extracellular space. In high grade gliomas, the T2W hyperintensity comprises not only

vasogenic edema but also infiltrating tumor cells outside of contrast enhancing lesions, whereas the T2W hyperintensity that surrounds brain metastases is purely vasogenic (6). Therefore, the aim of this study was to examine whether ADC can be used as a noninvasive parameter to distinguish peritumoral brain tissue in metastases from peritumoral tissue in cerebral gliomas.

Material and methods

Patients

This is a single-center retrospective study in which we retrospectively reviewed MR images of patients with cerebral gliomas and metastases. The study was approved by our Institutional Review Board. Written informed consent for this study was waived because of the retrospective nature of our clinically acquired data. All patients were referred for MR imaging by a single academic neurosurgical clinic in a period of five years.

Seventy-one patients (42 male, 29 female, mean age 52.8 ± 3.39) were involved in the study, including a group of 22 patients (12 male, 10 female, mean age 55.20 ± 10.37) with histologically proven intracranial metastases and 49 patients (30 male, 19 female, mean age 50.40 ± 12.53 years) with gliomas (Tables 1 and 2). All patients underwent conventional MRI and DWI up to seven days before surgical intervention at our institution. Histopathologic diagnosis was based on the World Health Organization (WHO) criteria using surgical specimens.

Of 71 patients, gliomas were diagnosed in 49 patients [30 males (71.4%) and 19 females (64.9%)]. According to the WHO classification, there were 4 (9.5%) patients with astrocytoma grade I, 12 (16.9%) patients with astrocytoma grade II, 13 (18.3%) patients with anaplastic astrocytoma and 20 (28.1%) patients with glioblastoma multiforme. Brain metastases were diagnosed in 22 patients (12 males and 10 females). Metastatic brain tumors included lung carcinoma in 5 (7%) patients, breast metastases in 5 (7%) patients, genital metastases in 3 (4.2%) patients, melanoma metastases in 4 (5.6%) patients, and mastases of unknown origin in 5 patients.

Imaging and data analysis

Whole-brain imaging was conducted on the same 1.5-T MR (Avanto, Siemens, Erlangen, Germany) with the standard protocol: sagital T1W, axial T1W, T2W, FLAIR, coronal T2W and postcontrast T1W. Intravenous gadolinium based contrast agent was administered in a dose of 0.1 mmol/kg of body weight. DWI was performed in the transverse plane with a single-shot gradient-echo echo-planar pulse sequence with next parameters: TR 3600, TE 99, b values of 0 and 1000, section thickness 5mm, intersection gap 10%, FOV 230, averages 3, concatenations 1, base resolution 128 and voxel size 1.8x1.85. The diffusion gradient was encoded in three orthogonal directions.

The ADC maps were calculated from isotropic DWI using software DP Tools. Two radiologists conducted the quantitative analysis by the use of three operator defined region-of-interest (ROI) measurements. Oval ROIs with diameters of 1cm were placed within peritumoral area. ADC values (avoiding calcifications and cystic or necrotic areas) were obtained as the mean of measurements from three ROIs within peritumoral brain tissue. ADC values were compared with the histopathologic findings after surgery, using the World Health Organization (WHO) criteria

None of the patients had begun corticosteroid treatment or radiation therapy, and none had previous brain biopsy at the time of MRI. Tumors with large calcifications, hemorrhages or both were excluded.

Statistical analysis

Data are shown as the arithmetic mean (Xsr), and a standard deviation (SD), the minimum values (min.), the maximum (max.) values and index structure (%). Comparison of the ADC values between patients with different histological diagnoses was performed by ANOVA and Bonferon's post hoc test. P values less than 0.05 were considered to indicate statistically significant differences. Comparison of the ADC values between peritumoral brain tissue with histopathological diagnosis and contralateral healthy brain tissue was performed by (Student t test - P). The statistical analysis of data was performed by using SPSS 10.0 software package.

Results

Demographic characteristics (age and sex) of patients and histopathologic diagnosis of brain gliomas and metastases are shown in Tables 1 and 2. The average age of patients with gliomas was 50.40 ± 12.53 years. The youngest patient was 19 and oldest 77 years old. Patients with glioblastoma multiforme (54.08 ± 2.83) and anaplastic astrocytomas were older than those with the astrocytoma grade I (49.00 ± 2.83) and astrocytoma grade II (39.80 ± 9.63). The average age of patients with metastases was 55.20 ± 10.37 years.

Data of ADC (mm^2/s) value in peritumoral brain tissue in comparison with histopathologic diagnosis are shown in Table 3 and Table 4. The ADC values in peritumoral edema showed statistical difference between different grades of gliomas ($p < 0.001$). There was a decrease in ADC values with increasing of glioma grade. ADC values in peritumoral edema were higher in the most benign astrocytoma Gr I (0.000755).

Peritumoral edema of the most malignant glioblastoma multiforme showed the lowest ADC values (0.000076). Peritumoral edema of brain metastases had significantly higher ADC values than that of gliomas (0.000750 vs. 0.000340) ($p < 0.05$). The ADC values of peritumoral brain tissue of lung meta-

stases (0.000947 ± 0.000043 mm^2/s), melanoma (0.000842 ± 0.000018 mm^2/s), metastases of unknown origin (0.000626 ± 0.000011) and breast metastases (0.000783 ± 0.000048 mm^2/s) were significantly higher than the ADC values of peritumoral brain tissue of astrocytoma grade I (0.000775 ± 0.000013 mm^2/s), grade II (0.000411 ± 0.000005 mm^2/s), grade III (0.000121 ± 0.000004 mm^2/s) and glioblastoma multiforme (0.000076 ± 0.000011 mm^2/s). There was no significant difference between peritumoral ADC values of Astocytoma Gr I and malilignant melanoma metastases.

Table 1. Histopathologic diagnosis and sex of examined patients

Histopathological diagnosis	Sex		Total
	Male	Female	
Astrocytomas grade I	4 (9.5 %)	-	4 (5.6%)
Astrocytomas grade II	6 (14.2%)	6 (31.5%)	12 (16.9%)
Anaplastic astrocytomas	8 (19%)	5 (26.3%)	13 (18.3%)
Glioblastoma multiforme	12 (28.5%)	8 (42.1%)	20 (28.1%)
Gliomas total	30 (71.4%)	19 (64.9%)	49 (69.1%)
Lung metastases	5 (11.9%)	-	5 (7%)
Breast metastases		5 (17.8%)	5 (7%)
Melanoma metastases	2 (4.7%)	2 (7.1%)	4 (5.6%)
Genital metastases	2 (4.7%)	1 (3.5%)	3 (4.2%)
Metastases of unknown origin	3 (7.1%)	2 (7.1%)	5 (7%)
Metastases total	12 (28.6%)	10 (35.2%)	22 (30.9%)

% - index of structure

Table 2. Histopathologic diagnosis and age of examined patients

Histopathologic diagnosis	Parameter				
	Xsr	SD	Med.	Min.	Max.
Astrocytomas grade I	49.00	2.83	49.00	47.00	51.00
Astrocytomas grade II	39.80	9.63	43.00	29.00	52.00
Anaplastic astrocytomas	52.00	19.56	61.00	24.00	72.00
Glioblastoma multiforme	54.08	9.59	55.00	38.00	77.00
Gliomas total	50.40	12.53	51.00	24.00	77.00
Lung metastases	52.50	6.36	52.50	48.00	57.00
Breast metastases	52.50	19.09	52.50	39.00	66.00
Melanoma metastases	51.00	0.00	51.00	51.00	51.00
Genital metastases	64.50	17.68	64.50	52.00	77.00
Metastases of unknown origin	55.50	3.54	55.50	53.00	58.00
Metastases total	55.20	10.37	52.50	39.00	77.00

Xsr – arithmetic mean; S – standard deviation: Min. – minimum value;
Max. – maximum value; M – mediana

Table 3. The value of ADC (mm2/s) in peritumoral brain tissue in comparison with histopathologic diagnosis

Histopathologic diagnosis	Parameter				
	Xsr	SD	Med.	Min.	Max.
Astrocytomas grade I	0.000755	0.000013	0.000761	0.000701	0.000789
Astrocytomas grade II	0.000411	0.000005	0.000640	0.000000	0.000992
Anaplastic astrocytomas	0.000121	0.000004	0.000000	0.000000	0.000604
Glioblastoma multiforme	0.000076	0.000011	0.000075	0.000071	0.000081
Gliomas total	0.000340	0.000009	0.000598	0.000000	0.000992
Lung metastases	0.000947	0.000043	0.000958	0.000892	0.000990
Breast metastases	0,000783	0.000048	0.000800	0.000694	0.000831
Melanoma metastases	0.000842	0.000018	0.000848	0.000815	0.000859
Genital metastases	0.000556	0.000011	0.000812	0.000000	0.000862
Metastases of unknown origin	0.000626	0.000019	0.000000	0.000000	0.000000
Metastases total	0.000750	0.000027	0.000815	0.000000	0.000990

Table 4. Comparison of the value of ADC (mm2/s) in peritumoral brain tissue between different histopathologica diagnoses (ANOVA and Bonferon's post hoc test - P)

Histopathologic diagnosis	Astrocytomas grade II	Astrocytomas grade II	Glioblastoma multiforme	Lung metastases	Brest metastases	Malignantmelanoma	Genital metastases	Unknown origin metastases
Astrocytomas grade I	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.9999	< 0.001	< 0.001
Astrocytomas grade II		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Anaplastic astrocytomas			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Glioblastoma multiforme				< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Discussion

Our study revealed significant difference in ADC values between peritumoral edema of brain metastases and gliomas. The minimum ADC values of the peritumoral edema in brain metastases were significantly higher than those in gliomas. The present findings can provide additional diagnostic information for distinguishing gliomas from metastases.

Gliomas and intracranial metastases are the most common mass lesions in the brain and 6 % of patients with newly diagnosed invasive cancer are expected to develop subsequent brain metastases as a progression of their original cancer diagnosis (5). Primary tumors which tend to produce brain metastases are most often localized in the lungs, breast, skin (melanoma), genitourinary tract, colon, rectum and paranasal cavities (4).

On conventional MRI, high grade glioma and solitary metastatic brain tumor often display similar signal intensity characteristics and contrast enhancement patterns (5). DWI has important advantages as it does not require contrast medium, it is a very quick technique and it provides qualitative and quantitative information that can be not only helpful but also essential in the evaluation of tumors (4). In many cases, a biopsy is performed for histologic confirmation even if there is a history of a known primary malignancy (5). If it is possible to obtain these data with MRI examination, this non-invasive modality will become more important both in the determination of the biopsy-sampling area within

the tumor and in the planning of the future therapies (7).

Alongside with tumor detection and characterization, DWI is a potential means of valuating the response of tumors and adjacent areas of vasogenic edema to drugs treatment (7, 8). Additionaly, preoperative DWI distinction between glioma and metastases is important for their surgical approach and therapeutic procedures, which could be completely different [1].

As intratumoral heterogeneity may further complicate the selection of a voxel of interest, the peritumoral region may provide more reliable and reproducible results due to its relative homogeneity (1). Because of their infiltrative nature, peritumoral edema of high grade gliomas represents a combination of neoplastic cell infiltration and vasogenic edema (1, 5). Peritumoral edema of intracranial metastases is purely vasogenic (5), originating from increased extracellular water from the leakage of plasma fluid from altered tumor capillaries, but no tumor cells are present (1). Therefore, the key to distinguishing between these two entities appears to lie in detecting the changes within the peritumoral area, that is, the area beyond the enhancing margin on the imaging (5). It is believed that ADC can be an important diagnostic and prognostic biomarker which shows good negative correlation with cellularity and grade of tumor malignancy. The nest of aberrant tumours` cells, which shows the most aggressive biological behaviour, within heterogeneous tumors, correspond to the lower ADC values (9).

Published data on intracranial tumors indicate that high ADC values were attributable to low cellularity, necrosis or cysts, and lower values to dense, highly cellular tumor (10). ADC is a direct reflection of tumor cell density (11). The results of previous research suggest that ADC values correlate with tumor cellularity for gliomas (12). Some authors (13) have proposed the use of ADC maps in malignant gliomas to demonstrate the boundaries between areas of tumor tissue (with decreased ADC due to elevated cellularity) and peritumoral areas (with increased ADC due to the presence of vasogenic edema).

Miquelini et al. found differences in the ADC values of apparently normal peritumor white matter between glioblastomas and cerebral metastases. The minimum ADC value measured in the apparently normal peritumor white matter was higher for the glioblastomas than for the metastases (14).

Sinha et al. (15) found that in high grade tumors, ADC values in the enhancing tumor region were larger than those in the peritumoral edema region. The lower ADC in tumor tissue may reflect a decreased volume of extracellular space due to higher cell density and increased intracellular viscosity, with a subsequent restriction of water motion (13). Mean diffusivity values in T2W hyperintense regions of presumably noninfiltrating neoplasms (specifically, metastases) were significantly higher (198% of the normal value) than mean diffusivity values in hyperintense regions touching gliomas (158% of the normal value) (8). Previous studies have shown that tumoral ADC is not useful for distinguishing between glioblastomas and metastatic tumors (6). However, Krabbe et al. (16) and Chiang et al. (17) found that the ADC values of cerebral metastasis are significantly higher than those of high grade astrocytoma. On the other hand, several studies have shown that peritumoral ADC is useful for distinguishing between glioblastomas and metastatic tumors (18). Our findings are in accordance with the previously reported ones. The areas of peritumoral neoplastic cell infiltration could be distinguished from predominantly peritumoral edema only if abnormalities were located in the white matter aligned in the direction of the diffusion-weighted gradient, published by Tien et al. (19). Kono et al. (20) do not support the hypothesis that peritumoral neoplastic cell infiltration can be depicted by ADCs or ADC maps. We found that the minimum ADC value of peritumoral edema in glioblastomas was significantly lower than the one in metastases. This finding may be helpful for preoperative differentiation between glioblastomas and metastases. A higher minimum ADC value in the peritumoral regions of metastases suggests that there are higher intracellular and extracellular water fractions than in glioblastomas (5). We also found that the mean minimum ADC values in the peritumoral regions of low grade gliomas were significantly higher than those in high grade gliomas.

Well differentiated adenocarcinoma showed hypointensity similar to gray matter which is attributed to inherently low T2 and high water diffusion in tissues and, as a result, both structures exhibited reduction of signal intensity on DWI. Well differentiated adenocarcinomas showed significantly lower SI than poorly differentiated adenocarcinomas and other histologic types of tumors. Therefore, this research suggests that the degree of differentiation may be relevant in their SI on T2-weighted images. The well differentiated adenocarcinomas can be derived from the lung, ovary and uterus, while metastatic brain lesions originate from the colon, lung or breast (21).

Our study had one limitation. The number of patients with brain metastases included in the study was relatively small. Further research with a larger number of patients with different kinds of brain metastasis is needed to confirm our results.

Conclusion

DWI with calculation of ADC maps can be regarded as a reliable useful diagnostic tool, which can provide additional diagnostic information for distinguishing gliomas from metastases. The values of ADC are significantly higher within the peritumoral edema surrounding brain metastases than in gliomas. Further research with a larger number of patients with different kind of brain metastasis is needed to confirm our results.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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"APPARENT DIFFUSION COEFFICIENT-(ADC)" PERITUMORSKOG TKIVA KAO DIFERENCIJALNO DIJAGNOSTIČKI MARKER MOŽDANIH METASTAZA U ODNOSU NA GLIOME

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Peritumorski edem kod visokogradusnih glioma predstavlja kombinaciju tumorske infiltracije i vazogenog edema, dok je peritumorski edem kod intrakranijalnih metastaza čisto vazogenog porekla.

Cilj ove studije bio je da ispita da li ADC može biti korišćen kao neinvazivni parametar u diferencijaciji peritumorskog edema moždanog tkiva kod metastatskih promena i kod cerebralnih glioma.

U ovoj prospektivnoj studiji analiziran je 71 bolesnik, 22 sa histoloski potvrđenim intrakranijalnim metastazama i 49 sa gliomima. Svi bolesnici su podvrgnuti konvencionalnom MRI i DWI sedam dana pre neurohirurške intervencije. ADC vrednosti su dobijene u tri regiona od interesa u okviru peritumorskog moždanog tkiva i upoređivane sa histopatoloskim nalazima.

Prosečne minimalne vrednosti ADC u peritumorskom tkivu niskogradusnih glioma su bile značajno veće (< 0,001) u odnosu na vrednosti nađenih kod visokogradusnih glioma. Prosečne minimalne vrednosti ADC u peritumorskom tkivu kod cerebralnih metastaza su bile značajno više nego one nađene kod visokogradusnih glioma. ADC vrednosti peritumorskog moždanog tkiva kod metastaza karcinoma pluća (0,000947 ± 0,000043 mm^2/s), melanoma (0,000842 ± 0,000018 mm^2/s) i karcinoma dojke (0,000783 ± 0,000048 mm^2/s) bile su značajno veće nego ADC vrednosti peritumorkog moždanog tkiva kod astrocitoma gradus I (0,000775 ± 0,000013 mm^2/s), gradus II (0,000411 ± 0,000005 mm^2/s), gradus III (0,000121 ± 0,000004 mm^2/s) i glioblastoma multiforme (0,000076 ± 0,000011 mm^2/s).

Minimalne vrednosti ADC peritumorskog edema kod moždanih metastaza bile su značajno veće u odnosu na vrednosti kod glioma. ADC vrednosti mogu imati dodatnu dijagnostičku vrednost u razlikovanju glioma u odnosu na moždane metastaze.

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Ključne reči: *MRI, moždane metastaze, DWI, moždani gliomi*

Review article

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HISTORICAL ASPECTS OF MEDICAL SIMULATION

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Medical simulation is a technique that replaces and enhances real experiences that has been used in education of health-care professionals since ancient ages. The wise, educated men of that time understood the importance of medical simulation by using simple models and techniques aiming at studying various medical fields, especially anatomy, physiology, obstetrics, and surgery. Simulation is a technique that replaces and enhances real experiences. It can evoke and replicate significant aspects of the real world in a completely interactive fashion. It has been widely used in the military and aviation industry, and in the last decades the use of simulation in medicine has also been established.

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Key words: *simulation, historical aspects*

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Introduction

Medical simulation is a technique that replaces and enhances real experiences that has been used in education of health-care professionals since ancient ages. The wise, educated men of that time understood the importance of medical simulation by using simple models and techniques aiming at studying various medical fields, especially anatomy, physiology, obstetrics, and surgery (1).

In 1908 a sculpture was excavated in Willendorf, Austria, dating back to the Paleolithic period. The figurine was cut from stones, with traces of red colour, disproportional physical attributes, representing a female figure.

It has been suggested that she is a fertility goddess. The figurine known as the Venus of Willendorf is estimated to have been carved around 24000 BC and is considered a forerunner of modern simulation mannequin. This type of figurine is a common art found in sites throughout Eurasia (2).

Figure 1. Sculpture of Vennus of Willendorf

(Preuzeto sa: https://arthistoryproject.com)

Numerous discoveries, especially scientific studies of mummies from ancient Egypt, revealed that people from such a civilization possessed a substantial knowledge of human anatomy, as additionally supported by carved figures from that time.

One of the earliest models in anatomy learning dates back to the ancient Maya civilization and a 'memento mori' clay sculpture from 300-750 BC, designed to remind us that life is short and death is inevitable.

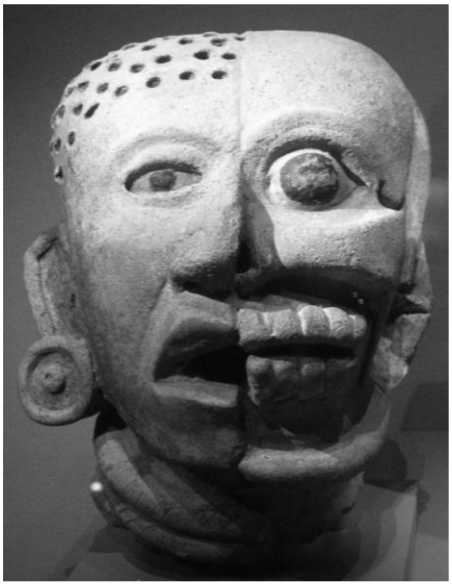

Figure 2. Pre-Columbian Mayan clay sculpture

(Preuzeto sa: http://commons.wikimedia.org/wiki/File:Ngv,_veracruz,_testa_con_vita_e_morte,_300-600_dc.jpg)

During the Song dynasty in China, the imperial court physician Wang Weiyi (987-1067) was responsible for standardizing teaching of acupuncture. He designed two life-sized bronze figures for studying acupuncture points (3). Under the Qing dynasty, examinations of female patients were performed indirectly by using miniature naked female models made of ivory. At that time only men were allowed to enter medical profession, but they were prohibited from touching female patients during examinations, except for pulse palpation, following a strict code of ethics. Family members of a female patient described the type of discomfort and indicated the painful location using these naked female figures. Then the doctor would make his medical opinion (4, 5).

The Sushruta Samhita medical text written in Sanskrit between the 4th and 6th century BC and discovered via the Silk Road in Asia had a great importance in the development of surgical technique simulators (6). The text describes how to repeatedly practice surgical skills and procedures using various experimental models for trying each surgical procedure. It was not until the 19th century that surgical stimulators were described again.

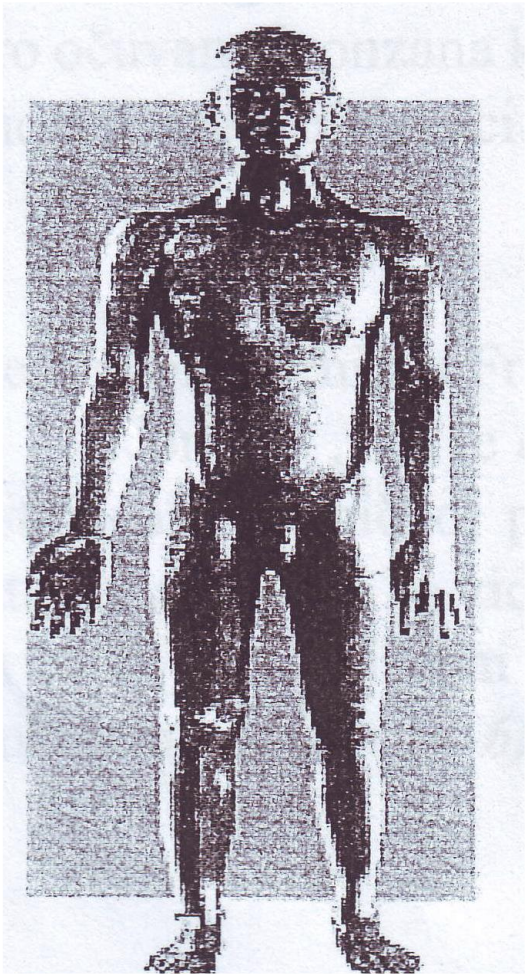

Figure 3. A figure of a man with acupuncture points

(Preuzeto sa: http://acupunctureforall.blogspot.com/2012/02/history-of-traditional-chinese-medicine.html)

In 1868 New York Medical Association Journal issued a report on Dr. Howard lecture "On the Radical Cure of Hernia", using a mannequin to demonstrate a new hernia operation technique (7).

In the new era, medical simulations started to be utilized in the 17th century, first in Italy, then in Germany, France, and Great Britain. Specialized workshops "La Specola" that made anatomical models out of wax for students' education appeared in Italy in the 18th century. Today, they are preserved as a precious testimony in the eponymous museum in Florence. The popularity of these models tended to increase at that time. They were changed due to pathological changes and as a result of different illnesses patterns and were made out of wax or other materials. Anatomical models were exhibited to the public in Europe and America in the first half of the 19th century, but popular interest in anatomical mo-

dels waned in the late 19th century and they were viewed only by students from medical schools.

An Italian painter and an architect, Ludovico Cardi Cigoli, created the first anatomical model in wax in 1598, called "Anatomia del Cigoli" – 'The Skinned Man'. He used a technology of wax body parts used as votive offerings in Catholic churches. The statuette is preserved in the National Museum of Florence together with a bronze copy.

Models similar to aforementioned ones, known as 'ecorches', were also made in France. These figures in standing artistic poses showed the bodies without skin, exposing the muscles and blood vessels that were used for medical students' education.

Figure 4 "Ecorche"

(Preuzeto sa: http//www.d-anatomystore.com/ecorche-male-anatomical-model)

Carved models of males and females in pairs, with anatomical structure of removable internal organs and a fetus attached to the uterus in models of females were used in Europe in the 17th and 18th century (8).

The first movable musculoskeletal model was mentioned in the 'Satyricon', the oldest existing novel in the world, written by Petronius in 61 AD. This satirical novel illustrates Roman life during the reign of Nero. During a dinner ceremony, a slave brought in a silver skeleton with movable joints. An educational model was made in Italy in the late 16th and early 17th centuries.

The earliest described medical simulation for trauma cases dates back to the 16th century, when king Henry II of France was injured. It was a serious eye-socket trauma and the king was treated by the royal court master surgeon Ambroise Pare who consulted Vesalius, the great anatomist. They performed experiments on executed criminals to find a cure for the king. The experiments were unsuccessful and the King soon died (8).

In the 18th century, Giovanni Galli, a surgeon from Bologna, developed the first obstetric simulator for the benefit of obstetricians and midwives. The simulator had a glass uterus and a flexible foetus. A female model with a glass uterus was also used in London in 1739 for obstetrics technique education (9).

Simulation of physiological processes started in the 18th century by using different mechanisms and machines and is associated with Abraham Chovet, a surgeon who designed a model of the fetal circulation in 1733. The circulation of blood was simulated through glass veins and arteries. In 1787 Dr. Cutler gave a description of the first cardiovascular simulator in a report to the Biological Society in Washington (9).

Ophthalmic surgery simulation is associated with the Ophtalmophantome, invented by Dr. Sachs in 1820. The mask phantom had a cadaver's or an animal's eye (10).

In 1879 Bacchi described laryngo-phantoms for exercising laryngoscopy. The first simulator for securing airways and endotracheal intubation was demonstrated by professor Otto Heubner in Vienna on the 5th of January, using O'Dwyer's tubes on cadavers (11).

In 1902 Killian demonstrated direct bronchoscopy to remove foreign bodies and he invented bronchoscopy simulator.

A computerized, realistic patient simulator, known as 'Sim One', was first used in 1966 for anesthesia training. The computer-controlled simulator was constructed to show drug dosage, blood pressure and heartbeat values. Replicated mannequin responses regarding the vital parameters and numerous side-effects were computer-controlled. The Sim One simulator was a phenomenon ahead of its time, nearly two decades before the introduction of computer technology and bioengineering in medicine.

Simulation is a technique that replaces and enhances real experiences. It can evoke and replicate significant aspects of the real world in a completely interactive fashion. It has been widely used in the military and aviation industry, and in the last decades the use of simulation in medicine has also been established.

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ISTORIJSKI ASPEKT SIMULACIONE MEDICINE

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Simulacija u zdravstvu je tehnika koja zamenjuje i pojačava stvarna iskustva, a koristi se u obuci zdravstvenih radnika i saradnika od davnina. Učeni ljudi toga doba su shvatili značaj korišćenja simulatora, jednostavnih modela i prostih tehnika u cilju izučavanja različitih oblasti medicine, posebno anatomije, fiziologije, akušerstva i hirurgije. Simulacija je tehnika koja zamenjuje i pojačava stvarna iskustva. Može da izazove i predstavi značajne aspekte stvarnog sveta na potpuno interaktivan način. Ima svoje potvrđeno mesto u avijaciji, vojsci, industriji, a poslednje decenije i u medicini.

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Ključne reči: *simulacija, istorijski aspekt*

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EFFECTS OF MICROWAVE RADIATION AND MELATONIN ON THE ACTIVITY OF ALKALINE AND ACID DNASE IN THE RAT BRAIN

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The toxic effect of the microwave radiation (MW) on human health usually manifests with the occurrence of various unspecified such as irritability, neurovegetative dystonia and insomnia. In the brain microwave radiation leads to thermal damage, oxidative stress induction and molecular changes in DNA. Melatonin is a neurohormone and a powerful antioxidant that reduces the damage of brain cells. The goal of this research is to analyze DNA fragmentation through the activity of alkaline and acid DNase in conditions of exposure to MW in the brain tissue and to monitor the melatonin effect on the activity of these enzymes. Wister rats were divided into four experimental groups: I(control), II(Mel)-the animals were given melatonin daily (2mg/kg), III(MW) animals were exposed to the MW for 20, 40 and 60 days (4h daily), IV(MW+Mel)-the rats that were given melatonin and were exposed to the MW as well. Animals were sacrificed after 20, 40 and 60 days of the experiment. In the brain of the rats that were exposed to microwave radiation a significant increase in the alkaline DNase activity (after 60 days) ($p < 0.05$) and acid DNase (after 20 days) ($p < 0.001$) were observed when compared to the control group. In animals that were exposed to microwave radiation and that were given melatonin a significant decrease in the acid DNase activity was observed in the brain when compared to the irradiated animals that were not given melatonin. It can be concluded that melatonin exerts significant anti-apoptotic and neuroprotective effect in the brain of animals exposed to the microwave radiation.

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Key words: *Melatonin, Microwave radiation, DNase, Brain*

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Introduction

The toxic effect of the microwave radiation (MW) on human health usually manifests with the occurrence of various unspecified symptoms, such as irritability, neurovegetative dystonia, and insomnia (1). Experimental animals exposed to relatively low microwave radiation intensity show signs of long-term memory and orientation capabilities disorders (2). Frequent exposures to the microwave radiation cause acute diseases and weakening of the immune system. It has been shown that if the person sleeps in the microwave radiation zone the organism recovery is not satisfying, the process of sleeping is superficial and intermittent and the person wakes up tired and unwilling. Usually, these persons complain at exhaustion, depression, nervousness, allergic manifestations, insomnia, a non-refreshing dream, nightmares, headaches, numbness in arms and legs.

Microwave radiation absorbed by the tissue converts into heat and manifests thermal effect. High temperature within tissues and organs leads to the damage of its function. This primarily refers to those tissues and organs with limited circulation and therewith constrained capability of releasing excess heat (usually refers to eyes and testicles) (3).

The non-thermal effects of microwave radiation lead to the induction of oxidative stress, as well as changes in DNA molecules and proteins in the brain tissue of experimental animals (4). Microwave radiation leads to the development of numerous disorders at the cellular level, such as the increased release of calcium ions in the culture of human neuroblastoma cells (5), the reduction in melatonin secretion or the disbalance in the dopamine-opiate system (6).

Numerous studies have shown the ability of microwave radiation to cause DNA damage and thus cause the carcinogenic effect. Lai and Singh (1995) have shown that microwave radiation dosage dependently causes interruptions of one or both DNA chains in brain cells of experimental animals. Later studies confirm that this is a consequence of damage to DNA reparation mechanisms, leading to cell apoptosis (7). It has been found that this DNA damage can be prevented by the use of anti-oxidants, immediately before and after exposure. This suggests that free radicals may play a significant role in the pathogenesis of DNA damage caused by microwave radiation. These effects are particularly expressed in the nerve tissue since neurons have a lower ability to repair DNA molecules.

Numerous studies suggest that long-term damage to DNA molecules by microwave radiation can lead to the formation of various neurodegenerative diseases, such as Alzheimer's and Huntington's disease. Since nerve cells do not show the ability to divide, malignancy is unlikely to occur and is most often the result of DNA damage, a disorder in cell function and/or the occurrence of cell death, which can lead to the development of neurodegenerative diseases or the acceleration of their development. On the other hand, another type of brain cells – glial cells may be malignantly altered due to DNA damage.

Long-term exposure to microwave radiation leads to the apoptosis in the brain tissue. The final result of the apoptosis is the fragmentation of DNA molecules by the endonuclease (DNase), which breaks down DNA chains at precisely defined sites. Endonucleases are enzymes that catalyze the proliferation of inter-nucleotide bonds simultaneously at several sites within the nucleic acid molecule. DNases are hydrolytic enzymes which disrupt both native and denaturated DNA molecules. Recently, DNasees have been increasingly referred to as chief executors of apoptosis responsible for the interleukemal fragmentation of the DNA cell into apoptosis. DNases responsible for the apoptosis may be classified into three groups: 1) alkaline DNase (DNase I), 2) CAD (caspase 3-dependent DNase) and 3) acidic DNase (DNase II) (Counis et Torriglia, 2000).

Melatonin is a neurohormone and is primarily synthesized and released from the pineal gland during the night, as its synthesis and secretion is inhibited by the light. Because its blood concentrations are significantly higher during the night and much lower throughout the day, it is often called "hormone of darkness" (8). It has been proven that this hormone recovers or even prevents the ageing process and the cancer development. In a lesser extent, melatonin is synthesized in extra-pineal tissues such as the retina, lens, brain tissue, thymus, respiratory epithelium, bone marrow, digestive tract epithelium, ovary, testicle, placenta, lymphocytes, and skin. Melatonin shows its effects by activating two G-protein-linked receptors (MT1 and MT2) (9). Melatonin receptors are located on the cell membrane and in the nucleus of CNS cells. However, some effects of melatonin are independent of the receptor, so that it can bind to calmodulin, thereby exhibiting antagonistic effects on the intracellular Ca-binding protein (10).

The Aim of study

This study aims to analyze DNA fragmentation, measuring the activity of alkaline and acidic DNase in conditions of exposure to microwave radiation in the brain tissue, and monitor the effect of melatonin on the activity of these enzymes.

Material and Methods

Experimental model

In the experiment white male rats of Wistar species (aged 8 to 10 weeks) were used, weighing about 200 grams, grown at the Institute for Biomedical Research, Medical School in Niš. The work with experimental animals was in accordance with the decisions of the Ethics Committee of the Medical Faculty in Niš.

For the purpose of the experiment, an experimental model was used for the exposure to microwave radiation, consisting of a mobile test phone and a PC measuring controller. Using this PC measuring device, the mobile phone is brought into a state of emission that corresponds to the normal mode of operation during a telephone conversation. The mobile test phone (Nokia Mobile Phones Ltd.) was located in a plexiglass box that was placed in the middle of the cage at the height of the floor. All animals were in plexiglass cages 30x40x40 cm in size.

Animals were exposed to microwave radiation in all experimental groups 4 hours a day, then moved to a room without sources of the electromagnetic field. Exposure to microwave radiation lasted for 20, 40 and 60 days. The electromagnetic field parameters in the cage were measured using the SPECTRAN HF 6080 instrument, manufactured by AARONIA AG (Germany). The range of the measured values of power, electric and magnetic fields were: E=9,884-18,356 V/m (electric field) and B=4,68-8,69 μT (magnetic field). Based on these parameters, the Specific Absorption Rate (SAR) for the whole body of the rat was calculated from 0.043 to 0.135 W/kg.

Laboratory animals (total 84) were divided into 4 experimental groups: I group (Control) – animals were intraperitoneally (i.p.) daily administered per 1,0 ml of saline; II group (Mel) - animals are given daily melatonin at a dose of 2 mg/kg body weight, intraperitoneally; III group (MW) – the animals were exposed to the microwave radiation of the mobile phone 4 hours a day, and 30 minutes before radiation they were applied to 1.0 ml of saline solution (i.p.); IV group (MW+Mel) – animals that are daily administered melatonin (at a dose of 2 mg/kg) are exposed to microwave radiation every day for 4 hours. Seven animals from each group were successively sacrificed after 20, 40 and 60 days of the experiment.

The animals were sacrificed after the experiment, in Ketamine Anesthesia (2 ml/kg BM), after a starvation period of 15 hours. After sacrificing experimental animals, the brain tissue was washed multiple times in a cold isotonic NaCl solution, immediately frozen at -20 °C and kept until homogenization. A 10% homogenate was then prepared in distilled water at 0 °C (on ice) using a homogenizer (IKA® Works de Brasil Ltd Taquara, RJ 22713-00).

Biochemical methods

Measurement of the activity of DNase (alkaline and acidic). The activity of DNase (alkaline and acidic) was determined by the method of Bartholeynes et al. (1975), using DNA as a substrate (Sigma-Aldrich, St. Louis, MO, USA) (11). This method is based on the spectrophotometric measurement of "acid-soluble nucleotides" extinction of which is read at 260 nm in the UV spectrum. The activity of the alkaline DNase was determined at an optimum pH of 7.4 with the use of TRIS-HCl buffer, with the addition of the Mg^{2+} ion activator, and the acidic DNase activity using the acetate buffer pH 5.0. The unit of activity of these two enzymes is defined by an increase in the absorbance of 0.001/min in a sample containing 0.132 mg of DNA at pH 7.4 or pH 5.0 and 3 ml of the reaction mixture. The DNase activity is expressed in international units per gram protein (U/g protein).

Determination of protein concentration. The amount of total protein in brain tissue was determined by the Lowry method (1951), with bovine serum albumin as standard (12).

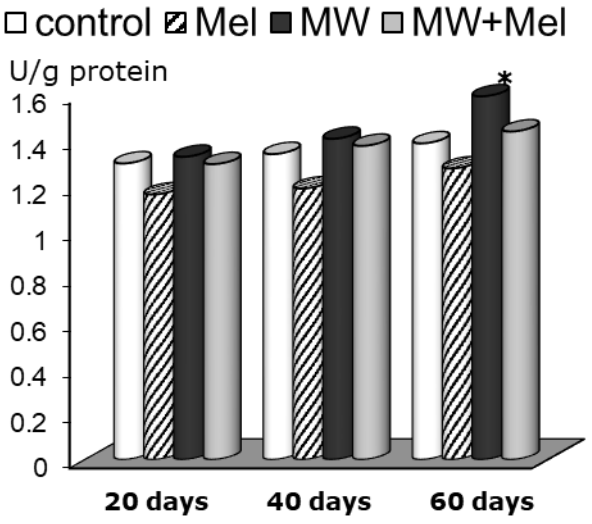
Statistical analysis

Statistical processing was done with Excel 7.0 and SPSS 11.0 in the Windows 2000 environment, with results displayed graphically.

Results

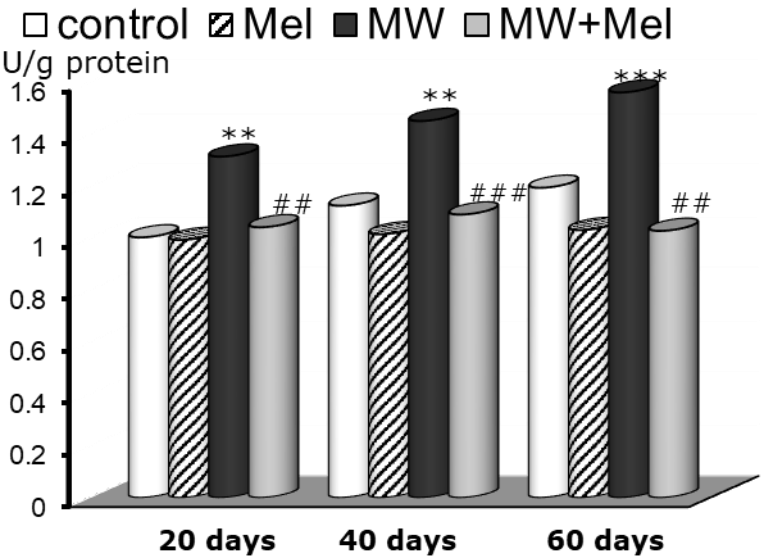
The effects of microwave radiation on the apoptosis process in the brain tissue of rats were being monitored by measuring the activity of alkaline and acidic DNase. The results are shown in Figure 1 and 2.

Alkaline DNase activity in brain tissue of rats exposed to microwaveradiation is shown in Figure 1. There was a significant increase of the enzyme activity in the brain of rats exposed to microwave radiation, after just 60 days of radiation exposure, as compared to the control group and the group to which melatonin was administered ($p < 0.05$). Administration of melatonin in animals that were exposed to microwave radiation caused no statistically significant change in alkaline DNase activity in brain tissue, as compared to animals which were exposed to radiation and not treated with melatonin (Figure 1).



* p<0,05 vs. control and Mel

Figure 1. The effect of melatonin and microwave radiation on the alkaline DNase activity in the rat brain (U/g protein)



***p<0.001 vs. control and Mel; ##p<0,01 vs. MW, ###p<0.001 vs. MW

Figure 2. The effect of melatonin and microwave radiation on the acid DNase activity in the rat brain (U/g protein)

Figure 2 shows the effects of melatonin on acid DNase activity in the brain of rats exposed to microwave radiation. As compared to the control group and the group in which melatonin was administered, there was a significant increase of acid DNase activity in the brain of rats exposed to microwave radiation, after 20, 40 and 60 days of radiation exposure ($p < 0.001$). Daily administration of melatonin to rats exposed to microwave radiation, in doses of 2mg/kg body weight, significantly decreased acid DNase activity in brain tissue, as compared to animals that were radiated and not treated with melatonin ($MW+Mel_{20}$ - 1.04 ± 0.03 vs. 1.31 ± 0.17, $p < 0.01$; $MW+Mel_{40}$ - 1.09 ± 0.06 vs. 1.45 ± 0.07, $p < 0.001$; $MW+Mel_{60}$ - 1.03 ± 0.13 vs. 1.56 ± 0.14 U/g protein, $p < 0.001$).

Discussion

Programmed cell death (apoptosis) in the CNS is a process mediated by various intracellular enzymes, inclucluding endonucleases which have an important place as enzymes that catalyze the internucleosomal fragmentation of DNA molecule (13, 14). Fragmentation of DNA molecule during apoptosis is a multistage process. In the initial stage, chromatin breaks into large fragments 50 to 1000 kb. Such fragmentation is essential for the continuation of apoptosis (15). At a later stage, apoptosis is associated with the internucleosomal DNA degradation, which is characterized by the production of standard fragments. The activity of DNases in the brain tissue is a marker of the apoptosis process since these enzymes are responsible for the hydrolytic internucleosomal fragmentation of DNA molecule.The results of our study show that the activity of alkaline DNase in the brain tissue is slightly increased during exposure to microwave radiation, after 60 days of exposure (Figure 1). The activity of acid DNase during exposure to microwave radiation in the brain was significantly increased after 20 days of exposure. Acid DNase activity is, according to our results, time dependent (Figure 2). Numerous studies have confirmed the significant role of acid DNase in the apoptosis process (16, 17). Otherwise, the acid DNase hydrolyzes native and denatured DNA molecules, by degrading the bond between the carbon in the 5'-pentose position and phosphorus, thereby forming 3'-nucleotides. Its presence in the nucleus, cytosol, and lysosomes has been confirmed. This enzyme breaks down a lower number of internucleotide bonds than alkaline DNase (18). The high activity of the acid DNase after 20 days of exposure in the brain tissue of the irradiated animals, in contrast to the alkaline DNase activity, can be explained by the fact that the pH in the brain apoptotic cells was most likely acidic.

Studies of Lai and Singh from 1995 and 1996 adduce that acute exposure to 2,45 GHz microwave radiation (strength density 2 mW/cm², SAR 1,2 W/kg) leads to a significant increase in single-stranded and double-stranded DNA interruptions in brain cells of experimental animals (7) These damages primarily relate to the tertiary and quaternary chromatin structure. In addition to the direct genotoxic effect of physical and chemical agents, single-stranded DNA interruptions are also an intermediate step during DNA reparation due to DNA-DNA and DNA-protein cross-linking. Single-stranded breaks of DNA also occur during reparation of double-stranded interruptions through recombination (19). The activity of endonucleases can be used as a measure of the reparation intensity, because it has been significantly increased in the brain tissue, during the exposure of animals to microwave radia-

tion. In this way, the phenomenon of DNA fragmentation under the conditions of chronic exposure to MT radiation can be explained.

The first experimental studies on the metabolic effects of melatonin in the brain tissue, following the chronic exposure to the microwave radiation, were published in 1996 by Lai et al. (1996), and they showed that melatonin, as a powerful antioxidant, reduces brain damage and successfully prevents disturbed functions of the central nervous system (19). This effect is also reflected in the prevention of morphologically visible damage to the cells of the central nervous system (20). This effect of melatonin is facilitated by its free passage through the blood-brain barrier, which is impermeable to other antioxidants.

The results of numerous studies indicate that lipid peroxidation caused by free radicals is one of the pathogenetic mechanisms involved in cell damage after exposure to microwave radiation. It has been proven that an elevated level of oxidative stress in brain tissue caused by microwave radiation has been successfully normalized after melatonin administration (21). The evidence for this claim lies in the fact that exposure to microwave species leads to an increase in reactive oxygen radicals (ROS) and a decrease in the concentration of melatonin in the brain. The decline in melatonin concentration is due to its increased take-up by tissues exposed to oxidative stress. It has been shown that treating rats with melatonin prior to exposure to microwave radiation blocks the side effects on the brain tissue (22).

Our research shows that the application of melatonin to irradiated animals significantly reduces the activity of acid DNase in the brain tissue (Figure 2), so this result can be interpreted as an antiapoptotic effect of melatonin by preventing DNA fragmentation. Numerous studies have shown the neuroprotective effect of melatonin, which is reflected in the inhibition of the apoptosis process, increase in the number of viable neurons, reduction of reactive gliosis, reduction of the oxidation of neuronal lipids and oxidative damage to DNA molecules (23, 24). It has been shown that melatonin induces gene expression of proapoptotic Bcl-2 proteins, which improves the survival of neurons (25, 26). Melatonin reduces the level of ROS and the intensity of apoptosis in rat astrocytes during laser radiation (27). Also, this neurohormone prevents the disturbance of the structure of DNA molecules caused by the action of free radicals and expresses an antiapoptotic effect in astrocytes of the rat brain (26).

Conclusion

By analysing the obtained experimental results, it can be concluded that DNA fragmentation in the apoptosis process measured through the activity of the alkaline and acid DNase is increased (in particular, the acid DNase activity) in brain tissue under the condition of exposure to microwave radiation, while the melatonin application has a significant anti-apoptotic and neuroprotective effect.

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EFEKTI MIKROTALASNOG ZRAČENJA I MELATONINA NA AKTIVNOST ALKALNE I KISELE DNAZE U MOZGU PACOVA

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Toksično dejstvo mikrotalasnog zračenja (MW) na zdravlje ljudi najčešće se ispoljava pojavom različitih nespecifičnih simptoma kao što su: razdražljivost, neurovegetativna distonija i nesanica. Mikrotalasno zračenje dovodi do termalnih oštećenja, indukcije oksidativnog stresa, promena na DNK molekulima u moždanom tkivu. Melatonin je neurohormon, koji kao snažan antioksidans smanjuje stepen oštećenja ćelija mozga. Cilj ovog istraživanja bio je da se analizira DNK fragmentacija, kroz aktivnost alkalne i kisele DNaze, u uslovima ekspozicije mikrotalasnom zračenju u tkivu mozga, i prati uticaj melatonina na aktivnost ovih enzima. Wister pacovi su bili podeljeni u četiri eksperimentalne grupe: I (kontrola), II (Mel) – životinjama je svakodnevno davan melatonin (2 mg/kg), III (MW) - životinje su 20, 40 i 60 dana izlagane MW (4h/dnevno), IV (MW+Mel) - pacovi kojima je aplikovan melatonin izlagani su MW. Životinje su žrtvovane nakon 20, 40 i 60 dana eksperimenta. U mozgu pacova koji su izlagani mikrotalasnom zračenju došlo je do značajnog porasta aktivnosti alkalne DNaze (nakon 60 dana) ($p < 0,05$) i kisele DNaze (nakon 20 dana) ($p < 0,001$) u odnosu na kontrolu. Kod životinja koje su izlagane mikrotalasnom zračenju i kojima je aplikovan melatonin došlo je do značajnog sniženja aktivnosti kisele DNaze u moždanom tkivu u odnosu na ozračene životinje koje nisu tretirane melatoninom. Može se zaključiti da aplikovanje melatonina životinjama koje su izlagane mikrotalasnom zračenju ima značajan anti-apoptotički i neuroprotektivni efekat u moždanom tkivu.

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Ključne reči: *melatonin, mikrotalasno zračenje, DNaze, mozak*

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QUALITY OF LIFE AND SEVERITY OF FATIGUE IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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In order to assess adequately the success of treatment in patients with systemic lupus erythematosus (SLE), it is necessary to evaluate their quality of life and severity of fatigue. This study aimed to investigate the quality of life of SLE patients, severity of fatigue they experience, and correlations between disease activity, organ damage and quality of life. The study involved 85 patients with SLE in whom the diagnosis was made based on the revised 1997 ACR criteria and 30 healthy examinees. The disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), organ damage was evaluated using the SLICC/ACR damage index (SDI), quality of life using the Medical Outcome Survey Short Form 36 (SF-36), and severity of fatigue using the Fatigue Severity Scale. The quality of life of SLE patients measured with SF-36 was significantly lower than that in healthy individuals ($p < 0.001$) and in most of the surveyed domains was not correlated with disease activity. The domain of physical functions in SLE patients demonstrated poorer results compared to mental functions, and the average results were below 52% in all SF-36 domains. Poorer quality of life was associated with a higher organ damage index (SDI), with the exception of emotional status domain. There was a significant difference in the severity of fatigue between SLE patients and controls ($p < 0.001$). Fatigue was positively correlated with organ damage ($p < 0.01$), and was not correlated with disease activity. Quality of life and severity of fatigue are associated more with organ damage than with disease activity in SLE patients.

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Key words: *systemic lupus erythematosus, quality of life, severity of fatigue*

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Introduction

SLE is a chronic inflammatory autoimmune disease characterized by multisystem clinical manifestations and serological finding of a multitude of antibodies (1). In order to assess adequately the success of treatment in SLE patients, in addition to the measurement of disease activity and degree of organ damage, the patient perception of one's own physical and mental health and degree of integration into the socity is necessary as well (2). The most commonly used standardized questionnaire for health-related quality of life (HRQoL) assessment, involving physical, psychological, mental and social domains, is the Medical Outcome Survey Short Form 36 (SF-36) (3, 4). The results obtained so far have been conflicting regarding the correlation of disease activity index, degree of organ damage in SLE patients and quality of life (4-8). Since fatigue is one of the primary symptoms in SLE patients, the severity of fatigue should be adequately assessed as well.

Aim of the paper

The aim of this paper was to assess the quality of life in individuals with SLE using the SF-36 questionnaire, as well as the severity of fatigue using the Fatigue Severity Scale (FSS). We also examined the correlation between disease activity index (Systemic Lupus Erythematosus Disease Activity Index – SLEDAI), organ damage index (Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for SLE – SLICC /ACR Damage Index – SDI) and quality of life.

Material and methods

Our cross-sectional study involved 85 patients with SLE aged over 18 years, hospitalized in the Institute „Niška Banja", in whom the definitive diagnosis of SLE had been made in accordance with the revised 1997 ACR criteria, with the presence of at least 4 out of the total of 11 criteria for the disease (9). Thirty healthy examinees constituted our control group. In all patients, the degree of disease activity was assessed using the SLEDAI activity index (10); the degree of organ damage was assessed using the SDI organ damage index (11); quality of life was assessed using the SF-36 questionnaire (12); and severity of fatigue was assessed using the FSS scale (13) (all standardized questionnaires). The SLEDAI index estimated disease activity in 9 organ systems based on the presence or absence of 24 variables during the examination. The values ranged from 0 to 105. The SDI index estimated organ damage in 9 organ systems and 3 disease complications. Each component was precisely defined in the glossary for SLICC/ACR damage index and was assigned a number of points. The SF-36 questionnaire consisted of 36 questions grouped in 8 domains, as well as the question about status changes. These domains were as follows: physical functioning, limitations related to physical difficulties, limitations related to emotional difficulties, vitality and energy, emotional status, social functioning, pain and general health. It also included three general, summative domains originating from the mentioned eight particular domains: physical health, mental health and general health. All the responses were assigned from 0 to 100 points, in accordance with the supplemented key, with more points indicating better quality of life. The fatigue scale contained 9 statements with possible answers graded from 1 to 7 (with 1 indicating „I completely disagree" and 7 indicating „I completely agree"). The average value was calculated from the sum of the values obtained for each question, i.e. statement, related to the severity of fatigue and degree of its impact on physical activity and motivation. Fatigue was considered serious if the average FSS scale value was over 4.

The entry and tabular representation of data was done using the MS Office Excel software package. Statistical calculations were performed using the SigmaStat 3.5 software. Attributive parameters were expressed as percentages, and continual (measurable) parameters were espressed as mean values (X) and standard deviations (SD), median (Md), coefficient of variation (CV) and 95% confidence interval (95% CI). Coefficient of variation was determined as the measure of homogeneity of the studied examinee samples related to the studied parameters. A homogenous sample was considered the one in which CV was 30 as a maximum. The correlation of continuous variables was established using the Pearson's coefficient of linear correlation (r).

Results

The study included 85 patients with SLE and 30 healthy controls. The average age of SLE patients at the time of the study was 45.3 ± 9.7 years (range, 22 to 64 years). The average age of control group subjects was 44.7 ± 9.5 years. In SLE group, there were 78 women (91.8%) and 7 men (8.2%), with female-to-male disease ratio of 11.1:1. Both groups were homogenous as to the age and gender distribution. The average disease duration in the studied group was 10.4 ± 8.0, and average age at the disease onset was 34.9 ± 9.4 years. The average period from the onset of symptoms of SLE to diagnosis was 13.1 ± 15.3 months (Table 1). At diagnosis, most of the patients fulfilled 4 and 5 criteria for the disease; 5 criteria (min 4; max 9) was the median, and the mean value of the number of criteria was 5.2 ± 1.2.

General disease manifestations, such as weakness, exhaustion and fatigue, were most common and present in 83 patients (97.6%). Arthritis and arthralgias were present in 80 patients (94.1%), skin changes in 76 (89.4%), photosensitivity in 61 (71.7%), serositis in 40 (47.1%), and hematological manifestations in 50 patients (58.5%). Lupus nephritis was found in 32 patients (37.6%), and neuropsychic manifestations in 16 (18.8%). The mean value of SLEDAI was 11.4 ± 7.5, with median value of 8 (min 0, max 36). The mean value of SDI was 1.8 ± 1.9, and median was 1 (min 0, max 9).

In 58 patients (68.2%) SDI was ≥1, and 27 patients (31.8%) had no organ damage. In 21 patients (24.7%) SDI value was 1; 20 patients had SDI of 2 or 3 (23.5%); and 17 patients (20.0%) had SDI ≥ 4. As for organ damage, neuropsychic and musculoskeletal changes were the most common and present in 23 patients (27.1%). In 21 patients (24.7%) cardiovascular changes were found, and eye lesions were present in 14 patients (16.5%). Renal and pulmonary changes were present in 13 patients (15.3%), skin changes in 3 patients (3.5%), and gastrointestinal changes in 2 patients (2.4%). Malignancies were present in 5 patients (5.9%), and diabetes mellitus in 2 patients (2.4%).

In all the domains of the SF-36, quality of life was significantly worse in the group of SLE patients compared to controls ($p < 0.001$). The domain of physical functions in SLE patients had poorer results compared to mental functions (36.7 vs. 49.1), and average results were below 52% in all the SF-36 domains (Table 2). Examining the correlation between the SLEDAI and quality of life as expressed in the SF-36, a negative correlation was found only between the limitations due to physical difficulties and SLEDAI ($r = -0.216$; $p < 0.05$). There was a negative correlation between all the SF-36 domains and SDI damage index ($p < 0.001$ for most of the domains), with the exception of emotional status and SDI, where no correlation was found (Table 3).

Fatigue was the predominant symptom in SLE patients, present in 97.6% of the cases. There was a significant difference in the severity of fatigue be-

tween SLE patients and controls (30 healthy examinees). The mean value of fatigue calculated using the FSS scale in SLE group was 5.8 ± 1.6 versus 3.1 ± 0.8 in controls ($p < 0.001$) (Table 4). Serious fatigue, expressed as the mean value of > 4 on the fatigue scale, was present in 70 patients with SLE (82.4%) and in only 5 controls (16.7%). There was not any correlation between the SLEDAI activity index and severity of fatigue assessed based on the FSS fatigue scale. We found a positive correlation between the SDI damage index and FSS scale value ($r = 0.324$; $p < 0.01$).

Table 1. Demographic characteristics of the subject

	Controls (n = 30)	SLE (n = 85)
Gender (M/F)	3/27	7/78
Age (years)	44.7 ± 9.5	45.3 ± 9.7
Disease duration (years)		10.4 ± 8.0
Age at diagnosis (years)		35.9 ± 9.7
Time to diagnosis (months)		13.1 ± 15.3

Table 2. Quality of life calculated using the SF-36

SF-36	Controls (n = 30)	SLE (n = 85)
Physical functioning	85.8 ± 13.1	38.9 ± 31.6*
Limitations related to physical difficulties	81.2 ± 19.3	42.4 ± 32.9*
Limitations related to emotional difficulties	75.5 ± 16.1	51.4 ± 29.6*
Vitality and energy	64.9 ± 19.3	31.7 ± 27.5*
Emotional status	70.1 ± 15.3	44.6 ± 27.9*
Social functioning	81.7 ± 14.2	51.3 ± 32.8*
Pain	77.4 ± 17.1	40.8 ± 30.0*
General health	74.2 ± 15.4	29.9 ± 22.3*
Physical health	76.7 ± 14.7	36.7 ± 26.7*
Mental health	75.7 ± 13.4	49.1 ± 26.9*
Total health	76.4 ± 13.8	41.0 ± 25.2*

* - $p<0.001$ vs. controls

Table 3. Correlation between all the SF-36 domains and SDI

SF36	SDI
Physical functioning	$r = -0.418$ $p < 0.001$
Limitations related to physical difficulties	$r = -0.411$ $p < 0.001$
Limitations related to emotional difficulties	$r = -0.384$ $p < 0.001$
Vitality and energy	$r = -0.313$ $p < 0.01$
Social functioning	$r = -0.341$ $p < 0.01$
Pain	$r = -0.382$ $p < 0.001$
General health	$r = -0.352$ $p < 0.001$
Physical health	$r = -0.410$ $p < 0.001$
Mental health	$r = -0.336$ $p < 0.01$
Total health	$r = -0.407$ $p < 0.001$

Table 4. The mean value of fatigue calculated using the FSS scale

FSS	Controls (n = 30)	SLE (n = 85)
FSS total score	27.8 ± 7.4	51.8 ± 14.3*
FSS mean value	3.1 ± 0.8	5.8 ± 1.6*

* - p<0.001 vs. controls

Discussion

SLE is a chronic disease which affects physical, social and psychological status of the affected. Although the survival rate of SLE patients has been dramatically improving over the last 50 years, quality of life of the affected is still relatively poor. Fatigue, fibromyalgia, depression and cognitive dysfunction significantly contribute to their poor quality of life. HRQoL, as it appears, is not so much associated with disease activity or organ damage, but the issue has still been debated in the literature (4-8, 14, 15). Various questionnaires have been used in quality of life assessments. Some of them are SLE-specific, but the one most widely used (and not only in SLE patients) is the SF-36 questionnaire (4, 16). Touma et al. have reported their results which sug-gest that SF-36 and LupusQoL are of similar value in the assessment of quality of life in SLE patients and that they represent sensitive enough quality of life measures for SLE patients with disease progression and exacerbations (17).

The studied group of 85 patients with SLE hospitalized for treatment in the Institute „Niška Banja" was a representative patient sample, similar by their demographic characteristics to other reported patient cohorts (18). The results of this study showed a significantly worse quality of life in all the SF-36 surveyed domains in SLE patients compared to control examinees ($p < 0.001$), which agreed with the literature data (5, 6, 14, 15, 19). We were also able to show that the average quality of life result was below 52% in all 8 examined domains, and that physical function domains yielded worse results compared to mental function domains, which was in accordance with the results obtained by a group of Portuguese authors (14). They also reported that there was no correlation between the SLEDAI clinical activity index and cumulative damage (SDI) with quality of life measured by the SF-36 questionnaire.

In the present study, examining the correlation between the SLEDAI disease activity index and quality of life assessed by SF-36, a negative correlation was found only between limitations due to physical activity and SLEDAI ($p < 0.05$), which was similar to the results of other studies, being mostly unable to find any correlation between quality of life and disease activity (5, 14, 15, 20).

The present study showed a significant negative correlation between all the SF-36 domains and SDI damage index ($p < 0.001$), except for the emotional status and SDI values, where there was no correlation, meaning that organ damage was associated with poorer quality of life. Some of the studies have reported the association of organ damage with quality of life in SLE patients (21). A study which investigated quality of life, degree of disease activity, organ damage, depression and fatigue in SLE patients, showed an association of quality of life, depression and fatigue, as well as daily glucocorticoid dose, and there was not any association of quality of life with the degree of disease activity and organ damage. SLE patients had stronger depression compared to control subjects. Similar to the results of this study, quality of life was markedly worse compared to the control group of healthy individuals (15).

Similar to this study, the study of 125 patients with SLE by Moldovan et al. in California (PATROL study) showed that the SLEDAI disease activity index was not correlated with quality of life measured using the SF-36, either among Latin Americans or among caucasians. Depression was significantly correlated with most of the SF-36 domains, except with general health, and age was significantly correlated only with the domain of physical function. Their conclusion was that depression had a considerable impact on quality of life in SLE patients, and disease activity did not have such an impact (20). The results of a cross-sectional study of prevalence and predictors of depression in 61 SLE patients have shown that depression and anxiety were common in SLE patients. Moreover, a high degree of anxiety and younger age may increase the risk of depression (22). There have been reports suggesting the loss of working ability in SLE patients, which is directly related to disease activity, more advanced age, incidence of thromboses and musculoskeletal manifestations, as well as with a greater number of clinical manifestations (23). Preservation of physical and mental functions in SLE patients or their better quality of life may help them to regain their working and general productivity, as the recommendations suggest (24). Furthermore, some more recent reports have indicated the importance of physical activity and physical exercise in people affected by SLE (25, 26). It is thought that one of the principal causes of morbidity in SLE patients is chronic, debilitating fatigue which reduces their quality of life, makes them unable to work and raises health care costs. The factors associated with fatigue include lack of physical activity, obesity, sleep disturbances, depression, anxiety, mood disorders, cognitive dysfunction, vitamin D deficiency or insufficiency, pain, effects of drugs, fibromyalgias, and other comorbid conditions. The results of a study showed that fatigue in SLE patients was similar to that in Lyme disease or multiple scle-rosis, and was significantly more severe than that in general population (27).

Some studies have reported a beneficial effect of aerobic or strength training on the improvement of health outcomes, including fatigue and quality of life parameters (27, 28).

The results of the present study showed that the severity of fatigue assessed using the FSS was significantly higher in the group of SLE patients compared to healthy controls ($p < 0.001$), which agreed with other studies' results (27). Serious fatigue, expressed as the mean value > 4 on the fatigue scale, was present in 82.35% patients with SLE and in only 16.67% healthy control group subjects. A significant correlation was also demonstrated between the SDI damage index and fatigue scale ($r = 0.324$; $p < 0.01$) (29), and there was no correlation between the SLEDAI disease activity index and fatigue. Fatigue was the predominant and most commonly encountered symptom in the studied group of patients, present in 83/85 (97.6%) patients. In other reported studies as well, the percentage of patients with SLE experiencing fatigue was rather high, and the impact of fatigue on numerous aspects of life (emotions, cognitive functioning, occupation, everyday social and family interactions and activities) has been stressed (29). Petterson et al., in their cross-sectional study of 324 patients with SLE, demonstrated that fatigue, pain, and musculoskeletal distress were the predomi-nant symptoms in about half of their patients. Only the patients reporting fatigue as the predominant symptom had lower mental and physical aspects of quality of life, which led to the conclusion about the importance of therapeutic interventions regarding these symptoms in order to improve the quality of life in SLE. Further, the same authors reported that the patients without these symptoms had better quality of life, lower grade depression and anxiety and lower activity of their disease (30).

The results of the present study showed that damage to the cardiovascular system is rather common and present in a quarter (24.7%) of the studied SLE patients. This agreed with other studies' results, suggesting that the list of potential consequences of severe fatigue and low-level physical activity in SLE was extended to involve an increased risk of cardiovascular diseases as well, among other factors due to an elevated proinflammatory biomarker-proinflammatory high density lipoprotein (HDL)-together with increased presence of carotid plaques (31, 32).

Conclusion

The quality of life of SLE patients measured by the SF-36 index was significantly lower compared to healthy individuals, and in most of the examined domains it was not correlated with disease activity. Lower quality of life was associated with a higher organ damage index (SDI) (with the exception of the domain of emotional status). Fatigue is a predominant symptom in patients with SLE. Quality of life and severity of fatigue were associated more with organ damage than with disease activity in SLE patients.

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KVALITET ŽIVOTA I INTENZITET ZAMORA KOD BOLESNIKA SA SISTEMSKIM ERITEMSKIM LUPUSOM

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U cilju adekvatne procene uspešnosti lečenja obolelih od sistemskog eritemskog lupusa (SLE) neophodna je procena kvaliteta života i intenziteta zamora. Cilj ove studije bio je ispitati kvalitet života kod obolelih od SLE, intenzitet zamora, kao i korelaciju između aktivnosti bolesti, oštećenja organa i kvaliteta života. Istraživanje je obuhvatilo 85 bolesnika sa SLE, kod kojih je dijagnoza postavljena na osnovu revidiranih ACR kriterijuma iz 1997. godine, kao i 30 zdravih ispitanika. Aktivnost bolesti ispitana je pomoću upitnika Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), oštećenje organa pomoću indeksa SLICC/ACR damage index (SDI), kvalitet života pomoću upitnika The Medical Outcome Survey Short Form 36 (SF-36), a intenzitet zamora korišćenjem Skale zamora (Fatigue Severity Scale). Kvalitet života bolesnika sa SLE meren indeksom SF-36 je značajno lošiji u odnosu na zdrave osobe ($p < 0,001$) i u većini domena nije u korelaciji sa aktivnošću bolesti. Domen fizičkih funkcija kod bolesnika sa SLE je pokazao niže rezultate u odnosu na mentalne funkcije, a prosečni rezultati su bili ispod 52% u svim domenima upitnika SF-36. Lošiji kvalitet života udružen je sa većim indeksom oštećenja organa SDI, izuzev u domenu emotivnog statusa. Postoji značajna razlika u intenzitetu zamora između SLE bolesnika i kontrolne grupe ($p < 0,001$). Zamor je u korelaciji sa oštećenjem organa ($p < 0,01$), a nije u korelaciji sa aktivnošću bolesti. Kvalitet života i intenzitet zamora povezani su više sa oštećenjem organa nego sa aktivnošću bolesti kod bolesnika sa SLE.

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Ključne reči: *sistemski eritemski lupus, kvalitet života, intenzitet zamora*

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PROBIOTICS: RATIONAL APPLICATIONS, PATIENTS' OPINION AND HEALTHCARE PROFESSIONALS' ROLE IN THEIR PROPER SELECTION AND USE

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Probiotics have gained worldwide use in great spectrum of indications. The aim of this study was to analyze the current market of probiotic-enhanced dietary supplements and fortified foods alongside patient's awareness of probiotic use. In addition, this work reviews contemporary knowledge on rational probiotic application in regard to recommendations from 1-4th Yale/Harvard workshop on probiotic use. Structured questionnaire was used to determine the attitude, knowledge level and habits of consumers in Niš, Serbia, in relation to products containing probiotics. A total of 363 individuals (age 18 - 80; 187 female and 160 male) responded to questionnaire which, in addition to the items of attitude, also assessed the health of consumers and acceptance and frequency of consumption of these products. In general, the attitude of respondents was positive and main named indications were different gastrointestinal disorders. Our results show that further information on rational use and potential positive health of probiotics. The healthcare professionals, especially physicians and pharmacists, should play the key role in patients' education.

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Key words: *probiotics, respondents' attitude, health benefits*

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Introduction

"Probiotics will be to medicine in the twenty-first century as antibiotics and microbiology were in the twentieth century." (As Dr. Michael McCann, MD, physician and researcher)

According to currently widely accepted definition, probiotics are live microorganisms which when administrated in adequate amounts confer a health benefit on the host (1). Probiotics and fermented foods (specifically fermented milks that are usually easily digestible) have a long history of safe use; they provide beneficial microorganisms to the human diet and may benefit human health in many ways (2). Probiotic microbial strains directly isolated from the fermented foods and beverages are shown to have anti Helicobacter pylori activity. Nair et al. Hypothesized that regular consumptions of these probiotics may have protective effect against peptic ulcer and gastric cancer for some populations (3). Since resident microbiota from mouth to rectum is an important factor for homeostasis and for the pathophysiological course of events, probiotics are reasonable and promising means of intervention (4).

The panel of International Scientific Association of Probiotics and Prebiotics recommended that the term "probiotic" should be used only for products that deliver live microorganisms with a suitable viable count of well-defined strains with a reasonable expectation of delivering benefits for the wellbeing of the host (2, 5).

Multi-strain probiotics appear to show greater efficacy than single strains, including strains that are components of the mixtures themselves. However, it is still unclear whether this is due to synergistic interactions between strains or a consequence of the higher probiotic dose used in some studies (6).

Each probiotic strain is a unique organism itself with specific properties that cannot be extrapolated from other, even closely related, strains (7). There are extremely large variations between the most popular taxa used as probiotics: Lactobacillus and Bifidobacterium. But the phylogenetical differences are also substantial between many of the different Lacto-

bacillus spp. (i.e. between L. acidophilus, L. Fermentum, L. reuteriand, L. plantarum) (4). Thus, probiotic performance of strains differs and host-related factors also have a great influence on overall health outcomes (7).

The applications of probiotics among others are: childhood and adult diarrhea, systemic immunemodulation, prevention of eczema in infants, the metabolic syndrome, liver injury, inflammatory bowel disorders, irritable bowel syndrome, management of side effects from antibiotics, colorectal cancer and radiation-induced enteritis (4, 5, 8). Bearing in mind huge differences between different types of probiotics, it is to be expected that the human body can respond differently to the different species and strains of probiotics (4).

In order to made recommendations for probiotics clinical use the first workshop meeting at Yale University (in conjunction with Harvard University), which included experts in the field of probiotic organisms, occurred in 2006.Second Yale/ Harvard workshop was held two years later, third in 2011 and fourth in March 2015 (9-12). At the last meeting the liver disease was included in to recommendations for the first time.

Since decision which probiotic to use in which clinical condition has remained confusing in some clinical conditions even for healthcare professionals, in this article we adopted and listed recommendations from these workshops on probiotic use (Table 1.) alongside brief overview of probiotic containing products from Serbian market (Table 2.).

The aim of this study was to analyze the market of probiotic-enhanced dietary supplements and fortified foods alongside patient's awareness and attitudes on probiotic use. In addition, this work reviews contemporary knowledge on rational probiotic application alongside recommendations from 1-4th Yale / Harvard workshop on probiotic use.

Table 1. Yale/ Harvard workshop recommendations for probiotic use (9 - 12)

Clinical Condition	No*	Specific probiotic strain
DIARRHEA		
Infectious childhood—treatment	1	*Lactobacillus* GG (LGG), *Saccharomyces boulardii*, *Lactobacillus reuteri* SD2112
Prevention of infection	2	*S. boulardii*, LGG
Prophylaxis of antibiotic-associated diarrhea	1	*S. boulardii*, LGG, combination of *L. casei* DN114 G01, *L. bulgaricus*, *Streptococcus thermophilus*
Prevention of *Clostridium difficile* associated diarrhea	2/3	LGG, *S. boulardii*
Prevention of recurrent *Clostridium difficile* associated diarrhea	2/3	*S. boulardii*, LGG, fecal microbiota transplant
INFLAMMATORYBOWEL DISEASE		
Pouchitis	1/3	VSL#3
Ulcerative colitis • Inducing remission • Maintenance • Crohn's	 2 1 3	 *Escherichia coli* Nissle, VSL#3 *E. coli* Nissle, VSL#3 *E. coli* Nissle, *S. boulardii*, LGG
IRRITABLE BOWEL SYNDROME	2 3	*Bifidobacterium infantis* B5624, VSL#3 *B. animalis*, *L. plantarum* 299V
NECROTIZING ENTEROCOLITIS	2/3	*L. acidophilus* NCDO1748, *B. bifidium* NCDO1453
IMMUNE RESPONSE	1	*L. rhamnosus* GG, *L. acidophilus* LAFT1, *L. plantarum*, *B. lactis*, *L. johnsonii*
ATOPIC ECZEMA ASSOCIATED WITH COW'S MILK ALLERGY	1	LGG, *B. lactis*
RADIATION ENTERITIS	3	VSL#3, *L. acidophilus*
VAGINOSIS AND VAGINITIS	3	*L. acidophilus*, *L. rhamnosus* GR-1, *L. reuteri* RC14
LIVER DISEASE		
Hepatic encephalopathy	1	VSL#3
Nonalcoholic fatty liver disease	3	VSL#3, combinations of *L. plantarum*, *L. delbrueckii*, *L. bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidium*, *S. thermophilus*, *B. longum*
Nonalcoholic fatty liver disease in children	3	VSL#3, LGG
Alcoholic liver disease	3	VSL#3, LGG, *L. acidophilus*, *L. bulgaricus*, *B. bifidium*, *B. longum* with oligosaccharides

* No. 1 - effectiveness denotes recommendation based on strong, positive, well-conducted, controlled studies;
No. 2 – positive, controlled studies but also the presence of some negative studies and
No. 3 – some positive studies but clearly an inadequate amount of work to establish the certainty of 1 or 2.

Examinees and methods

This study was conducted in a territory of Niš, Serbia by recording products from market and using face-to-face interview. Our 10 minute, 17-item questionnaire, inspired by similar research of Chin-Lee et al. (13), was designed in order to assess the knowledge, attitude and experience of patients towards probiotics use. The questionnaire consisted of three sections, containing both open and closed forms of questions. The first part consisted of questions related to the socio-demographic characteristics of each individual, while the second one comprised questions dealing with the knowledge on probiotic term and composition, (possible) usage of probiotics and symptomatology indications for probiotic use. The third group of questions dealt with the source of information/ recommendation and the origin of probiotic product; this part also revealed patients previous experience and attitudes towards the usage of probiotics and the data about the effects observed during its consumption. A total of 363 examinees patients (randomly chosen individuals from 18 to 80 years of age) were invited to complete a questionnaire while waiting in the community pharmacy. All questionnaires were completed anonymously, on a voluntary basis, and individual responses were not linked to specific patients. Patients were able to skip questions they did not wish to answer although its consequences exclusion from the study. Data obtained by filling out the questionnaire were statistically analyzed by SPSS software, 10th version. A probability value of $p < 0.05$ or less was deemed of significance.

Results

Analysis of marketed products (probiotic-enhanced dietary supplements and fortified foods) showed different content of broad spectrum of bacterial and yeast strains (Table 2.).

Table 2. Overview of probiotic containing products (dietary supplements and food) on Serbian market

Product	*Lactobacillus helveticus*	*Lactobacillus rhamnosus*	*Lactobacillus casei*	*Lactobacillus plantarum*	*Lactobacillus acidophilus*	*Lactobacillus salivarius*	*Lactobacillus bulgaricus*	*Lactobacillus reuteri*	*Lactococcuslactis*	*Lactobacillus paracasei*	*Bifidobacterium infantis*	*Bifidobacterium longum*	*Bifidobacterium breve*	*Bifidobacterium lactis*	*Bifidobacterium bifidum*	*Pedicoccuspentosecans*	*Streptococcus thermophilus*	*Saccharoyzcesboulardii*	*Bacillus subtilis*	CFU per dose
Biogaia protectis®								+												$1*10^8$
Bulacol®250																		+		$5*10^9$
Esenbak kolic®												+				+				$3*10^{11}$
Fermental®		+			+		+								+		+			$> 2*10^9$
Flobian®				+																$20*10^9$
Medicobiotic		+			+							+						+		$5*10^9$
Multilac®	+	+	+	+					+			+	+		+		+			$4,5*10^9$
Multilac® Baby		+	+	+	+	+			+	+		+			+					$1*10^9$
Probiotic®		+			+							+								$5*10^9$
Probalans imuno®*																			+	$2*10^9$
Probiochocco+Zn**					+									+						10^9
Probiodrops®	+	+																		$15*10^9$
Prolife®					+		+								+		+			$2,1*10^9$
ProbioKid immuno®					+						+				+					$5*10^9$
Food																				per 100 ml
AB jogurt					+										+					min 10^8
Balans +					+										+					min 10^8
Probiotik jogurt KPlus					+										+					min 10^8

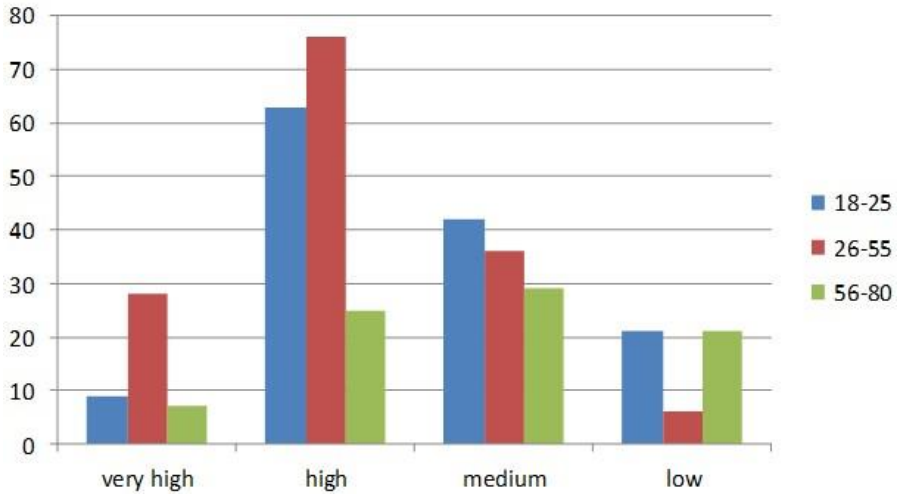
A total of 363 individuals (age from 18 to 80) responded to questionnaires. Since 16 questionnaires were incomplete and excluded from study, investigation included 347 examinees. Socio-demographic data are presented in Table 3. There were more female than male examinees (187 versus 160) and most (40%) participants were in the age group from 26 to 55. The most common education level was Bachelor's /Master's degree and only 9% (31) of examinees were medical professionals. According to our results there were no gender differences in probiotics use.

Table 3. Demographics of population – examinees

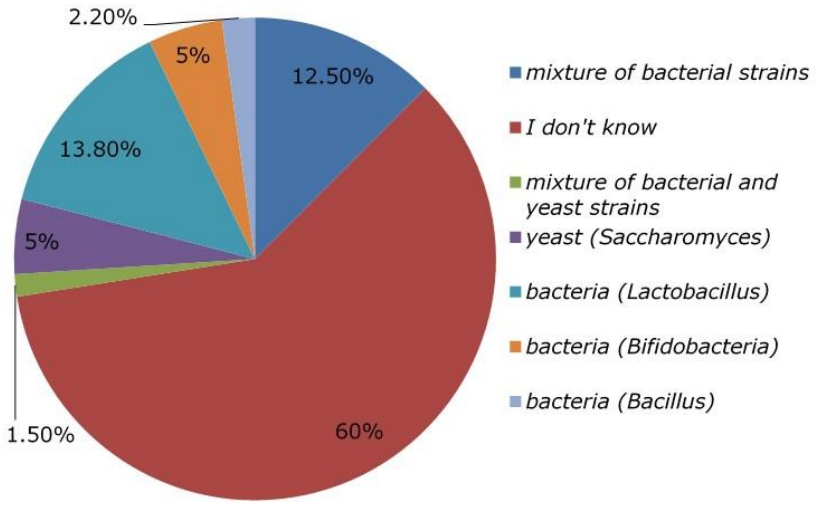
Demographic characteristic	% (n)
Age	44*
18-25	38 (132)
26-55	40 (139)
56-80	22 (76)
Female	54 (187)
Male	46 (160)
Education level	
High school diploma	15 (52)
Bachelor's /Master's degree	63 (219)
Doctorate or professional degree	22 (76)
Profession	
Pupils/students	33 (115)
Medicinal professionals	9 (31)
Non-medicinal professions	58 (201)

* - mean value of examinees age

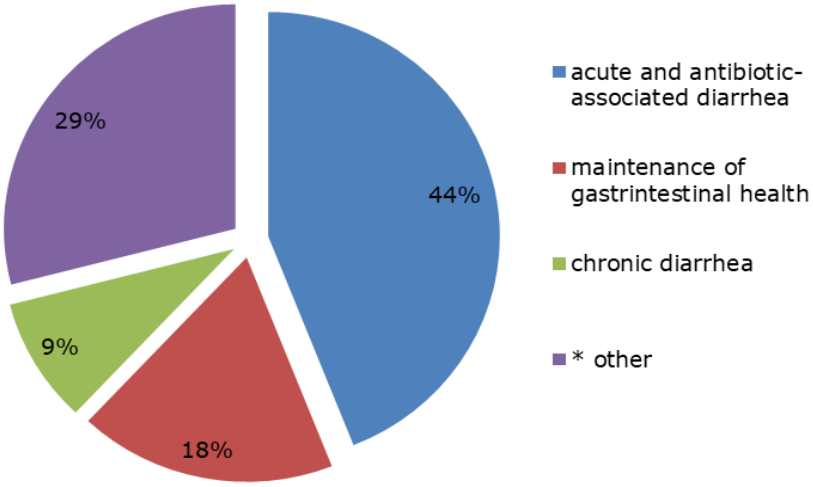
On Graphs 1 and 2 are presented main results of questions concerning overall knowledge on term probiotic. Indications for probiotic use according to our examinees are shown on Graph 3.



Graph 1. Level of specific knowledge concerning probiotics shown by different examinees age groups



Graph 2. Content of the probiotic products that were used, according to examinees



Graph 3. Indications for probiotics use identified by respondents
(*eczema, allergy, low immune function, dyspepsia, high cholesterol levels)

Discussion

Results of our analysis of probiotic containing products from the Serbian market (on the July 2016) that are presented in Table 2, shows that the largest number of products contains few milliards of colony forming units (CFU) per dose. On the other hand, food products with probiotics contains $1*10^9$ (one milliard) of CFU in 1 liter. Thus, patient should take more that 1 lit. of probiotic drink instead of 1 dose of probiotic-enhanced dietary supplement in order to obtain equivalent amount of CFU.

Bearing in mind recommendations from Table 1, in order to get the maximal health benefits, specific probiotic strain should be applied in specific medicinal condition.

Although probiotics have a long history of use, and offer a promising strategy for preventing and treating a large number of diseases, there are still doubts about their proper application and choosing the right probiotic preparations. Examinees were divided into four groups based on their personal perceptions on probiotic term knowledge – from low to very high level (Graph 1.). Graph 2 presents patients' specific knowledge on probiotic/prebiotic contents (Graph 2.). Since less than 10% of examinees were medical professionals we believe that our findings reflect real state of average patient awareness on this topic. Statistically significant difference was observed in the awareness of meaning and importance of probiotic use in relation to the education level of examinees ($p < 0.05$). There was no significant difference between knowledge levels of diffe-

rent age groups. From 243 respondents that have ever consumed probiotic products, about 42% were consumed both probiotic-enriched food and supplements while 45% were used only supplements. Most of the examinees that have previously used probiotic preparation didn't know the composition of probiotic products they used: even 60% answered "I don't know". Recognized probiotic strains were: Saccharomyces, Lactobacillus, Bifidobacterium and Bacillus. Around a quarter of respondents was familiar with named bacterial strains and believed that one of them is unique one used in probiotics formulation. Despite the result of our study that less than a half of examinees get familiar with term and use of probiotics through TV or internet promotion, named strains are frequently present in media and consequently adopted by auditorium. Our survey brings forward conclusion that of those patients with some knowledge of probiotics, the most common source of their information were medicinal professionals (45%) followed by family member or friend recommendation. On the contrary, the similar survey find out that the most popular sources of such data were TV, radio and internet (13).

Although previous investigations showed higher frequency of probiotic and prebiotic use in female population, there was no gender difference in our study. Also, there was no statistical significance in probiotic/prebiotic use experiences among three different age groups of examinees. In their questionnaire-based study, Schultz and al. (14) reported lower rate of previous experiences with probiotic use (around one-third of all respondents) toward our survey (52%). It could be, at least partly, explained by the date their study took place (2011.) and by growing popularity of probiotic applications.

The usually named indications for probiotic use are different gastrointestinal disorders. Probiotics indeed have been used in a variety of gastrointestinal illnesses with varying degrees of success and supporting evidence. Some of the proposed mechanisms of its action are multiple and include suppression or displacement of pathogenic bacteria, enhancement of innate immunity, and promotion of epithelial barrier function (15, 16). Despite the heterogeneity in effectiveness among the patients, the antibiotics, and the probiotic strains or blends, the pooled evidence suggests that adjunct probiotic administration is associated with a reduced risk of antibiotic-associated diarrhea (17).

Patients with inflammatory bowel disease and irritable bowel syndrome (in multicenter study conducted by Mercer et al. in 2012) doubts about the data on probiotics obtained from internet and/or media. They have expressed the need for reliable information from health care professionals (18). Our examinees (around half of all of them) also showed the highest level of trust in the information provided by health professionals. However, Williams et al. (15) tried to determine how perceive and use probiotic-based therapies in practice and concluded that gastroenterologists practice patterns did not consistently correlate with published, expert-panel-generated recommendations for evidence based probiotic use.

Only 22 of total 347 examinees linked probiotics and immunology, despite the fact that gut microflora is extremely important for immunological function of GIT (70% of organism's immune cells are located in intestinum). In survey of Green et al. (19) respondents were often aware of this probiotic's indication. Since probiotic research is still in its infancy, World Allergy Organization emphasizes that the full implications of probiotic supplementation for the treatment of allergic disease remain to be worked out. Further epidemiologic, immunologic, microbiologic, genetic, and clinical studies are necessary to demonstrate probiotic supplements efficiency in preventing allergy (20).

Probiotics could be locally applied in terms of prevention of recurrent vulvovaginal infections (21). A minority of our female respondents were aware about its positive effects when applied with the antimicrobial treatment in order to prevent vaginal candidiasis.

However, overall findings of this survey suggest that attitude towards probiotics at baseline was positive, as was patient experience with applied preparation. And furthermore, based on the results of our study obtained from voluntary subjects, we can say that average consumer/patient is interested in hearing more about possibilities for rational probiotic application, especially if that information is coming from healthcare professionals.

Conclusion

The great variety of dietary supplements is available today. A growing number of them are probiotic-enriched products. With the right selection and application probiotics could have a health-promoting effect. According to our results there is a certain level of awareness of the population on probiotic supplements and their positive effects, especially those on gastrointestinal health. Of course, this conclusion should be taken with reserve since our examinees were of higher education level than average ones. The healthcare professionals should play the key role in right selection and rational use of probiotics as well as in providing relevant public information on benefits of consuming probiotic products. Since probiotics enriched dietary supplements are over-the-counter products, further education of patients by health care professionals is preferred for recognizing their proper indications and applications.

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PROBIOTICI: RACIONALNA PRIMENA, STAVOVI BOLESNIKA I ULOGA ZDRAVSTVENIH PROFESIONALACA U NJIHOVOJ PRAVILNOJ SELEKCIJI I PRIMENI

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Upotreba probiotičkih dijetetskih preparata je poslednjih godina u porastu, kao i njihova širina indikacijskog područja. Cilj ovog istraživanja bio je analiza postojećih prehrambenih proizvoda i dijetetskih suplemenata koji sadrže probiotike na našem tržištu. Pored toga, rad evaluira nivo znanja i uverenja bolesnika u vezi sa upotrebom probiotika, a u odnosu na postojeće preporuke u literaturi (preporuke radionica o upotebi probiotika u organizaciji Yale/Harvard). Upitnik je sačinjen sa ciljem spoznaje stavova i nivoa znanja ispitanika o probioticima. Ispitanici koji su birani slučajno na teritoriji grada Niša (187 žena i 160 muškaraca, uzrasta od 18 do 80 godina) odgovarali su na pitanja vezana za učestalost i način upotrebe probiotika i ulogu zdravstvenih profesionalaca u sticanju informacija o mogućim pozitivnim zdravstvenim efektima upotrebe probiotičkih preparata. Uočen je pozitivan stav ispitanika prema upotrebi probiotičkih preparata, a najčešće navođene indikacije za primenu su različiti poremećaji gastrointestinalnog trakta. Rezultati rada ukazuju na potrebu za informisanjem o racionalnoj primeni i mogućim pozitivnim zdravstvenim ishodima upotrebe probiotika. Zdravstveni profesionalci, posebno lekari i farmaceuti, treba da imaju odlučujuću ulogu u podršci i daljoj edukaciji bolesnika.

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ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST ORAL PATHOGENES – INFORMATIVE ARTICLE

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Periodontal disease and Dental caries associated with dental plaque are the most common bacterial diseases, but also, significant oral health problem is *Candidiasis. Candida albicans,* is an opportunistic pathogen that can, under certain conditions proliferate and cause infections. The need for prevention and alternative forms of treatment and products for oral diseases comes from the rise in disease incidence, increased resistance by pathogenic bacteria to currently used chemotherapeutics. The products derived from medicinal plants have proven to be a source of biologically active substances, and thanks to their active principles, products based on medical herbs are more prevalent in modern phytotherapy. Essential oils are complex natural mixtures of volatile secondary metabolites – aliphatic and aromatic, terpinen and phenyl-propane compounds isolated from plants. The main constituents of essential oils are terpenes and sesquiterpenes including carbohydrates, alcohols, ethers, aldehydes and ketones, which are responsible for the fragrant and biological properties of plants. Different oils produce various pharmacological effects such as anti-inflammatory, antioxidant and anticancerogenic properties, but also oils are biocides. There are numerous *in vitro* studies that dealt with the research activities of natural herbal substances against oral bacteria that are known to be etiological factors in the development of oral and dental diseases. The phenolic major compounds of essential oils have been suggested to have a potential antifungal activity. There is ample of evidence that plant extracts and essential oils have the potential to be developed into agents that can be used as preventative or treatment therapies of oral diseases.

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Introduction

Periodontal disease and Dental caries are the most important global oral health problems (1). Diseases associated with dental plaque are probably the most common bacterial diseases occurring in man. Dental caries means the destruction of hard dental tissue and, if left untreated, may progress and cause death of vital pulp tissue, with possible spread of infection to the periapical region. The disease process is related to the acidogenic bacterial plaque (*Streptococcus mutans, Streptococcus sobrinus, Lactobacillus spp.*). Periodontal disease is initiated by microorganisms of dental plaque: Aggregatibacter actinomycetemcomitans, Porhyromonas gingivalis, Prevotella intermedia. In the oral cavity, bacteria are mainly accumulated on tooth surfaces above and below the edge of the gingiva, where they form a biofilm composed of bacterial microcolonies, extracellular layers, fluid channels, and communication systems. Adherent, gelatinous biofilm on the teeth, dental restorations, prostheses and implants can contain more than 700 different bacterial species. Dental biofilm can be prevented by regular daily tooth brushing and by effective chemotherapeutic agents (2-5). Also, significant oral health problem is *Candidiasis* caused by *Candida* species. *Candida* species is commensal yeasts in healthy people and can cause systemic infection under immune compromised conditions (6). *Candida albicans,* a fungus norma-

lly found in the human body and lives in balance with other microorganisms, is an opportunistic pathogen that can, under certain conditions proliferate and cause local and systemic infections mostly in immune compromised patients and those exposed to long-term therapy with antibiotics to eliminate bacteria, which also eliminates "friendly" *Lactobacillus spp.* that normalize the level of *Candida*. A similar situation occurs during pregnancy, due to hormonal imbalance and improper nutrition. Smokers, diabetics, or patients with partial and total prosthesis often suffer from candidiasis (7,8).

The global need for prevention and alternative forms of treatment and products for oral diseases that are safe, efficient and economical comes from the rise in disease incidence, increased resistance by pathogenic bacteria to currently used chemotherapeutics and antibiotics, opportunistic infections in immune compromised individuals and financial reasons in developing countries. Despite the few agents that are commercially available, these chemicals can alter oral microbiota and have unwanted side-effects such as vomiting, diarrhea and tooth staining. For example, the resistance observed in most (if not all) antibiotics that are commonly used in the treatment of oral infections (penicillins and cephalosporins, erythromycin, tetracycline and derivatives, metronidazole). Other antibacterial agents used in the prevention and treatment of oral diseases, including cetylpyridinium chloride, amine fluorides, chlorhexidine and other products containing such agents may exhibit toxicity, causing staining of the teeth or in the case of ethanol (commonly found in mouthwashes) are associated with increased risk of cancer of oral localization. Chlorhexidine is one of the most widely used biocides in antiseptic products. It can cause brown stains on the teeth, tongue and on restorations, burning sensation at the tip of the tongue and tastes change. In dental practice, the most commonly used antifungals are nystatin and fluconazole. It is believed that the presence of *C. albicans* in subgingival sites is in the form of biofilms, which could explain the resistance to antifungal therapy. Thus, the search for alternative products continues and natural phytochemicals isolated from plants are considered good alternatives to synthetic products (1, 3, 5, 6, 9).

Herbal medications have been used for centuries in the prophylaxis and treatment of many diseases. They have been used for thousands of years and there is numerous archaeological evidence that people in the prehistoric era used medicinal plants so that their application can be considered universal for all time. Some of the earliest known records dealt with the subject of healing with medicinal herbs. In rural areas of developing countries it is still the primary source of medicine care, and to 80% of people use traditional medicines for their health care. The natural products derived from medicinal plants have proven to be an abundant source of biologically active substances, many of which have been the basis for the development of new chemicals for the pharmaceutical industry. Thanks to their active principles, as well as the beneficial effect and efficiency, products based on medicinal herbs are more prevalent in modern phytotherapy (1, 2, 8, 10). With the development of experimental pharmacognosy, these products get scientific proof of its effectiveness, and, in today's world of medicine and dentistry, there is a growing interest in their application due to their therapeutic effect based on completely natural basis.

Effects of essential oil research

Essential oils are complex natural mixtures of volatile secondary metabolites - aliphatic and aromatic, terpinen and phenyl-propane compounds isolated from plants by hydro - or steam distillation and by expression (citrus peel oils). The main constituents of essential oils are terpenes and sesquiterpenes including carbohydrates, alcohols, ethers, aldehydes and ketones, which are responsible for the fragrant and biological properties of aromatic and medicinal plants. For centuries essential oils have been isolated from different parts of plants and also are used in various applications. Different oils produce pharmacological effects, demonstrating anti-inflammatory, antioxidant and anticancerogenic properties. Others are biocides against a broad range of microorganisms such as bacteria, fungi, viruses, protozoa. In dentistry, medical plants with essential oils are particularly used as flavour and odour corrigents in substances for oral hygiene such as toothpastes and mouthrinses. The mechanism of action of terpenes is not fully understood, but it is assumed that the degradation of lipophilic components of cell membranes involved in the antibacterial activity. For the antimicrobial assessment of essential oils, conventional methods of testing antibiotic abilities are usually applied. There are two basic techniques used for the assessment of both antibacterial and antifungal properties of essential oils: the agar diffusion method and the dilution method (2, 8, 10, 11).

Antimicrobial effects of essential oils in vitro

There are numerous *in vitro* studies that dealt with the research activities of natural herbal substances against oral bacteria. These studies have focused on bacteria that are known to be etiological factors in the development of oral and dental diseases. Earlier studies have clearly shown that many substances have the potential to be used in the dental industry, based on their effects on cariogenic bacteria and those that cause periodontal disease. Thus, for example, the researchers examined the effects of Tea tree essential oil in dentistry. During the 1930-ties, clinical efficacy of Tea tree oil was confirmed in different medical fields (8, 12-14). Tea tree essential oil is a highly lipophilic substance. Its mechanism is explained with lipophilic terpene (terpinen-4-ol), which penetrates microorganism cell membranes and acts against its structural permeability. In this way, Tea tree oil can affect the metabolism of some microorganisms with bactericidal or fungicidal effect. Even though the composition of Tea tree oil that provides optimal antimicrobial activity is still uncovered, there is strong evidence that terpinen-4-ol is the most

important component (8, 15, 16). Hammer and colleagues presented a study that aimed to test the activity of Tea tree essential oils (*Melaleuca alternifolia*) against 161 isolates of oral bacteria from 15 genera. Minimum inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) ranged from 0.003% to 2% (v/v). MIC values for *Actinomyces spp*, *Lactobacillus spp*, *Streptococcus mitis* and *sanguis* were 1% (v/v) and 0.1% (v/v) for *Prevotella spp*. Isolates of *Porphyromonas*, *Prevotella* and *Veillonella* had the lowest MICs and MBCs, and the isolates of *Streptococcus*, *Fusobacterium* and *Lactobacillus* had the highest. Time kill assays with *Streptococcus mutans* and *Lactobacillus rhamnosus* showed that treatment with more than 0.5% Tea tree caused decreases in viability of more than 3 colonies formed after only 30s, and viable organisms were not detected after 5 min. These studies indicate that a large number of oral bacteria are sensitive to Tea tree oil, suggesting that it can be used for oral hygiene (17).

In addition to Tea tree essential oil study that has been published, numerous other in vitro studies demonstrate the antimicrobial activity of essential oils of various medical herbs. Cha and colleagues have presented a study that aimed to investigate the composition and antibacterial activity of essential oils obtained from Cryptomeria japonica on oral bacteria. The chemical structure of the essential oil was analyzed by gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS). Sixty-eight compounds accounting for 95.82% of the oil were identified. The main components were α-pinene (6.07%), sabinene (8.86%), terpinen-4-ol (9.77%), α-terpineol (6.13%), elemol (11.17%) and 10(15)-cadinen-4-ol (7.16%). The essential oil and some of its major components were tested for antimicrobial activity against 15 different species of oral bacteria. The essential oil of *C. japonica* exhibited considerable inhibitory effects against all bacteria tested (MICs, 0.025 – 0.05 mg/ml; MBCs, 0.025 – 0.1 mg/ml), while its major components showed various degrees of growth inhibition. The study used the following oral bacterial cultures: *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus sobrinus*, *Streptococcus ratti*, *Streptococcus criceti*, *Streptococcus anginosus*, *Streptococcus gordonii*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia* and *Porphyromonas gingivalis*. The reference strains used in this study were: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pyogenes*. Brain-heart infusion broth supplemented with 1% yeast extract was used for all bacterial strains except for P. intermedia and P. gingivalis. For them, brain-heart infusion broth containing hemin and menadione was used. The MICs were determined for the essential oil and some of its major components by the broth dilution method, and were carried out in triplicate. The antibacterial activities were assessed after incubation at 37°C for 18 h (facultative anaerobic bacteria), for 24h (microaerophilic bacteria), and for 1–2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MICs plates, the MBCs were determined on the basis of the minimum concentration of the essential oil that kills 99.9% of the test bacteria. Ampicillin and gentamicin were used as standard antibiotics in order to compare the sensitivity of the test bacteria to essential oils and some of its major components (18). Takarada and colleagues have published a study which investigated the antibacterial effects of essential oils on the following oral bacteria: *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Streptococcus mutans* and *Streptococcus sobrinus*. Cariogenic and periodontopathic bacteria are present in dental plaque as biofilms. They tested manuka oil, tea tree oil, eucalyptus oil, lavandula oil and rosmarinus oil and determined the minimum inhibitory and minimum bactericidal concentration. The essential oils inhibited the growth of the bacteria tested, of which the most effective was manuka oil. MBC values showed that lavandula oil acts bacteriostatically, and the remaining oils, bactericidally. Periodontopathic bacterial strains were completely killed when treated with 0.2% manuka oil, tea tree oil or eucalyptus oil for 30s. Tea tree oil and manuka oil showed significant adhesion-inhibiting activity against *P. gingivalis*, and all the essential oil tested inhibited the adhesion of S. mutans. This study demonstrated that, among the essential oils, manuka oil and tea tree oil had strong antibacterial effects on cariogenic and periodontopathic bacteria. For safety reasons, the authors also investigated the effects of essential oils on cultured human umbilical vein endothelial cells and found that, at a concentration of 0.2% they had little effect on cells (19). Gursoy et al. have published a study whose aim was to investigate the bacterial growth inhibiting and anti-biofilm effects of *Satureja hortensis L.* (summer savory), *Salvia fruticosa M.* (sage), *Lavandula stoechas L.* (lavender), *Myrtus communis L.*, and *Juniperus communis L.* (juniper) essential oils. Chemical compositions of the essential oils were analyzed by gas chromatography–mass spectrometry, minimum inhibitor concentrations with the agar dilution method, and anti-biofilm effects by the "microplate biofilm" assay. They also tested the toxicity of each essential oil on cultured keratinocytes. The bacterial strains used in this study were: *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*; *Porphyromonas gingivalis*; *Parvimonas micra* (formerly *Peptostreptococcus micros*); *Tannerella forsythia* (formerly *Bacteroides forsythus*); *Fusobacterium nucleatum*; *Prevotella intermedia*; *Prevotella nigrescens*. The strains were revived from frozen (-70°C) stocks and subcultured for purity. Bacteria were grown on Brucella blood agar enriched with hemin, and incubated at 37°C in an anaerobic chamber for 5 days. Of the 5 essential oils, *S. hortensis L.* essential oil had the strongest growth inhibition effect. Subinhibitory dose of *S. hortensis L.* essential oil had antibiofilm effects only against *Prevotella nigrescens*. Essential oils did not affect the viability of keratinocytes

at the concentrations of 1–5 µl/ml, but at the concentration of 5 µl/ml epithelial cells detached from the culture well bottom. The results from this study suggest that *S. hortensis L.* essential oil inhibits the growth of periodontal bacteria in the concentration that is safe on keratinocytes, but, in the subinhibitory concentration its anti-biofilm effect is limited (5). In "*in vitro*" studies are also used commercially available solutions based on essential oils such as Listerine TM (Essential Oils Rinse), which are often compared with other available anti-plaque mouth rinses. Pan and colleagues have performed a study that aimed to compare the antimicrobial activity of commercially available antiseptic mouth rinses against saliva-derived plaque biofilms in static and flow-through biofilm systems in vitro. The test treatments were the following commercially available mouth rinses: an essential oil (0.064% thymol, 0.092% eucalyptol, 0.060% methyl salicylate and 0.042% menthol) rinse (EO, Cool Mint, Listerine, Johnson and Johnson); a 0.12% chlorhexidine rinse (CHX, Peridex, 3M Pharmaceuticals), seven 0.05% cetyl pyridinium chloride rinses (CPC1, PLAX global mouth rinses, Colgate Palmolive); a 0.05% cetyl pyridinium chloride/0.05% chlorhexidine rinse (CPC/CHX, Perio-Aid, Dentaid); an amine fluoride/stannous fluoride rinse (AFSF, Meridol, GABA); a 0.07% cetyl pyridinium chloride rinse (CPC2, Crest Pro Health, Clean Mint, Proctor & Gamble); Sterile water or phosphate buffered saline (PBS) and 70% ethanol (Etoh) served as the negative control and positive controls respectively. So, nine mouth rinses were tested in a recirculating flow-through biofilm model (RFTB) with viability assessment by ATP bioluminescence. Five mouth rinses were evaluated in a batch chamber slide biofilm (BCSB) model, using live/dead staining and confocal laser scanning microscopy. In the RFTB model, essential oil and chlorhexidine – containing rinses showed equivalent antimicrobial activity and were more efficient than a range of cetyl pyridinium chloride (CPC1) formulations. In the BCSB model, twice daily mouth rinse exposure demonstrated that the EO rinse was significantly more effective than rinses containing amine and stannous fluorides (AFSF), a combination of CPC/CHX, and another CPC formulation (CPC2). Essential oil showed biofilm kill ability comparable to the CHX rinse (20). Filoche et al. have published a study that aimed to compare antimicrobial effects of essential oils alone and in combination with chlorhexidine digluconate against planktonic and biofilm cultures of *S. mutans* and *Lactobacillus plantarum*. The essential oils included cinnamon, tea-tree (*Melaleuca alternifola*), manuka (*Leptospermum scoparium*), *Leptospermum morrisonii*, *arnica*, *eucalyptus*, *grapefruit*, the essential oil mouth rinse *Cool Mint Listerine* and two of its components, menthol and thymol. Cinnamon had the highest antimicrobial potential (1.25 – 2.5 mg/ml). *Manuka*, *L. morrisonii*, tea-tree oils, and thymol also showed antimicrobial potency but to a lesser extent. The effect of combination of the essential oil–chlorhexidine was greater against biofilm cultures of both *S. mutans* and *L. plantarum* than against planktonic cultures. The amount of chlorhexidine required to achieve a similar growth inhibition against the biofilm cultures was reduced 4–10-fold in combination with cinnamon, manuka, L. morrisonii, thymol, and Listerin (21).

The antimicrobial activities of the essential oil individual components also were published. Carvacrol is one of the most common essential oils components which exhibit antibacterial activity, and is the major component of oregano and thyme (10). Botelho et al. presented a study whose main objective was to examine the composition and antimicrobial activity of *L. sidoides* essential oil. *Verbenaceae*, popularly known as "*Alecrimpimenta*" is a typical shrub commonly found in the Northeast of Brazil. The leaves of *L. sidoides* are extensively used in popular medicine for the treatment of skin wounds and cuts. In this study, the essential oil was obtained by hydro-distillation and analyzed by GC-MS. Twelve compounds were characterized, having thymol and carvacrol as major constituents. The antimicrobial effects of the essential oil and the main components was tested against cariogenic bacterial strains of the genus *Streptococcus* as well as *Candida albicans* using the broth dilution and disk diffusion assays. The essential oil and its major components (thymol, carvacrol) demonstrated potent antimicrobial activity against the microorganisms tested with minimum inhibitory concentrations ranging from 0.625 to 10.0 mg/ml. The most sensitive microorganisms were *C. albicans* and *Streptococcus mutans*. The essential oil of *L. sidoides* and its major components exert promising antimicrobial effects against oral pathogens (22). Didry and colleagues have investigated the antimicrobial activity of thymol, carvacrol, eugenol and cinnamaldehyde alone or combined on eight oral bacteria. The strains were obtained from culture collection or isolated from human clinical samples. Strains used were *Streptococcus mutans*, *S. sanguis*, *S. mitis* and *S. milleri* for aerobes, *Peptostreptococcus anaerobius*, *Prevotella buccae*, *P. oris* i *P. intermedia* for anaerobes. After determination of minimum inhibitory concentration of components, investigators studied the effect of their combinations. The components showed an inhibitory effect on seven bacteria and a synergistic effect was observed with some combinations. The four compounds can be used alone or combined, as eugenol and thymol, eugenol and carvacrol, thymol and carvacrol, during the treatment of oral diseases (23).

Antimicrobial effects of oral care products containing essential oils

Oral hygiene is the key to oral health. Mechanical methods for oral hygiene, brushing and flossing are defined by clinicians as the gold standard methods of plaque control. However, despite this emphasis on mechanical methods of plaque control, gingival inflammation prevalence is still high. Therefore, other agents for the maintenance of oral hygiene such as mouth rinses with anti-plaque and anti-gingivitis properties that can add to the effects of me-

chanical plaque control may have clinical relevance (24). It is well established that antibacterial mouth rinses are effective in reducing plaque on tooth surfaces. Mouth rinses may contain fluorides, alcohols, detergents and other antimicrobial substances. Such synthetic antimicrobial agents include povidone iodine products, chlorhexidine and cetylpyridinium chloride. Toothpastes also contain fluoride and other substances including triclosan and zinc citrate. Natural antimicrobial substances are now attracting attention as useful antimicrobial agents to be added into mouth rinses and toothpastes. For example, extracts of tea tree oil, peppermint, green tea and manuka honey have recently been added into such products in order to improve their antimicrobial properties. Mouth rinses containing essential oils are generally recognized as safe and effective products and recommend for oral hygiene. Numerous studies have been conducted which included a commercially registered mouth rinses containing essential oils. Listerine TM (Essential Oils Rinse) contains the active ingredients thymol, eucalyptol, methyl salicylate and menthol and has been in widespread use for many years. Thymol and eucalyptol are antimicrobial, while methyl salicylate and menthol act as a cleaning agent and anesthetic respectively. *In vitro* and *in vivo* studies have demonstrated the potential of essential oil mouth rinse use in the control of plaque-related diseases (1, 2, 25).

The clinical efficacy of EO and chlorhexidine rinses in the reduction of plaque and gingivitis has been extensively assessed. Since there is the fact that chlorhexidine eventually leads to the appearance of side effects that have already been mentioned, mouth rinses based on essential oils tend to be increasingly used. A number of recent studies add to the evidence that essential oils may be suitable additives in products used for the maintenance of oral hygiene or prevention of dental disease. Fine DH and colleagues have conducted two studies to determine the antimicrobial effect of rinsing with an essential oil-containing mouth rinse 12 hours after a single rinse and 12 hours after 2 weeks of twice daily rinsing, during the daytime and overnight. It was a randomized, double-blind controlled study with "crossover" design. Following baseline sampling of bacteria from supragingival plaque and the dorsum of the tongue, subjects began twice-daily rinsing with either an essential oil mouth rinse containing 0.09% zinc chloride (Tartar Control Listerines Antiseptic) or a negative control rinse. Bacterial sampling was repeated 12 h after the first rinse and again 12 h after the final rinse 14 days later. Samples were plated on Schaedlers medium (total anaerobes), Schaedlers Nalidixic/Vancomycin medium (Gram-negative anaerobes), and OOPS medium (volatile sulphur compound (VSC)-producing organisms). This study demonstrated that rinsing with the essential oil mouth rinse can have long-lasting effects in reducing anaerobic bacteria overall as well as Gram-negative anaerobes and VSC producing bacteria (26). Fine et al. have published a study that aimed to investigate the effect of rinsing with an essential oil-containing mouth rinse on levels of specific supra and subgingival bacteria in subjects with gingivitis. Fifteen subjects meeting entry criteria completed this randomized, controlled, double-blind, crossover study. Subjects were required to have ≥ 1000 target microorganisms per ml in all samples taken from two subgingival sites. Following sampling of supra and subgingival plaque, subjects began twice-daily rinsing for 14 days with either an essential oil-containing mouth rinse (Cool Mint Listerines Antiseptic) or a negative control. Then, plaque was again sampled on the fifteen day, and the procedure repeated after a week washout period with subjects using the alternate rinse. The study showed that compared to the negative control, the essential oil mouth rinse produced significant reductions in supragingival plaque levels of *Veillonella sp*, *Capnocytophaga sp*, *Fusobacterium nucleatum*, and total anaerobes and respective reductions in subgingival plaque (27). Charles and colleagues have presented a study that aimed to compare the anti-plaque and anti-gingivitis effectiveness and the side-effect profiles of an essential oil-containing mouth rinse and a chlorhexidine-containing mouth rinse. One hundred and eight eligible patients from 20 to 57 years were randomly assigned into 3 groups: essential oil mouth rinse (Listerines Antiseptic); 0.12% chlorhexidine mouth rinse (Peridex); or 5% hydroalcohol negative control. At baseline, subjects received a complete oral soft tissue examination and scoring of the Loe–Silness gingival index, Quigley–Hein plaque index Volpe-Manhold calculus index and Lobene extrinsic tooth stain index. After a complete dental treatment patients began to take twice daily their respective mouth rinse as an adjunct to their usual mechanical oral hygiene procedures. Subjects were reexamined at 3 and 6 months. This six-month controlled clinical study demonstrated that the essential oil mouth rinse and the chlorhexidine mouth rinse had comparable anti-plaque and anti-gingivitis activity. The chlorhexidine mouth rinse group had significantly more calculus and extrinsic tooth stain than either the essential oil mouth rinse group or the control group (28).

The effects of essential oils on Candida species

Candidiasis is a term for a fungal infection caused by Candida species. The spectrum of diseases caused by Candida includes infections of skin, mucous membranes and internal organs. Although over hundred species of Candida are known, not all are pathogenic to humans. Significant pathogens are *Candida albicans*, *Candida tropicalis*, *Candida parapsillosis*, *Candida guilliermondi*, *Candida krusei*, and *Candida stellatoidea*. *C. albicans* is the most common type of Candida species. Candida species are harmless commensals, and are a part of the normal flora of the pharynx, intestine, vagina, perianal skin folds, and mouth. The dorsum of the tongue is the primary oral reservoir for these yeasts, but they also can be on mucosal surfaces and in dental plaque. It was suggested that when *C. albicans* accessed the periodontal tissues, they may be damaged by the

production of metabolites by these yeasts (6, 11, 29). The efficacy of essential oils of various medical herbs against Candida species has been extensively assessed (7, 12, 30-32).

Oropharyngeal candidiasis is the most common opportunistic infection that occurs in the oral cavity. The expression of *Candida albicans* virulence in the oral cavity is strongly associated with the decline of the immune system, especially in patients with AIDS. Certain conditions, such as hyposalivation, diabetes mellitus and prolonged antibiotic and corticoid therapy can predispose to oral candidiasis. Specific features of these fungi that contribute to the development of oral candidiasis include its ability to colonize the oral mucosa and form germ tubes. It is accessible a large number of antifungal agents for the treatment of infection caused by Candida. The main agents belong or to the polyenes, such as amphotericin B and nystatin, or to the azoles, such as itraconazole and fluconazole. Studies have shown the resistance of *Candida albicans* to azole, and also on the nephrotoxicity and hepatotoxicity associated with the polyenes, especially with the use of amphotericin B. To avoid all these drawbacks, it is necessary to search constantly for new and effective medicines to treat this fungal infection (29, 33).

The antifungal effect of essential oils of many aromatic plants has been described in various studies. Anticandidal activity is also well described. The phenolic major compounds of essential oils have been suggested to have a potential antifungal activity. Chami and colleagues have published a study that aimed to evaluate the therapeutic efficacy of carvacrol and eugenol, the main components (phenols) of essential oils of some aromatic plants, in the treatment of experimental oral candidiasis caused by *Candida albicans* in immunosuppressed rats. Anticandidal activity was analyzed by microbiological and histopathological techniques, and it was compared with nystatin, which was used as positive control. Microbiologically, carvacrol and eugenol reduced the number of colony forming units sampled from the oral cavity of rats treated for eight consecutive days, compared to the untreated control group of rats. Treatment with nystatin gave similar results. Histologically, the untreated group of animals showed numerous hyphae on the epithelium of the dorsal surface of the tongue. In the group of animals treated with carvacrol hyphae were not seen on the epithelium, whereas in rats treated with eugenol has seen only a few focal areas on the dorsal surface of the tongue. In the group treated with nystatin hyphae were found in the folds of the tongue mucosa. Therefore, the histological data were confirmed by the microbiological tests for carvacrol and eugenol, but not for the group treated with nystatin. Carvacrol and eugenol could be considered as strong antimycotic agents and might be proposed as therapeutic products for oral candidiasis (33). Jandourek et al. have published a study with the objective to evaluate the efficacy of melaleuca oral solution in AIDS patients with fluconazole-resistant oropharyngeal candida infections. The study comprised thirteen patients with AIDS and oral candidiasis documented to be clinically refractory to fluconazole, as defined by failure to respond to a minimum of 14 days of ≥ 400 mg fluconazole per day. In addition, patients had in vitro resistance, which is defined by minimal inhibitory concentrations of ≥ 20 μg/ml. Patients were given 15 ml melaleuca oral solution four times daily to swish and spit for 2-4 weeks. Evaluations were performed weekly for 4 weeks and at the end of therapy for clinical signs of oral candidiasis. A total of 13 patients who participated in the study, 12 were evaluable. After 2 weeks, seven out of 12 patients had improved, none were cured, and six were unchanged. After 4 weeks, eight out of 12 patients showed a response (two cured, six improved), four were non-responders, and one had deteriorated. A mycological response was seen in seven out of 12 patients. A follow up evaluation 2-4 weeks after therapy was revealed that there were no clinical relapses in the two patients who were cured. Therefore, it is estimated that melaleuca oral solution may be an effective alternative in AIDS patients with oropharyngeal candidiasis refractory to fluconazole (34). Shrestha et al. have published *in vitro* study in which they assessed the antifungal effect of mouth rinses containing chlorhexidine and thymol. The fungistatic activities of chlorhexidine - and thymol-containing mouth rinses were assessed by means of the minimum inhibitory concentration and the fungicidal activity was determined by a time-kill assay. It was found that chlorhexidine-containing mouthwash was able to kill all species of *Candida albicans* and *Candida tropicalis* in shorter period compared to the thymol-containing mouthwash. Hexidine showed an MIC of 1:32 for both Candida species, whereas Listerine respectively showed MICs of 1:8 and 1:16 for *C. albicans* and *C. tropicalis*. Antimicrobial agents used in the study had good *in vitro* activity against the two Candida species, but better antifungal and fungicidal activity showed mouth rinses containing chlorhexidine. However, both antimicrobial agents can be proposed for use as topical antimycotic agents (29).

Conclusion

A large number of essential oils have positive characteristics, such as antioxidant, anti-inflammatory and antimicrobial properties. The main components of essential oils, mono - and sesquiterpenes including carbohydrates, alcohols, ethers, aldehydes and ketones – are responsible for the fragrant and biological properties of aromatic and medicinal plants. Despite the development of antibiotics, bacterial and fungal infections are still a major health problem, and the presence of species that are resistant to multiple products poses great challenge. Recently, there has been a growing interest in natural products due to their accessibility and better biodegradebility. There is ample of evidence that plant extracts, essential oils and purified phytochemicals have the potential to be developed into agents that can be used as preventative or treatment therapies for oral diseases. While it is encouraging to see a number of

clinical trials of such products, further studies of the safety and efficacy of these agents will be important to establish whether they offer therapeutic benefits, either alone or in combination with conventional therapies, that can help to reduce oral diseases.

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ANTIMIKROBNO DEJSTVO ETERIČNIH ULJA NA ORALNE PATOGENE

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Parodontopatija i dentalni karijes, čiji je nastanak povezan sa dentalnim plakom, predstavljaju najčešća bakterijska oboljenja. Takođe, značajni oralni zdravstveni problem predstavlja i kandidijaza. *Candida albicans* je oportunistički patogen koji pod pojedinim okolnostima može da proliferiše i uzrokuje infekciju. Potreba za prevencijom i alternativnim oblicima lečenja i produktima za oralna oboljenja proizilazi zbog povećanja učestalosti oboljenja i povećane rezistencije patogenih bakterija na primenu hemoterapeutika koji se trenutno upotrebljavaju. Dokazano je da su produkti dobijeni od lekovitog bilja izvor biološko aktivnih supstanci, a zahvaljujući svojim aktivnim principima, produkti na bazi lekovitog bilja zastupljeniji su u modernoj fitoterapiji. Eterična ulja su kompleksne prirodne mešavine isparljivih sekundarnih metabolita – alifatičnih i aromatičnih, terpenskih i fenil-propanskih jedinjenja izolovanih iz biljaka. Glavni sastojci etarskih ulja su terpeni i seskviterpeni, uključujući i ugljene hidrate, alkohol, etar, aldehide i ketone, koji su odgovorni za mirisna i biološka svojstva biljaka. Različita ulja imaju brojne farmakološke efekte, kao što su antiinflamatorno, antioksidativno i antikancerogeno dejstvo, ali su takođe i biocidi. Postoje brojne *in vitro* studije koje su se bavile istraživanjem aktivnosti prirodnih biljnih supstanci usmerenih na oralne bakterije za koje je poznato da su etiološki faktori u nastanku oralnih i dentalnih oboljenja. Navedeno je da glavne fenolne komponente eteričnih ulja imaju potencijalnu antigljivičnu aktivnost. Postoje brojni dokazi da biljni ekstrakti i eterična ulja imaju potencijal da se razviju u agense koji se mogu koristiti u preventivi ili u lečenju oralnih oboljenja.

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Ključne reči: *parodontalna bolest, eterična ulja, zubni karijes, kandidijaza, fitoterapija*

Review article

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MATHEMATICAL DETERMINATION IN NATURE-THE GOLDEN RATIO

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Although the deterministic equation of life predicted and recognized by Albert Einstein is not yet defined, there are clear marks and signs of mathematical regularity which appear in nature showing fascinating accuracy. One of those "God's imprints" in the world that surrounds us is the Fibonacci Sequence, which represents more likely a starting point in revealing certain universal formula of life. The main idea of this paper is to enlighten two points: the Fibonacci sequence and the Golden Ratio, as well as to stress out the importance of reveling the perfect mathematical patterns that exist both in the organic as well as in the inorganic world. Thus, we might be able to understand the physical, spiritual and mental aspect of a human nature more clearly and completely.

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Key words: *Fibonacci sequence, Golden ratio, Anatomy, Biology, Mathematics*

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Introduction

"Mathematics is the language in which God has written the Universe."
(Galileo Galilei)

Many early civilizations, such as Chinese, Islamic and Indian gave certain contribution to the development of mathematics. However, it was the ancient Greeks who established the basic principles of mathematical science as it is today. Among the greatest are Euclid with his field of geometry, Archiedes with the approximation of number and Ptolemy who constructed the exact mathematical model which contained all of the celestial bodies gravitating around the Earth. Ptolemy believed that the movements of celestial objects could be explained by certain mathematical terms, which he explained in his scientific piece of work entitled as "The Mathematical Collection." The well-known Euclid's axioms are as important today as they were when he recognized them over 2,000 years ago. Going as far as 228 B.C, and with only basic postulates of trigonometry, the astronomer Eratosthenes estimated the Earth's diameter with over 99 percent accuracy. The Greek discoveries are timeless (1).

Ancient Greeks were strongly convinced that the Universe was created with mathematical accuracy and they mostly applied mathematical principles to static objects. They measured angles of solid objects and calculated their volumes. Also, they widely used mathematics in philosophical purposes. For anyone who wanted to enter his famous Academy, yet was not interested in mathematics, Plato said: "He is unworthy of the name of man who is ignorant of the fact that the diagonal of a square is incommensurable with its side". It has remained as such ever since.

Most of the 17th century, Galileo Galilei put in the center of scientific attention some characteristics of the Nature that could possibly be measured, such as certain variables: weight and force, space and time, acceleration and velocity. Using these results, Galileo was able to establish mathematical equations which described phenomena more precisely and more clearly that had ever been possible before. He said:

"*Nature is written in that great book … I mean the universe, but we cannot understand it if we do not first learn the language and grasp the symbols in which it is written. the book is written in mathematical language, and the symbols are triangles, circles and other geometrical figures, without whose help it is impossible to comprehend a single word of it; without which one wanders in vain through a dark labyrinth.*"

Having in mind that the same mathematical law can explain multiple phenomena, scientists are able to discover relations among those phenomena

that might otherwise go undetected. For example, trigonometric functions could be applied to all wave motions: sound, light and radio waves, as well as waves in gas, in water and many other types of different wave motions. Mathematics can detect various patterns, designs and shapes in Nature left to us to be discovered (2).

Since the mathematical structures of each aspect of Nature, from celestial objects to plants are evident, we need to ask ourselves: "Does the Universe have a specific mathematical construction?" In addition to this idea, the mathematician Mario Livio studied the Nature and its shapes in his book "Is God a mathematician?" Many questions naturally were asked, such as: "Have we humans invented all of mathematics or it had already been out there, waiting to be revealed?" At this point, it is obvious that mathematically defined forms and shapes exist in the smallest as well as in the largest parts of Nature, in living and also in non-living objects. One of those revealing, hidden, perfect patterns of beauty, function and order is the Golden Ratio (3, 4).

Fibonacci Sequence and the Golden Ratio

Golden ratio (Divine proportion, Golden intersection) **ϕ** is the irrational number /2. It means that it cannot be expressed as a fraction and its approximate value is estimated to 1, 6180339887… The intellectual elite of wide domains of scientific interests has been deeply fascinated by the Golden ratio for more than 24 centuries. In fact, it is practically impossible to specify all of the organic and inorganic living structures where this extraordinary number is hidden. Furthermore, according to the scientific point of view, new aspects of the living nature in which the pattern of the gorgeous mathematics have appeared and are still being revealed. There are specific, precise and accurate relations among the existing forms in organic and inorganic aspects of nature which seem to be in perfect proportion and therefore they are called "Divine". In order to be attributed as "Divine", the proportion needs to have specific symmetry, beauty and harmony. It has to show unique specific proportion between the parts and the whole. In order to clarify this, let us assume that we have a line segment divided into two parts **a** and **b**. Divine (golden) ratio is such relation satisfying: the longer part divided by the smaller part equals the whole length of the given line segment divided by the longer part. Expressed mathematically the Golden proportion is determined as:

$$(\mathbf{a} + \mathbf{b})/\mathbf{a} = \mathbf{a}/\mathbf{b} = \boldsymbol{\phi},$$

where a denotes the longer part, and **ϕ** denotes the Golden number (Figure 1).

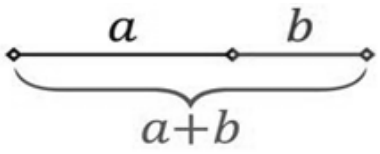

Figure 1. Golden ratio on the line

Pythagoras together with his followers constructed a regular pentagon based on the knowledge of the Golden intersection. They called it Health (Higias) and strongly believed that it represented a pure mathematical perfection. They connected health of the human body directly with the mathematical harmony of the Golden intersection. The Roman statesman, philosopher and mathematician Boethius (480-524 A.D.) showed that a soul and a body follow the same set of mathematical laws of proportion that governs the Cosmos itself.

The sequence of numbers that hides the Golden proportion given as: 0, 1, 1, 2, 3, 5, 8, 13, 21, 34, 55, 89, 144, has been described in the 12th century by the Italian mathematician from Pizza, Leonardo Fibonacci. Each member of this sequence may be calculated as the sum of the two previous members. A very interesting fact of the Fibonacci sequence is that (except for the first few numbers) the quotient of any member of the sequence with its previous one is a constant which tends to 1,6. Thus, we have next equalities: 5/3 = 1,66, 8/5 = 1,6, 13/8 = 1,6, 21/13 = 1,6… Further on, we have that 89:55 = 1,618, rounded to 3 decimal places. Investigating these interesting relations, Fibonacci revealed the sequence in various physical, chemical and biological phenomena. The fascinating moment is that we obtain a sequence of quotients which converges exactly to the Divine number **ϕ**. As the legend says Fibonacci came to this discovery by observing the behavior of rabbits, whose reproduction followed the dynamics of this particular sequence. Fibonacci numbers and the Golden ratio may be found as a pattern in almost every aspect of Nature (4).

Golden shapes

First let us analyze a shape which we are all familiar with. It is a spiral commonly seen in shells. For instance, we can focus on the Chambered Nautilus (Nautilus pompilius Linnaeus, 1758). It is probably the clearest example we could observe. As it grows, the spiral gets longer but it preserves its identical form. Its shape never changes because of the fact that it grows in the path of a spiral that is equiangular and logarithmic.

An amazing fact is that we may present Fibonacci sequence geometrically by drawing specific squares. We begin with 2 squares, with the side

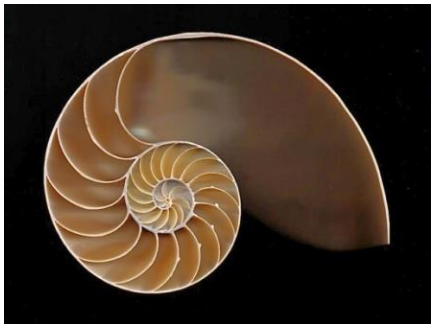

Figure 2. A cross-section displaying the rationed compartmentalization of the *Nautilus pompilius.* Source: tumblr

lengths 1 and draw them side by side. Then, above those two squares we draw a square with the side length 2 (1 + 1 = 2). We continue the procedure by drawing a square of the side length 3 (1 + 2 = 3), right to the obtained rectangle. By adding new squares on the picture, such that the side length of a new square equals the sum of the side lengths of two previous squares, we obtain squares with the side lengths equal to the Fibonacci numbers. Now, if we draw in the obtained squares specific arcs of a circle, we obtain the Golden spiral (Figure 3).

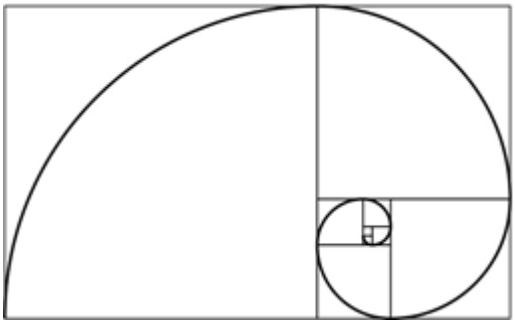

Figure 3. Golden spiral

This spiral follows a precise mathematical pattern. The perfection and beauty of this form we can find in nature approximating the shapes of various natural phenomena extremely well. Apart from shells, we find the Golden spiral in most diverse things, such as: the cochlea of the human ear, spiral seeds, growing fern leaves, ram's horn, sea-horse tail, tornados, the structure of DNA molecule, galaxies. Also, the Milky Way has a number of spiral arms, each of them having a 12 degrees of a logarithmic spiral roughly. The shape of the spiral is identical to the Golden one. Furthermore, the Golden rectangle can be drawn over any spiral galaxy (5, 6). There are numerous examples where this shape may be recognized: in whirlpools, in the tail of a comet as it winds around the sun, in the proportions of the face, in sunflower seed patterns, in the rhythm of heart beats. Really, by looking carefully at a sunflower you will detect two sets of spirals spiraling in opposite directions (rows of seeds or florets). Further on, daisies, dandelions and the ears of most mammals show the same pattern. The fascinating fact is that in the population of bees the ratio of the number of females and the number of males is always ϕ (6).

The length of human fingers, each part from the tip of the base to the wrist is larger than the previous one roughly by the ratio of ϕ. The measurement of the human navel to the floor and the top of the head to the navel presents the Golden ratio. Nevertheless, we are not the only examples of the Golden proportion in the animal world; starfish, dolphins, sea urchins, ants and honeybees also exhibit this proportion. The number of petals on some flowers follows the Fibonacci sequence. In the Darwinian processes it is believed that each petal is placed to allow for the best possible exposure to sunlight and other factors that are necessary for its existence (7).

Golden Ratio in Art, Beauty and Human body

Historically speaking, this specific number can be seen in many ancient architectural creations, such as the Parthenon and the Great Pyramid (8, 9). One has to wonder why Phidias as well as many of Greek sculptors and others in ancient Greece and Egypt use to apply this Golden ratio in designing many of their works of art. The answer may be found in the fact that this ratio seems to be remarkably attractive to the human eye. It also produces what is called a Golden rectangle. Take that the short side of the rectangle is 1, then the long side is 1.618. This rectangular shape is similar to the pattern that has been used in designing the famous Parthenon of Greece and in many other purposes, such as: vases, artistic pictures, windows, doorways and statues. The Golden ratio was used to reach balance and beauty in numerous Renaissance paintings and sculptures. Da Vinci himself applied the Golden ratio in order to define the proportions in his Last Supper, including the dimensions of the walls, backgrounds and the table. This ratio can also be seen in da Vinci's Vitruvian Man and the Mona Lisa. Other artists who also implemented the Golden ratio into their work are: Michelangelo, Rembrandt, Raphael, and Salvador Dali. The United Nations building is an example of Golden rectangle (9). When we talk of the Modern era we could recognize the approximate principle of the same Golden rectangle idea such as: postcards, credit cards, light switch plates, playing cards and writing pads. What makes the Golden proportion so unique? The fact is that art forms can be either of dynamic or static symmetry. In dynamic symmetry it is the proportioning of the areas that is being emphasized which implies changes and power movement, whereas in static symmetry the lines have definite measurements. Dynamic gives life and animation to a work of art, while the effect of stillness and quietness is the expression of static symmetry. This is the meaning of the Golden proportion.

Golden ratio, also called the "Divine proportion" establishes the standard of beauty. Physical attraction among humans depends on ratio (10, 11).

We are attracted to another person's body if that body is in certain proportion. If a face is in the proportion, it is more likely that we will notice it and find it beautiful. The results of recent studies have shown that when tested subject is being exposed to the photos of various random faces, the ones they seem most attractive are those with solid similarities to the Golden ratio. Faces qualified as the most attractive show proportions the width of the face compared to the width of the eyes, nose and eyebrows. All these make the Golden ratio. Even when being seen from the side, the human head represents the Divine proportion (11).

Scientists claim that proportional bodies seem to be healthy. This idea can be seen in the famous image showing a perfectly proportioned human body within a square and a circle, Leonardo da Vinci's Vitruvian Man presenting the Golden ratio in body dimensions: foot to navel: navel to head shows the Golden ratio. Other best known examples of human structure and physiology are: fingers, DNA molecules (a DNA molecule measures 21 angstroms by 34 angstroms at each full cycle of the double helix spiral; in Fibonacci series 21 and 34 are successive numbers). Obviously, human genomic structure is strongly associated with Fibonacci Series and Golden proportions (12, 13). Furthermore, Yamagishi et al. supported the evidence by revealing the existence of Fibonacci Series over whole genomic structure (12, 13, 14): fingerprint, human face and bronchial structure in the lungs. Even more subtle, divine aesthetics might also manifest itself in the essence of a wide variety of inner organ systems. Based on the central visual dynamics, Elliot and his team demonstrated the potential connection between aesthetic preference and Golden ratio. Really, they showed that Golden sectioning in brain activities probably have an impact on the efficiency of visual processing. Golden ratio has also been an object of interest in gynecology: mean values of length/width ratio were reported to have an inverse correlation with age and number of gravidity in a retrospective study investigating ultrasonographic (USG) measurements of non-pregnant uteri. Even more surprisingly, this ratio happened to be 1.618 at peak of fertility (for the age of 21), exactly in concordance with the Golden ratio. These results may suggest that divine aesthetics is more likely to show itself in organ systems at their full capacity and the peak of functioning confirming the measures of divine reflection.

Cardiovascular system might also be a major predilection site of divine aesthetics as measured with Golden ratio and its derivated forms (15). The ancient knowledge related the human heart with the spiritual and esoteric components of human nature including human soul. The results are published in large number of studies associating these concepts with anatomy, physiology, electrocardiogram (ECG) and echocardiogram of the heart, all exclusively yielding positive results (Table 1).

Table 1. Summary of cardiovascular indices conforming to the golden proportions in the normal healthy state

- Ratio of vertical/transverse dimensions in LV (16)
- Ratios of LVEDd/LVESd and LVESd/(LVEDd–LVESd) (17)
- Length/width ratio in mitral anulus (16)
- Angle between outflow tract and continuity of inlet tract in RV (16)
- Angle between continuity of proximal ascending aorta and pulmonary trunk (16)
- Branching pattern and culprit lesion location along the course of coronary arteries (18, 19)
- Time-dependent maturation of diastolic functions during fetal life (20)
- Ratios of diastolic/systolic and R–R/diastolic time intervals (21)
- Night-time ratios of SBP/DBP and DBP/PP (22)

LV: left ventricle; LVESd and LVEDd: end-systolic and end-diastolic dimensions of the left ventricle, respectively; RV: right ventricle; R: R wave in electrocardiogram; DBP: lowest blood pressure in diastole; SBP: highest blood pressure in systole; PP: pulse pressure.

In accordance with this, Henein et al. Investigated the potential existence of Golden ratio and Golden angle in numerous cardiac and vascular parameters measured echocardiographically in their studies (16). The authors analyzed two diverse racioethnic groups (30 healthy Chinese subjects vs. 30 healthy Swedish vs.) with regard to their left ventricle (LV) transverse and vertical dimensions measured echocardiographically and made a comparison between them. As it was expected, both dimensions were smaller by 5 ± 0.5 mm and 8 ± 1 mm, respectively, in the Chinese group (16).

However, LV ratio (the ratio of the above dimensions) in both groups converged to a constant numerical value of 1.618, the Golden number implicated the greater importance of proportions than the absolute values of cardiac structures in achieving functional perfection of the heart (16). Beside this, the authors investigated whether LV ratio demonstrated a significant alteration in the setting of heart failure in correspondence to the severity of the disease. Patients with mild and with terminal-stage heart failure appeared to have LV ratio around 1.64 and 1.4 respectively. Probably due to extensive structural remodeling in the second group, this indicated

more significant impairment and resulted with more globoid LV pattern in these patients. Moreover, in patients with severe heart failure having the LV ratio significantly deviated from normal value (1.618), 3 years follow-up overall survival rates were only around 50%, suggesting the prognostic significance of Golden ratio in heart failure patients (16). Similarly, Yetkin E. et al. researched in a retrospectively designed study in a population of 1412 subjects with normal left ventricular ejection fraction (LVEF) values, whether left ventricular diameters represent the concept of golden ratio (17). Mean values of end-diastolic and end-systolic dimensions of the LV (LVEDd and LVESd, respectively) appeared to be 4.54 and 2.81 cm, respectively, through M-mode echocardiogram along with a mean value of LVEF measuring 68% (17). Further calculation and analysis of LVEDd/LVESd and LVESd/(LVEDd – LVESd) ratios showed mean numerical values of 1.614 ± 0.08 and 1.624 ± 0.21, respectively, both in perfect harmony with the Golden ratio (17). Mitral annulus is well known to build a fibrous skeleton having a resilient nature against tensile forces and provides a substantial quantity of sustenance for mitral valves and base of the heart. Nevertheless, maintenance of its normal dimensions and functions strongly depends on LV structure. Annular dimensions ratio in normal mitral valve was reportedly 1.6 and it is approximately close to Golden ratio (16). Recently, many studies regarding blood pressure and coronary arteries have confirmed that the human cardiovascular system serve as the reflection of divine aesthetics.

Conclusion

Number **ϕ** is not just an obscure term found in science of mathematics and physics. This Divine proportion reveals that our Universe was intelligently structured and at the same time it expresses a beauty of creation. It surrounds us in our everyday lives, even in our aesthetic criteria. The Golden ratio also appears in all modalities of nature and science, in human body in a variety of its functional and structural performances. However, there still exists a long way to go regarding diagnostic and prognostic implications, physiological and pathophysiological of Golden proportions and their wide use in clinical practice. The following researches still need to explore the potential relation between divine aesthetics and medicine along with the clear implications in clinical setting. Also, it is necessary to develop new methods in order to establish the divine reflection in our modifiable physiological functions expressed as measure of Golden proportion.

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MATEMATIČKA DETERMINISANOST U PRIRODI - ZLATNA PROPORCIJA

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Iako još uvek nije definisana deterministička jednačina života koju je predviđao Albert Anštajn, postoje jasno određeni tragovi i simboli matematičke pravilnosti koji se pojavljuju u prirodi, sa neverovatnom preciznošću. Jedan od takvih "Božanskih otisaka" u svetu koji nas okružuje je i Fibonačijev niz, koji možda predstavlja osnovu za otkrivanje neke univerzalne životne formule. Cilj rada bio je da objasni pojam Fibonačijevog niza i zlatnog preseka, kao i važnost otkrivanja postojećih savršenih matematičkih obrazaca u organskom i neorganskom svetu. Na taj način, moguće je jasnije i celovitije sagledati i razumeti fizičku, mentalnu i duhovnu prirodu čoveka.

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Ključne reči: *Fibonačijev niz, zlatni presek, anatomija, biologija, matematika*

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THREE DIMENSIONAL PRINTING IN DENTISTRY

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Three-dimensional printing is a method that has found its application primarily in industry but also increasingly in medicine. In order to reach the desired shape of an object that would be formed using three-dimensional printing, specialized programs are used to draw three-dimensional objects, or special equipment designed for three-dimensional scanning. The three dimensional printing which is used in medicine can already be classified on the basis of the techniques and materials used in the preparation of the desired product. This technology can be used at every stage of making dentures, starting with the development of a study model, to the definitive development of mobile and fixed restorations. In order to obtain adequate prosthetic restorations bioprinting technology should be set aside from three-dimensional printing of non-living materials. In this way a series of tissues used in dentistry may be created: the printing of the skin and mucous membranes for covering defects on the face or mouth, the formation of bones and joints, artificial nerves, blood vessels, muscles, etc.

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Key words: *scanner, materials, 3D printing*

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A three-dimensional PRINTING OF NON BIOLOGICAL MATERIAL

Three-dimensional printing is a relatively new, additional method, which has found its application primarily in industry, to create elements that cannot be created with classic tools, or their production requires a large number of complex operations on multiple machines. To make such a structure it is required that the device for three-dimensional printing receive appropriate instructions. Therefore, a third coordinate is included in the programming, and three-dimensional object is a series of small interconnected points, the position of which is precisely defined in space.

Basically, the precision of such obtained object depends not only on the current definition of its shapes and a range of other factors, but on the characteristics of the printer, then on the quality of the program that controls the printer, material used, etc…

In mechanical engineering, in order to reach a desired shape of an object that would be formed using three-dimensional printing, specialized programs for drawing three-dimensional objects are used or, if such an object already exists, it is simply scanned in an appropriate scanner.

In medicine, the work could not be thus simplified. The problem in medicine, and especially in dentistry, is that it is most often an irregularly shaped object that cannot be easily measured or designed, nor imported in classical scanner. Therefore, it is often necessary to make scan of them, which is mainly done in two ways.

If it is necessary to perform replacement of invisible part of the body or the part that was lost (for example due to trauma), we use computer tomography which offers a three-dimensional image of the defect and surrounding structures, on the basis of which the image of the part is formed that needs to be developed to compensate existing defect. In this way such object can be developed before the beginning of surgical procedure or other intervention. In addition, it is possible to modify the obtained data in order to improve certain characteristics of the object being manufactured.

If it is a part of the body that is visible (nose, ear ...) the so-called optical scan can be used. Optical scanning is also used for elements in the oral

cavity, but since they are more or less inaccessible, scanning can be performed directly in the mouth (e.g. scanning of the brushed teeth), or by scanning model made on the basis of impression taken from the tissues of the oral cavity.

Based on the data obtained by scanning, picture of the scanned object is made and then the analysis is performed and a virtual object will be created.

The three dimensional printing which is used in medicine can be classified on the basis of the techniques and materials used in the preparation of the desired product (1).

The most commonly applied technique of a three-dimensional printing is a method that uses a photosensitive resin, and an ultraviolet laser for curing the resin. In this way, the object can be obtained which fully corresponds to the 3D drawing.

The second method instead of the photosensitive resin uses powder of a certain material, which under the influence of appropriate beam of light (ultraviolet, laser ...) polymerizes. This method of polymerization of the object is created layer by layer, until they come to their final assembly in the form of the desired object. After polymerization non polymerized powder is removed which can be mainly reused to create other object (2)?

There is a method that does not use powder in the layer but in front of light beam brings the material (usually in the form of wire of appropriate dimension), which immediately polymerizes, it adds to the second material and so on until obtaining the required shape.

Newer devices allow 3D printing of several types of materials, including metals and non-metals. So Steiner ProX™ 100 Dental system allows direct sintering of metal in order to create high quality metal dentures. This system enables 3D metal printing with obtaining chemically pure, very compact and highly precise parts (EN ISO 2768) with a tolerance of 20 microns in all three axes. It uses more than 15 materials including steel, stainless steel, super alloys, non-ferrous and precious metals as well as alumina (Figure 1.) (3).

Figure 1. Metal objects obtained by 3D printing

In order to obtain a metal object, such as, for example, skeleton of prosthesis, one method is the creation of a 3D object from plastic, then the object is flasked and casted, as well as any prosthetic restoration made using the classic method. Another method consists of making direct object of metal, for example, polymerizing layers of titanium powder.

Application of 3D technology enables the production of certain objects at a time when the need arises. This technology can be used at every stage of making dentures, starting with the development of a study model, to the definitive development of mobile and fixed restorations. So there would be no need to buy pre-made bars, attachments and other elements, but they would be made on the spot, according to the individual characteristics of each patient. In this way, in addition to greater adaptability of the casted elements to patient, the possibility of deficiency of prosthetic restorations classically made is reduced, due to imperfections and complexity of the material and the stages in the process of making the same.

The advantage of 3D printing that involves several materials with different characteristics is that it is possible to produce restorations with more elastic deeper layers and those on the surface more rigid, thereby reducing stress that the abutment teeth are subject to for prosthetic restorations as well as their supporting structures. This is consistent with the knowledge that the central part of the tooth - dentin is significantly softer and more flexible compared to tooth enamel that makes work surface of teeth.

This way of work has certain advantages compared to currently more common technologies related to the grinding of ceramic blocks or sintering base for ceramic prosthodontic works.

Since processing of expensive ceramic blocks may cause environmental pollution there is no need to use them anymore. In addition, this requires the use of complicated and expensive devices and tools for processing, which wear out and must be replaced, which will otherwise cause deviation in the dimensions of the created object. The truth is that 3D printers are currently expensive, but this is because

of their limited production. It is likely that in a short period of time their production will be doubled, the technology is getting better and lead to drastic cost reduction. It will also lead to the development of new, more adequate and reliable materials, as well as new and better printing techniques.

Bioprinting

Bioprinting technology should be set aside from three-dimensional printing of non-living materials for the purpose of obtaining adequate, usually prosthetic restorations. Having realized that the ink droplets from inkjet printers are of approximate size as human cells, Makoto Nakamura made the first bioprinter that was able to match individual living cells. Nowadays, bioprinters match and merge layer after layer of cells, those cells and layers merge with gel, thus forming tissue (4, 5).

In order to develop primarily vascular elements, the company Organovo and Invitech developed bioprinter NovoGen MMX, which has two heads, one that lays down cells, and another that lays down bonding material - Biogel. Firstly, a layer of biopaper based on water, collagen, gelatin and other hydrogelsis are applied. Then, the spheroids are injected into this layer, and finally, after joining of several layers binding agent is removed by evaporation or by other methods (Figure 2.).

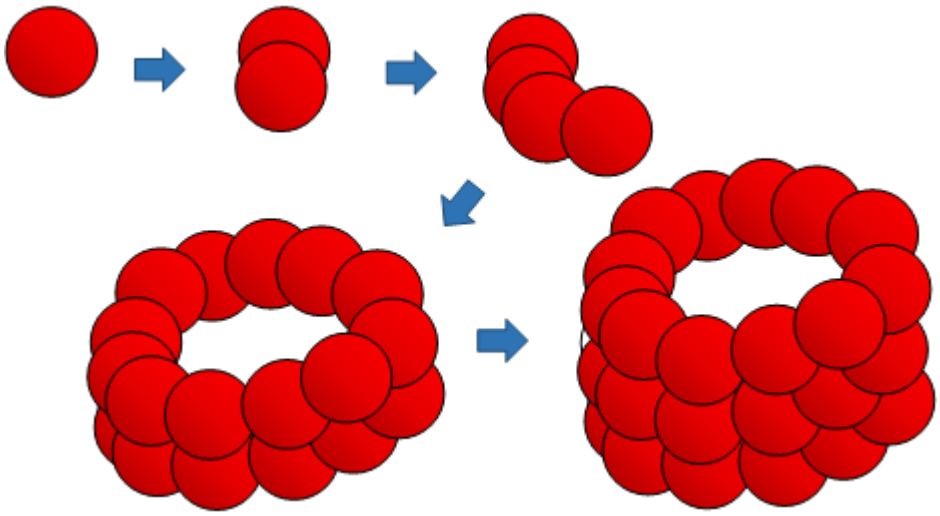

Figure 2. Composition of sferoids

Their research has shown that it is not necessary to completely print a certain part, but only to start the process, and the nature alone can complete it because the cells migrate to its natural place (as in wound healing or the development of an organism). The researchers said: "When certain cells reach appropriate place in some way they know themselves what to do." Thus, the heart tissue obtained by printing, 90 hours after merging began to beat rhythmically (6, 7).

Given that with the help of 3D printers formed tissues contain cells that were not in function such as muscle cells, the application of mechanical stimuli is provided in order to prepare these tissues for normal functioning (4).

Scientists from Australia and the United States, led by Dr. Luiz Bertassoni managed to create a network of blood vessels by bioprinting (8).

Russian researchers have started using the techniques of 3D printing parts of the human body or the whole organs by merging the so-called spheroids cells (conglomerate of stem cells of the patient himself, therefore, fully compatible with its organism), after which the accelerated maturation of cells in the stimulatoris approached (9).

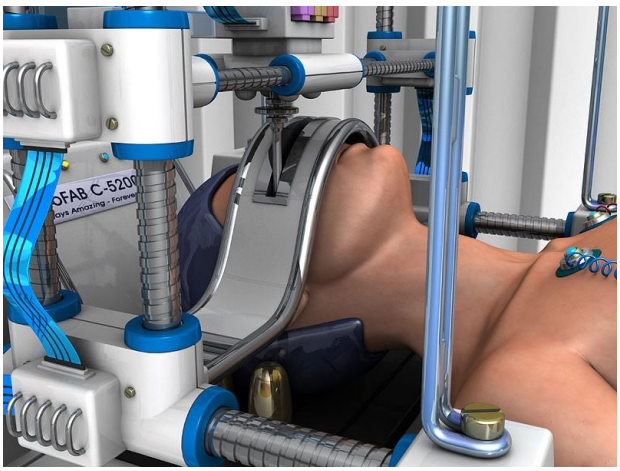

Figure 3. Bioprinter for the face

A group of scientists led by Jeremy Mao developed a printing technology of hard tissues - bones and teeth. Incisor made by this way was successfully implanted in the jaw bone of rat. Just nine weeks after implantation, there was a growth of fresh periodontal ligaments and newly formed alveolar bone. In another experiment, bioprinted skeletons at the site of the rabbit's hip joint were implanted. In the period of four months new, fully functional joints formed around the skeleton. Some of the rabbits began to walk only a few weeks after surgery (4, 5, 10).

Bioprinting can be used to create a series of tissues that may be applied in dentistry: the printing of the skin and mucous membranes to help them cover the defects on the face or in the mouth resulting from burns or mechanical effects, the formation of bones and joints, artificial nerves, blood vessels, muscles, etc.

One of the currently applicable printer is just doing a particular type of biomaterial, while others may combine different tissues and form organs from them. Science and technology united make progress with big steps, thus gradually erasing the border between classical treatment and production (4). It is estimated that printer designed for this purpose (Figure 3.), or a robotic arm carrying bioprinter-nozzle on the top, could make the missing part on the spot (eg. tooth, part of the alveolar process, or a soft tissue).

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STOMATOLOG I TRODIMENZIONALNO ŠTAMPANJE

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Trodimenzionalno štampanje je metoda koja je svoju primenu našla pre svega u industriji, ali sve više i u medicini. Da bi se došlo do željenog oblika nekog objekta koji bi se formirao trodimenzionalnom štampom, koriste se specijalizovani programi za crtanje trodimenzionalnih objekata, ili posebni aparati namenjeni trodimenzionalnom skeniranju. Trodimenzionalna štampa koja se koristi u medicini može biti klasifikovana na osnovu tehnike ili materijala koji se koristi u izradi željenog proizvoda. Ova tehnologija se može koristiti u svakoj fazi izrade protetske nadoknade, počevši od izrade modela za studije, do definitivne izrade mobilnih i fiksnih nadoknada. Od trodimenzionalne štampe neživih materijala, u cilju dobijanja odgovarajućih, najčešće protetskih nadoknada, treba izdvojiti tehnologiju bioštampe, koja se može koristiti za stvaranje niza tkiva za primenu u stomatologiji: štampanje kože i sluzokože, kako bi se njima prekrili defekti na licu ili u usnoj duplji nastali usled opekotina ili mehaničkog dejstva, stvaranje kostiju i zglobova, veštačkih nerava, krvnih sudova, mišića.

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Ključne reči: *skener, materijali, 3D štampa*

Original article

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PROTEIN, BODY FAT AND PROTEIN FAT INDEX (PFI): MODEL CHARACTERISTICS AND DIFFERENCES BETWEEN ATHLETES AND NON-ATHLETES OF BOTH GENDERS ESTIMATED USING MULTICHANNEL BIOELECTRIC IMPEDANCE

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The main objective of this research was to define the quantitative indicators for model characteristics and differences pertaining to body protein (Protein) structure as the basic component of contractile tissue, body fat mass (BFM) as the ballast tissue relevant to the basic motor skills and movement in humans, and protein fat index (PFI), a new index developed to define the relationship between ballast and contractile body tissues. The sample included 1,055 subjects (729 men and 326 women). The subjects were divided into subsamples according to types of sport, while the control groups were divided according to age and exercise levels. Body composition was estimated using InBody720, a segmental multichannel bioelectrical impedance analyzer.

The results revealed highly significant statistical differences between the variables relative to gender, men subsamples, and women subsamples (Wilks' Lambda = 0.403, p= 0.000; WL = 0.602, p = 0.000; WL = 0.427, p = 0.000, respectively). The difference between genders was most influenced by the Protein variable with 56.7%, followed by PFI with 21.9%, and least by BFM with 6.7%. In other words, the difference between men and women was 8.5 times higher in body protein mass, i.e. in basic contractile tissue, than in body fat mass, i.e. in ballast tissue. In men, the between-groups difference was most influenced by the BFM variable with 26.4%, followed by PFI with 18.8%, and least by Protein with 10.2%. In women, Protein and PFI accounted for 33.7% and 33.1% of the between-groups difference, respectively, while the effect of BFM was 25.1%.

Based on the results of this research, it can be argued that multichannel bioelectrical impedance, as a new method for body composition analysis, is discriminative and sensitive in measuring body protein and fat mass, and that PFI can be used as an integral indicator of the ratio between body protein and body fat components in scientific research and in practice, both in sports and in medicine.

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Key words: *bioimpedance, body composition, athletes, protein fat index*

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Introduction

Body composition is the term that defines the phenomenon of body composition, i.e. the set of substances that constitute the materially manifest structure of the human body (1). The macro-level composition of the human body is represented by four biologically measurable segments of matter:

- water, as liquid;
- the fat component, as the basic reserve of energy;
- the mineral component, as the solid body component; and,
- the protein component, as the basis for the contractile component responsible for locomotion, i.e. movement (2).

Quantitative characteristics and proportions of body composition are the subject of research in anthropological, medical and sports sciences, focusing on the methodological, metrological or healthcare aspects of the problem (3-7).

However, in addition to the key elements of body composition determined by the basic morphological variables, the growing area of research takes

into account the index variables, in which two or more data on body composition are integrated. Such integrated information enables the determination of not only the ratio or proportion of individual components but also of the segmented relationship between homogeneous components of body composition. This is equally important for research and clinical theory and practice (6-8).

Sport is an element of human social need to compete; as such, it represents a meaningful, long-term physical training and exercise system aimed at achieving an adequate level of general and specific competitive fitness, as well as an optimal level of efficiency in sports performance (9). Since athletes are systematically subjected to various physical efforts, the need for a specially tailored lifestyle, diet, work and rest regime has given rise to the special technology for continuous monitoring of their fitness level, health and morphological status (10-12). In monitoring the effects of training, a very important specific segment of the general technology considers the control and continuous monitoring of the level of adaptation with respect to body composition. This includes the adaptation of the tissue aimed at improving the contractile capacity, i.e. force or power, the increase in the resistance of bone tissue, or the improvement in a specific type of endurance (11-14).

Both in science and in sport, the use of multichannel bioelectrical impedance to estimate body composition is increasingly becoming the method of choice and a desirable standard in practice (7, 15-19). On the other hand, this method has offered an addition to the body of index variables already established in science and practice that define a particular aspect of body composition, such as BMI, FFMI, or FMI. Thus, the index space has been enriched by creating a provision for the definition of new indices of body composition bearing a greater innovative informational and scientific potential than the existing ones. One of such innovative indices is protein fat index, which provides a two-dimensional definition of the relationship between protein, as a purely contractile component of body composition, and total body fat mass, which is an energy reserve component from the biological aspect but is also seen as a non-contractile ballast mass in sports (12, 20).

The main objective of this research was to determine the general and the specific model characteristics of protein fat index (PFI), the new index developed to define the relationship between contractile and ballast body tissues, which can be regarded as an important conveyor of information on body composition in the system of sport and medical sciences. The secondary objective of the study was to contribute to the sports science by providing quantitative information and model values for the original PFI variables, namely, the data on protein mass and total body fat in the subsamples of athletes and non-athletes examined using the bioimpedance method.

Material and methods

This research was conducted using non-experimental scientific methods. The main testing technique involved taking quantitative measurements in the laboratory using multichannel segmental bioelectrical impedance. The chosen method type required parallel group testing (21), while the analytical method and mathematical modeling were used to gain new knowledge on the characteristics of the phenomenon under study (22).

Subject sample

The total effective sample was 1,055 subjects: 729 men (age 25.48 ± 8.33 years, body height 184.11 ± 8.52 cm, body mass 85.08 ± 14.16 kg, BMI 25.04 ± 3.39 kg m^2, length of training 12.12 ± 4.81 years), and 326 women (age 24.01 ± 5.26 years, body height 171.00 ±8 .86 cm, body mass 66.38 ± 11.75 kg, BMI 22.73 ± 3.61 kg m^2, length of training 12.12 ± 4.81 years) from the Republic of Serbia and the Republic of Slovenia. The subjects were divided into subsamples defined with respect to two criteria, as follows:

1. In athletes, with respect to the type of sport
- For men:
  - individual sports (athletics, swimming, cycling, tennis, rowing, kayaking),
  - combat sports (judo, karate, wrestling, kickboxing, fencing), and
  - team sports (water polo, basketball, volleyball, handball, football, rugby);
- For women:
  - individual sports (athletics, swimming, cycling, tennis, triathlon),
  - combat sports (judo, karate, wrestling), and
  - team sports (basketball, volleyball, handball, football).

2. In the control group of non-athletes, with respect to the level of physical exercise
- For men: adult working population not physically active, students of colleges with programmed physical exercise (Faculty of Sport and Physical Education-FSPE, Academy of Criminalistic and Police Studies-ACPS), students of colleges without programmed physical exercise (Faculty for Special Education and Rehabilitation-FASPER, Faculties of Medicine, Forestry, Pharmacy, Law, and Economics);
- For women: adult working population not physically active, students of colleges with programmed physical exercise (FSPE, ACPS), students of colleges without programmed physical exercise (FASPER, Faculties of Medicine, Forestry, Pharmacy and Law).

The research was conducted in accordance with the terms of the "Declaration of Helsinki: Recommendations guiding physicians in biomedical research involving human subjects" (23), with the approval and consent of the Ethics Committee of University of Belgrade Faculty of Sport and Physical Education. All participants were randomly selected and voluntarily participated in the study.

Measurement method

The method of measuring body composition using segmental multichannel bioelectrical impedance belongs to the latest, easily applicable non-invaive technology that provides valid data on body composition (18, 24). For the purposes of this study, measurements were carried out using InBody720 analyzer (Biospace Co. Ltd., http://inbody.rs/) with an integrated Tetrapolar 8-Point Tactile Electrode System, which uses DSM-BIA (Direct Segmental Multi-frequency Bioelectrical Impedance Analysis).

All measurements were conducted in the period 2013-2015 in the Methodical and Research Laboratory (MRL) of University of Belgrade Faculty of Sport and Physical Education and in the Laboratory for Physiology of University of Ljubljana Faculty of Sport, in accordance with the standard manufacturer's recommendations, and the recommendations found in previous studies (7, 16, 18), as follows:

- Measurement sessions were conducted between 08:00 and 11:00 in the morning;
- Subjects were instructed to fast after 22:00 h on the night before measurement, and not to consume food or beverages on the morning of the measurement session;
- Subjects were requested to avoid highly intensive or extensive training for 24 h before measurement, and to avoid any strenuous physical exercise for 12 h before measurement;
- Subjects were requested not to consume alcohol for 48 h before measurement;
- Subjects were instructed to void at least 30 minutes before measurement;
- Subjects were asked to remain in the stan-ding position for at least 5 min before measurement;
- Room temperature during measurement was between 20°C and 25°C;
- Menstruating women were excluded from the study.

Variables

Three variables were used for the purposes of this study: two primary variables and one derived index variable.

The primary variables were:

1. Protein – total body protein mass, expressed in kg; and,
2. Fat (BFM) – total body fat mass, expressed in kg.

The derived index variable was:

1. Protein Fat Index (PFI) – the index of total body protein and fat mass ratio, expressed in kg.

Statistical analysis

All raw data were subjected to descriptive statistical analysis to calculate the central tendency and dispersion (Mean, SD, cV%, Std. Error, Skewness, Kurtosis, Min and Max, and 95% confidence interval). In order to establish the normative classification for the given variables, the following percentile distribution values were quantified: 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 70.0, 80.0, 90.0, 95.0 and 97.5‰. Multivariate and univariate analyses of variance (MANOVA and ANOVA) were used to determine the differences between the variables with respect to subsamples and gender, while the independent-samples t-test with the Bonferroni correction was used to establish the difference between paired variables. The SPSS Statistics 17.0 was used for all statistical analyses. The criterion for the statistical significance of the differences between groups was set at 95% probability level, or $p > 0.05$ (25).

Results

Tables 1, 2 and 3 provide the basic results of descriptive statistical analysis and percentile distribution for the observed variables across the men and women subsamples.

Figures 1, 2 and 3 show comparative results for PFI, Protein, and BFM distributions with respect to gender.

Discussion

MANOVA and ANOVA results (Table 4) indicated that there was a highly statistically significant difference in the observed variables with respect to gender (Wilks' Lambda Value = 0.403, F = 519.74, p = 0.000), with respect to male subsamples (Wilks' Lambda Value = 0.602, F = 26.79, p = 0.000), and with respect to female subsamples (Wilks' Lambda Value = 0.427, F = 21.14, p = 0.000). The difference between genders accounted for 59.7% (Partial $Eta^2 = 0.597$), the between-groups difference for men accounted for 15.6% (Partial $Eta^2=0.156$), while the between-groups difference for women accounted for 24.7% of the explained variance (Partial $Eta^2 = 0.247$). In all analyses, the observed power was at the level of 100% (Observed Power = 1.000), indicating that the results can be accepted as a valid scientific truth. It can be argued that, on a general level, the observed variable that defines the ratio of protein and total body fat (PFI) as a function of gender was significantly discriminatory, and that it was nearly twice as discriminative across female subsamples as in men. Overall, the results showed that the differences in PFI were almost 2.5 to 4 times higher between the genders than across the same-gender subsamples.

Considering the effect of individual variables on the observed general difference, it can be maintained that the difference between genders was most influenced by Protein, the variable defining the protein body mass, which accounted for 56.7%, followed by PFI with 21.9%, and least by BFM with 6.7% (Table 4). In other words, the difference between men and women was 8.5 higher in body protein mass, i.e. in pure contractile tissue, than in body fat mass, i.e. in ballast tissue.

If the average PFI values are compared across gender and group specifics, it can be argued that gender dimorphism index for PFI was at the level of

132.07% for men in individual sports, 156.15% for men in combat sports, 92.95% for men in team sports, 92.81% for adult working men, as well as 43.81 and 81.62% for students with and without programmed exercise, respectively. Generally, dimorphism index value for PFI was at 136.33% for the total men sample (Tables 1 and 2, Men All PFI = 1.711, Women All = 0.724, which means that on average men had 136.33% more protein relative to total body mass than women in the total female sample).

Table 1. Descriptive statistics for the observed variables across the subject sample

Sample	Variable	Mean	Std. Dev.	cV%	Min	Max	Std. Error. Measur.		95% Confidence Interval	
							Aps.	Rel.	Lower Bound	Upper Bound
Men All (N = 729)	PFI	1.711	1.02	59.61	0.21	6.65	0.038	2.22	1.649	1.774
	Protein	14.63	2.08	14.22	8.60	23.50	0.077	0.53	14.49	14.77
	BFM	11.55	7.96	68.92	2.30	89.60	0.295	2.55	10.96	12.13
Individual Sports (N = 79)	PFI	2.453	1.66	67.67	0.98	6.46	0.128	5.22	2.28	2.63
	Protein	14.23	1.66	11.67	10.40	18.60	0.187	1.31	13.84	14.63
	BFM	6.75	2.49	36.89	2.30	14.80	0.280	4.15	5.22	8.29
Combat Sports (N = 148)	PFI	1.998	1.08	54.05	0.42	6.65	0.088	4.40	2.87	2.12
	Protein	14.81	2.24	15.12	10.70	22.80	0.184	1.24	14.52	15.10
	BFM	8.99	4.54	50.50	2.30	37.30	0.373	4.15	7.87	10.11
Team Sports (N = 213)	PFI	1.725	0.89	51.59	0.42	6.48	0.061	3.54	1.62	1.83
	Protein	15.55	2.08	13.38	10.10	23.50	0.142	0.91	15.31	15.79
	BFM	10.90	4.98	45.69	2.70	38.90	0.341	3.13	9.97	11.84
Adult Working (N = 111)	PFI	0.912	0.59	64.69	0.21	4.17	0.056	6.14	0.77	1.06
	Protein	13.93	1.86	13.35	8.90	19.90	0.177	1.27	13.60	14.26
	BFM	20.12	11.8	58.70	4.10	89.60	1.121	5.57	18.83	21.42
Students with programmed exercise (N = 110)	PFI	1.837	0.94	51.17	0.71	6.35	0.090	4.90	1.69	1.98
	Protein	14.07	1.57	11.18	11.20	18.20	0.150	1.07	13.74	14.41
	BFM	9.26	3.89	42.01	2.50	19.20	0.371	4.01	7.96	10.56
Students w/o programmed exercise (N = 68)	PFI	1.284	0.71	55.30	0.23	4.42	0.027	2.06	1.01	1.47
	Protein	13.91	2.19	15.74	8.60	21.60	0.265	1.91	13.49	14.34
	BFM	14.43	11.0	76.37	3.50	82.30	1.336	9.26	12.78	16.08

Table 2. Percentile distribution across the men and women subsamples

Percentiles M	***2.5***	***5.0***	***10.0***	***20.0***	***30.0***	***40.0***	***50.0***	***60.0***	***70.0***	***80.0***	***90.0***	***95.0***	***97.5***
Men All	0.44	0.57	0.73	0.97	1.17	1.33	1.50	1.67	1.90	2.24	2.92	3.63	4.71
Individual Sports	1.05	1.09	1.34	1.65	1.78	1.96	2.09	2.34	2.62	3.16	4.26	5.00	6.39
Combat Sports	0.83	1.04	1.18	1.27	1.45	1.55	1.67	1.89	2.13	2.41	3.15	4.71	5.37
Team Sports	0.73	0.80	0.90	1.12	1.26	1.40	1.50	1.61	1.78	2.25	2.89	3.52	4.04
Adult Working	0.33	0.34	0.40	0.50	0.60	0.68	0.78	0.90	1.03	1.18	1.42	1.93	2.83
Student w Prog. Ex.	0.75	0.78	0.83	1.14	1.28	1.49	1.67	1.82	1.99	2.25	3.28	3.45	4.82
Student w/o Prog. Ex.	0.35	0.48	0.59	0.77	0.83	0.99	1.09	1.31	1.46	1.75	2.19	2.69	3.44
Percentiles F	***2.5***	***5.0***	***10.0***	***20.0***	***30.0***	***40.0***	***50.0***	***60.0***	***70.0***	***80.0***	***90.0***	***95.0***	***97.5***
Women All	0.25	0.31	0.36	0.47	0.55	0.62	0.69	0.76	0.82	0.93	1.11	1.21	1.53
Individual Sports	0.46	0.47	0.57	0.71	0.74	0.80	0.90	1.06	1.14	1.64	1.84	1.99	2.07
Combat Sports	0.50	0.51	0.52	0.56	0.76	0.78	0.79	0.80	0.83	0.93	1.06	1.10	1.11
Team Sports	0.47	0.49	0.56	0.66	0.75	0.78	0.85	0.92	1.03	1.12	1.21	1.36	1.54
Adult Working	0.17	0.20	0.24	0.33	0.36	0.41	0.44	0.49	0.55	0.66	0.73	0.78	0.87
Student w Prog. Ex.	0.37	0.46	0.49	0.54	0.61	0.65	0.72	0.79	0.86	0.89	1.00	1.07	1.24
Student w/o Prog. Ex.	0.27	0.30	0.31	0.36	0.43	0.52	0.57	0.62	0.68	0.74	0.85	1.06	1.24

Table 3. Descriptive statistics for the observed variables across the female subsamples

Sample	Variables	Mean	Std. Dev.	cV%	Min	Max	Std. Error. Measur.		95% Confidence Interval	
							Aps.	Rel.	Lower Bound	Upper Bound
Women All (N = 326)	PFI	0.724	0.31	42.82	0.16	2.47	0.017	2.35	0.63	0.82
	Protein	9.88	1.50	15.18	6.90	14.90	0.083	0.84	9.67	10.09
	BFM	16.20	8.23	50.80	4.50	78.10	0.456	2.81	15.33	17.08
Individual Sports (N = 27)	PFI	1.057	0.46	43.52	0.46	2.07	0.457	43.24	0.76	1.35
	Protein	10.55	1.07	10.14	8.10	12.30	1.072	10.16	9.88	11.23
	BFM	11.52	3.86	33.51	5.70	21.60	3.857	33.48	8.89	14.14
Combat Sports (N = 12)	PFI	0.780	0.17	21.79	0.51	1.11	0.050	6.41	0.34	1.22
	Protein	9.05	1.39	15.36	7.50	12.30	0.402	4.44	8.04	10.06
	BFM	12.43	4.04	32.50	7.50	18.30	1.166	9.38	8.49	16.37
Team Sports (N = 80)	PFI	0.894	0.30	33.56	0.27	2.47	0.034	3.80	0.72	1.07
	Protein	11.25	1.29	11.47	8.50	13.70	0.144	1.28	10.86	11.64
	BFM	13.89	5.04	36.29	4.50	38.60	0.564	4.06	12.36	15.41
Adult Working (N = 63)	PFI	0.473	0.18	38.05	0.16	1.00	0.022	4.65	0.28	0.67
	Protein	9.54	1.47	15.41	7.30	14.90	0.185	1.94	9.10	9.98
	BFM	23.78	12.20	51.30	9.10	78.10	1.537	6.46	22.06	25.50
Students with programmed exercise (N = 85)	PFI	0.735	0.20	27.21	0.28	1.29	0.022	2.99	0.57	0.90
	Protein	9.38	1.09	11.62	7.00	12.40	0.119	1.27	9.00	9.76
	BFM	13.66	3.97	29.06	6.20	29.00	0.431	3.16	12.18	15.14
Students w/o programmed exercise (N = 59)	PFI	0.582	0.224	38.49	0.25	1.33	0.029	4.98	0.38	0.78
	Protein	8.99	1.07	11.90	6.90	11.60	0.139	1.55	8.53	9.44
	BFM	17.83	7.63	42.79	6.60	44.30	0.993	5.57	16.06	19.61

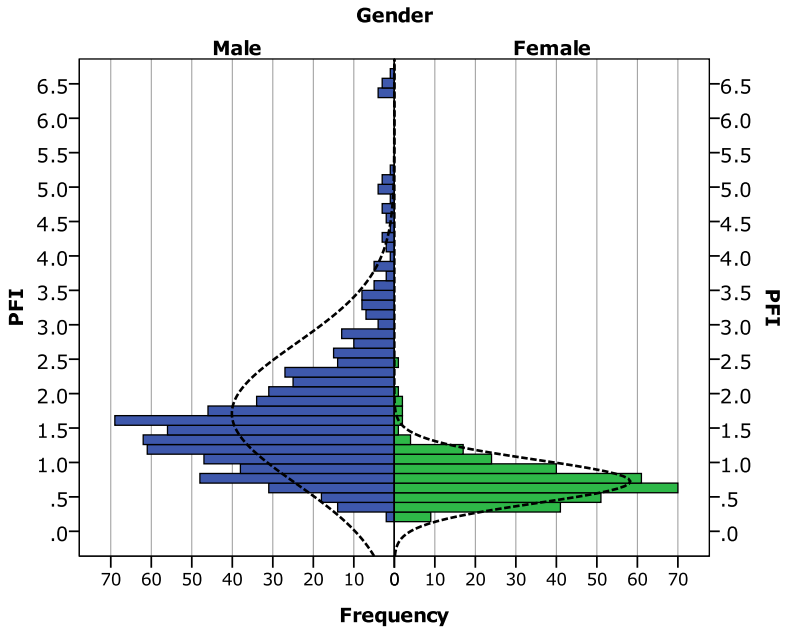

Figure 1. Comparative results for PFI distribution with respect to gender

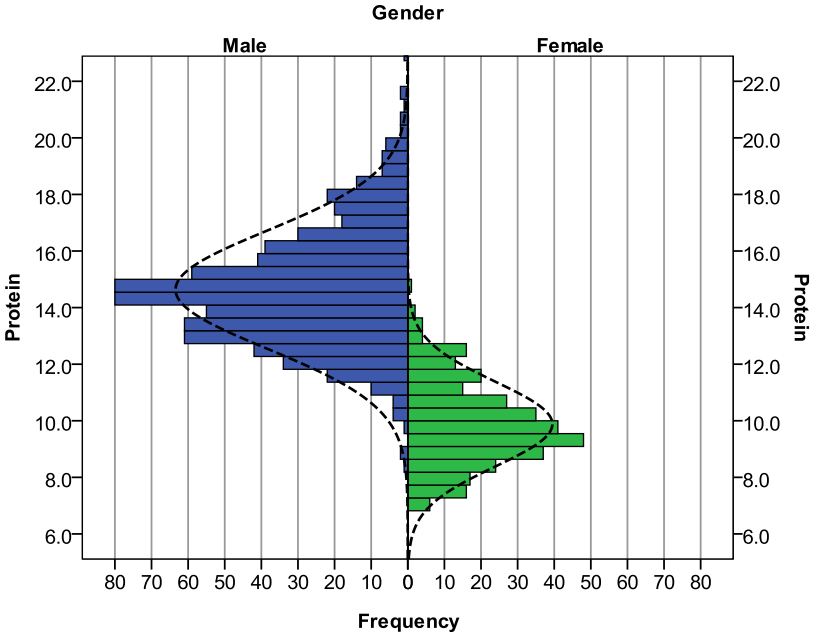

Figure 2. Comparative results for Protein distribution with respect to gender

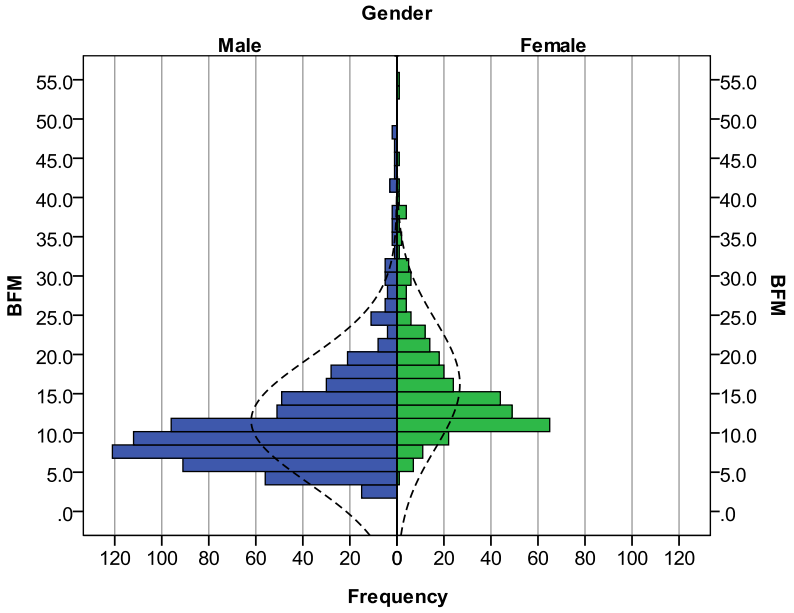

Figure 3. Comparative results for BFM distribution with respect to gender

Table 4. MANOVA and ANOVA results for the observed variables with respect to gender, male and female subsamples

Multivariate Tests[c]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta^2	Observed Power
Gender	Wilks' Lambda	0.403	519.74	3.00	1051.00	0.000	0.597	1.000
Males	Wilks' Lambda	0.602	26.79	15.00	1990.77	0.000	0.156	1.000
Females	Wilks' Lambda	0.427	21.14	15.00	878.26	0.000	0.247	1.000

Tests of Between-Subjects Effects - Gender

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta^2	Observed Power
Gender	Protein	5086.02	1	5086.02	1377.09	0.000	0.567	1.000
	BFM	4878.80	1	4878.80	75.45	0.000	0.067	1.000
	PFI	219.63	1	219.63	295.74	0.000	0.219	1.000
Groups - Male	Protein	321.71	5	321.71	16.38	0.000	0.102	1.000
	BFM	12176.74	5	12176.74	51.95	0.000	0.264	1.000
	PFI	140.81	5	140.81	33.41	0.000	0.188	1.000
Groups - Female	Protein	245.53	5	245.53	32.57	0.000	0.337	1.000
	BFM	5516.39	5	5516.39	21.40	0.000	0.251	1.000
	PFI	10.51	5	10.51	31.66	0.000	0.331	1.000

Discussion

In men, the between-groups difference was mostly affected by BFM, accounting for 26.4%, followed by PFI with 18.8%, and least by Protein with 10.2%. These results showed that the difference across male subsamples was highest for the fat, i.e. ballast, component and lowest for the protein, i.e. contractile component. Namely, the difference for the body fat component was 2.6 times higher than for the body protein component.

In women, the between-groups difference was mostly affected by Protein and PFI, accounting for 33.7 and 33.1%, respectively, while the value for BFM accounted for 25.1%. The results across women subsamples showed that the difference for the pro-tein, or contractile, component was 1.5 times higher than for the fat, or ballast, component.

The average PFI value for the total women sample was 0.724, and for men it was 1.711. The observed index value for women was at the level of 42.31% compared to men; namely, on average, the ratio of protein to fat in men was 2.36 times higher than in women. The average Protein value was 14.63 kg for men and 9.88 kg for women; while men had 1.48 times more contractile tissue, protein mass in women was 67.53% of that in men. In contrast, the results were reverse for the total body mass component, with the average of 16.20 kg fat in women and 11.55 kg fat in men. This means that there was 1.40 times more fat mass in women than in men, while the fat mass component in men accounted for 71.30% of that in women.

Overall, there was an inverse relationship between protein and fat in men and women.

The results revealed that in men, the average value for body protein mass was 14.64 kg; the highest value of 15.55 kg was observed in the subjects in team sports subsample, while the lowest values of 13.91 and 13.93 kg were found in students without programmed exercise and adult working subjects, respectively (Table 1). At the same time, the average value for total body fat was 11.55 kg in the total men sample; the highest value of an average of 20.12 kg was found in adult working subjects; and, the lowest value of 6.75 kg was found in individual sports athletes (Table 1).

In contrast to men, the average value for body protein mass in the total women sample was 9.88 kg; the highest value of 11.25 kg was found in the subjects in team sports subsample, while the lowest value of 8.99 kg was found in students without programmed exercise (Table 3). At the same time, the average value for total body fat mass was 16.20 kg in the total women sample; the highest value of an average of 23.78 kg was found in adult working subjects; and, the lowest value of 11.52 kg was obser ved in individual sports athletes (Table 2).

In men, the highest PFI value was found in individual sports subsample at 2.453 (the subjects had 2.453 kg protein per 1 kg fat), while the lowest index value of 0.912 was found in adult working subjects (Table 3). The same relationship structure for PFI was established in women, with the subjects in individual sports subsample having an average PFI of 1.057, while the subjects in adult working subsample had the lowest value of 0.473 (Table 3).

Considering the results for between-groups differences in men, PFI value showed higher statistical significance in individual sports athletes than in all other subsamples, at $p = 0.000$ (PFI individual sports = 2.453 vs relative differences in combat sports 1.988 (22.77% lower), team sports 1.725 (42.20% lower), adult working 0.912 (168.97% lower), students with programmed exercise 1.837 (33.53% lower) and students without programmed exercise 1.284 (91.04% lower)). With regard to the differences observed between other subject pairs, it should be noted that the PFI value for combat sports did not differ from team sports or students with programmed exercise. Similarly, no difference was found between male subjects in adult working subsample and students without programmed exercise. Across all other subsamples, there were statistically significant differences higher than $p > 0.005$.

With respect to between-groups differences in women, PFI value showed higher significance in individual sports athletes than in all other subsamples except in team sports athletes, at $p = 0.000$ (PFI individual sports = 1.057 vs relative differences in combat sports 0.780 (35.51% lower), adult working 0.473 (123.47% lower), students with programmed exercise 0.735 (43.81% lower), and students without programmed exercise 0.582 (81.62% lower)).

Considering the differences observed between other subject pairs, it could be argued that in women as well as in men the PFI value for combat sports did not differ from team sports, students with programmed exercise, or students without programmed exercise. Similarly, no difference was found between female subjects in adult working subsample and students without programmed exercise. Across all other subsamples, there were statistically significant differences higher than $p > 0.005$.

One of the few studies that used the same va-riables in estimating body composition in elite wrest-lers found that their average body protein mass was 15.00 ± 2.62 kg, their average body fat mass was 6.99 ± 3.28 kg, and their average PFI was 2.69 ± 1.54 kg (12). In the present study, the same vari-able values observed with respect to combat sports subsample were slightly lower for PFI, quite similar for Protein, and slightly higher for body fat; this was to be expected, as elite wrestlers are typically mus-cular with low percentage of body fat (%BF≈8.5%; %SMM≈52.8%) (12) so that their PFI is high due to low fat levels and high muscle mass.

Previous research has shown that the values for total body fat mass obtained using the bioimpedance method were approximately 15.4 ± 5.5 kg in physically active women (26), and approximately 21.9 ± 7.4 kg and 18.9 ± 6.8 kg in general population of adult women and men, respectively (27). Thus, it can be argued that since the results from the present study demonstrated acceptable external validity they can be used in defining the initial standard for the observed variables.

Conclusion

Measurement and control of body composition with the use of multichannel bioelectrical impedance is increasingly becoming the method of choice and the standard of practice in science and sport. The main objective of this research was to define the qu-antitative indicators for model characteristics and the differences relative to body protein structure as the basic component of contractile tissue, body fat as the ballast tissue relevant to the basic motor skills and movement in humans, and protein fat index (PFI), a new index developed to define the relationship be-tween ballast and contractile body tissues.

The results indicated that there was a highly statistically significant difference in the observed variables with respect to gender (Wilks' Lambda Value = 0.403, F = 519.74, p = 0.000), with respect to male subsamples (Wilks' Lambda Value = 0.602, F = 26.79, p = 0.000), and with respect to female subsamples (Wilks' Lambda Value = 0.427, F = 21.14, p = 0.000). It can be argued that, on the general level, the PFI as a function of gender was significantly discriminatory, and that it was nearly twice as discriminative across female subsamples compared to men. Considering the effect of individual variables on the observed general difference, it can be maintained that the difference between genders was most influenced by Protein, the variable defining the protein body mass, which accounted for 56.7%, followed by PFI with 21.9%, and least by BFM with 6.7%. In other words, the difference between men and women was 8.5 higher in body protein mass, i.e. in pure contractile tissue, than in body fat mass, i.e. in ballast tissue.

In men, the between-groups difference was mostly affected by BFM, accounting for 26.4%, followed by PFI with 18.8%, and least by Protein with 10.2%. These results showed that the difference across men subsamples was highest for the fat, i.e. ballast, component, and lowest for the protein, i.e. contractile component. Namely, the difference for the body fat component was 2.6 times higher than for the body protein component.

In women, the between-groups difference was mostly affected by Protein and PFI, accounting for 33.7 and 33.1%, respectively, while the value for BFM accounted for 25.1%. The results across female subsamples showed that the difference for the protein, or contractile, component was 1.5 times higher than for the fat, or ballast, component.

The results showed that the average values in men and women were 14.63 ± 2.08 and 9.88 ± 1.50 kg for body protein mass, 11.55 ± 2.08 and 16.20 ± 8.23 kg for total body fat mass, and 1.711 ± 1.02 and 0.724 ± 0.31 kg for PFI, respectively. With respect to PFI, the highest values in men and female subsamples were found in individual sports (2.453 ± 1.66 and 1.057 ± 0.46 kg, respectively), while the lowest values were found in adult working subjects (0.912 ± 0.59 and 0.473 ± 0.18 kg, respec-tively).

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PROTEIN, TELESNA MAST I PROTEINSKO-MASNI INDEKS (PROTEIN FAT INDEX– PFI): MODELSKE KARAKTERISTIKE I RAZLIKE IZMEĐU SPORTISTA I NESPORTISTA OBA POLA, MERENE METODOM MULTIKANALNE BIOELEKTRIČNE IMPEDANSE

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Osnovni cilj ovog istraživanja bio je definisanje kvantitativnih pokazatelja modelskih karakteristika i razlika u odnosu na proteinsku strukturu u telu (proteini), kao osnove kontraktilnog tkiva, telesnih masti (BFM), kao balastnog tkiva u kontekstu osnovne motorike i kretanja kod čoveka, kao i karakteristike novog indeksa, kojim se definiše odnos kontraktilnog i balastnog tkiva u organizmu tj. proteinsko masnog indeksa (PFI). Uzorak je bio sastavljen od 1055 ispitanika (729 muškaraca i 326 žena). Ispitanici su bili podeljeni na subuzorke, definisane u odnosu na kriterijum tipa sporta, a kontrolne grupe su bile podeljene u odnosu na kriterijum uzrasta i vežbanja. Merenja telesnog sastava realizovana su primenom segmentalne električne multikanalne bioimpedanse pomoću instrumenta InBody720.

Rezultati su pokazali da postoje visoko statistički značajne razlike između ispitivanih varijabli u odnosu na pol, ispitivanih subuzoraka muškaraca i ispitivanih subuzoraka žena (Wilks' Lambda = 0,403, p = 0,000; WL = 0,602, p = 0,000; WL = 0,427, p = 0,000, respektivno). Na razliku između polova najviše je uticala varijabla Proteini i to sa 56,7%, zatim PFI sa 21,9%, a najmanje BFM sa 6,7%. Drugim rečima, muškarci i žene se 8,5 puta više razlikuju u odnosu na masu proteina u telu, tj. osnovno kontraktilno tkivo, nego u odnosu na masno, tj. balastno tkivo. Kod muškaraca, na razliku između grupa najviše je uticala vrednost varijable BFM i to sa 26,4%, zatim vrednost PFI sa 18,8%, a najmanje vrednost variable Proteini sa 10,2%. Kod žena, na razliku između grupa najviše je uticala varijabla Proteini PFI i to sa 33,7 i 33,1%, respektivno, dok je varijabla BFM uticala sa 25,1%.

Na osnovu dobijenih rezultata ovog istraživanja može se tvrditi da je multikanalna bioelektrična impedansa, kao nova metoda merenja telesne strukture, diskriminativna i senzitivna u odnosu na merenje mase proteina i masti u telu, a da se PFI može koristiti kao integralni pokazatelj odnosa proteinske i masne komponente tela i u nauci i u praksi, kako u sportu tako i u medicini.

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Ključne reči: *bioimpedansa, telesna struktura, sportisti, proteinsko-masni indeks*

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BATUT, 1915.: ENLIGHTENED SONS, START THE MEANINGFUL FIGHT AGAINST THE TYPHUS!

Goran Čukić

A century has passed since the epidemic typhus in 1914/15, but the answer if the Serbian people were led to fight against the typhus has not been given yet. If so, was the fight successful? Did the doctors' reaction for not taking into consideration the activity of the State Committee for the suppression of communicable diseases was appropriate? Were there any steps which should be remembered by the citizens of Serbia, especially medical professionals? The Committee was in charge in 1915, and its decisions represent the official attitudes of Serbia, the ones that should be analyzed at first, while the after war historical-medical analyses are in the second place. As the president of the Committee, Velisav Vulovic was the coordinator of this body, and so had an influence on working of Serbian Military Medical Corps in 1915. The lack of factory autoclaves resulted in the scientific problem how to replace them. Vulovic is a forerunner of sanitary engineers in our country and improved the prototype of "a dry warm air chamber". "The disinfecting central" was built in Niš and started working before Hunter's mission. Its function was to act offensively by depediculation in suppressing epidemics of transmission diseases. It was used by army, hospitals, prisoners, and civilians. With the consent of dr Gencic, the chamber was applied in basic military units on the battling field. The successful action of Serbian medical staff in 1915 on suppressing epidemics was the beginning of the significant fight against typhus and its recurrence, and not only Serbian people but the world medicine owes them a debt of gratitude.

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Key words: *Velisav Vulović, The State Committee for the suppression of communicable diseases, dry warm air chamber, typhus fever, epidemics in 1915*

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Uvod

Dr Batut je pozvao proklamacijom „prosvećene sinove " da povedu svoj narod u smišljenu borbu protiv pegavca (1). Do 2015. godine ni u radovima o epidemijama 1914/15. nije pisano da je poslanik Velisav Vulović bio angažovan u sprečavanju epidemija - pegavca i rekurensa (2, 3). Zapostavljena je činjenica da je vodio skupštinsko i vladino telo Državni odbor za suzbijanje zaraznih bolesti od njegovog formiranja do okončanja epidemija 1915. godine. Da li je taj rad bio uspešan?

Sa pojavom epidemije velikih razmera i pridavanjem značaja Nikolovim ogledima, trebalo je imati taktičko sredstvo kojim se masovno uništavaju vaši. To je moglo da se izvrši: autoklavom fabričke izrade, improvizacijama autoklava ili upotrebom nekog novog sredstva, drugačijeg principa delovanja od autoklava. U Srbiji su korišćene improvizacije autoklava od dolaska pukovnika dr Hantera (4). Vršena je depedikulacija sa nekoliko tipova „srpskih buradi". Furune za pečenje hleba i sušare za voće su deo narodne tradicije. Narod ih je povremeno koristio za oslobađanje od šugarca, a uzgred i od vaši tela (5). Vaš tela je tada uništavana kao molestant i to nije imalo protivepidemijski karakter.

Srpski sanitet je pokušao da reši problem epidemija pre dolaska puk. dr Hantera. Srbija je formirala skupštinsko i vladino telo Državni odbor za suzbijanje zaraze (Odbor). Dezinfekciona centrala je napravljena u Nišu (6), a od značaja je ustanoviti da li je bila primenjena komora na vojištu, u rezervnim poljskim bolnicama i kod zdravih vojnika u borbenim jedinicama?

Materijal i metod

Korišćena je dominantno arhivska građa koja se odnosila na period pre 4.3.1915, pre dolaska puk. dr Viljema Hantera u Srbiju.

Primenjen je genetski (istorijski) metod, koji pridaje značaj razvoju predmeta spoznaje, a time i saznajnosti. Istoričar medicine je objedino a) „kako"

(kako nastaje događaj) i b) „zašto" je događaj bitan medicinskoj nauci. Analiza toka događaja može da bude retrogradna i anterogradna.

Rezultati i diskusija

Dr Hanter je smatrao da je „misao vodilja srpskih vlasti bila da se dobije što više toliko potrebne kliničke pomoći" [4:238]. Ovo bi bilo i danas aktuelno, jer se karantinska bolest suzbija izdvajanjem bolesnika i organizovanjem karantina. Odgovoriće se da li je srpski sanitet pre dolaska dr Hantera 4.3.1915. u srpskoj, prvoj fazi postojanja epidemija pegavca i rekurensa učinio dodatni korak usmeravanjem ka zaštiti zdravih.

U mnogim biografijama aktera: Subbotića (sic.), Batuta, Kujačića, Genčića, Protića itd. ne spominje se da su oni doprineli strategijskom i taktičkom opredeljenju, bitnom za suzbijanje pegavog tifusa u Srbiji 1915. godine.

Za državnog savetnika, Velisava Vulovića (1865-1931), zbog nedovoljne istraženosti, ne zna se šta je radio 1915. godine. Navodno je „za vreme Prvog svetskog rata boravio u Francuskoj" (7), ali je bio i na Krfu među poslanicima u izbeglištvu (8). „Boravku na dva mesta u isto vreme", pridružuje se i činjenica da se u citiranom radu ne spominju epidemije 1915. godine, kao da nisu postojale i bile predmetne poslanicima.

U nekrolozima objavljenim u dnevnoj štampi, od Vulovića se oprostilo biranim rečima. Bio je „(...) veoma plemenitog, otvorenog, čistog i nesavitljivog karaktera. Svoje mišljenje je sticao na osnovu ozbiljnog i savesnog proučavanja i branio je ta mišljenja odlučno, dosledno iskreno, spreman da se do kraja založi za odbranu ideja u čiju je ispravnost verovao. Zauzimao je visoke položaje, bio je retko ljubazan i predusretljiv, a u isti mah potpuno predan interesima posla." Vulović, po nekrologu objavljenom u „Pravdi", zaslužuje sećanje zbog tople pažnje, vezane kako za njegovu ličnost tako i za njegov javni rad (9), (Slika 1).

Slika 1. Velislav Vulović (1865-1931)

„Ni na jednom poslu Vulović nije gubio poverenje, jer je sve svršavao savesno i uspešno, iako je često dobijao poslove za koje nije bio stručnjak. No, po obavljenom poslu, nikada se nije videlo da su obavljeni nestručno. Dobijao je priznanja i za takve poslove" (10).

Ni biografije ni nekrolozi ne spominju V. Vuloviću 1915. godine angažovanje protiv epidemija pegavog tifusa i rekurensa.

Državni odbor za suzbijanje zaraznih bolesti postoji nezvanično od oko 21.1.1915. godine. Velisav Vulović je njegov predsednik tokom čitavog trajanja epidemija pegavca i povratnog tifusa, tj. tokom čitavog postojanja Odbora (3). Srpske novine, 10. 03. 1915. godine, informacijom iz Opšteg odeljenja Ministarstva građevina, obaveštavaju da je u Nišu, po odluci br. 2050 od 08.03.1915. Ministarskog saveta obrazovan Državni odbor za suzbijanje zaraznih bolesti. Članovi su bili dva lekara: Maočanin, Sondermajer; i tri poslanika: Jovanović, Vulović i Pavlović (11). Nameće se pitanje da li su u Skupštini, na dan legalnog osnivanja Odbora, 2.4.1915,

poslanici članovima ovog tela za dotadašnji rad lakonski izrekli „hvala im" (4). Brz zaborav znači nezahvalnost Odboru i njegovom predsedniku ili/i svrstavanje rada na suzbijanju epidemija među beznačajne aktivnosti (4).

Podfaza I – 15.01.1915.

Pukovnik dr Genčić, načelnik saniteta Vrhovne komande, sarađivao je sa Odborom, pa je to dokaz da je ovaj postojao pre 15.1.1915 (12). On povodom rasta epidemija u obraćanjima načelniku štaba Vrhovne komande od 15.1. i 30.3.1915. godine opisuje delatnost oko epidemija i rad Odbora.

U iscrpnom „Referatu" od 15.1.1915. godine ukazuje se na 12 strana teksta da su rekurens i pegavi tifus pre rata 1912. bili nepoznati u narodu i vojsci u starim granicama Srbije. Potom se navodi da su tada turski zarobljenici širili pegavi tifus. „Povratni tifus (rekurens), delimično i pegavi tifus, vladali su u vojsci i posle zaključivanja mira 1913. godine i to poglavito među trupama dislociranim na krajnjim južnim granicama i u garnizonima novih oblasti. Potrajala je u mesecu maju 1914. godine epidemija pegavog tifusa u Debru". Zatim iznosi da su činjeni napori „(...) da se ceo naš Zemaljski sanitet stavi na zdraviju osnovu i osposobi za borbu protiv epidemija običnog trbušnog tifusa i drugih zaraznih bolesti, koje su vladale u vojci i narodu (...)" (12). U narednom referatu od 30.3. 1915. precizirano je da za ratna iskušenja treba biti spreman: „Ceo Zemaljski sanitet, civilni i vojni, toliko je bio nespreman za borbu sa epidemijama, da i u miru nije uvek mogao sačuvati vojsku i narod od tifusa i drugih zaraznih bolesti, a kamoli u ratu" (13).

Dr Genčić 15.1.1915. obaveštava pretpostavljenog o bitnim činjenicama, da vaš prenosi pegavac i da bolnice nemaju dezinfekcione aparate (fabričke izrade, GČ) (12). Tražio je dr Genčić Odboru da kupi 20 autoklava. Iznosi da je i 100 nedovoljno za razmere epidemije (12).

Kako tada Hanter nije bio u Srbiji, predmetna je prva, „srpska faza", postojanja epidemija 1915. godine (14). Hanter je smatrao da je srpska epidemija bila osobena kao „(...) prva velika epidemija, koja je izbila i koja je suzbijena, od kako su istraživanja Nikola, nekoliko godina ranije od 1909. do 1911. godine, utvrdila prirodu zaraznih bolesti pegavog tifusa i povratne groznice koje se prenose vašima" (4). Nikol je eksperimente obavio na oglednim životinjama. Stanojević s toga ističe da je prvi pronašao da je šimpanza pogodna ogledna životinja u ispitivanjima pegavog tifusa [15:39].

Dr Genčić je napisao o odboru: „Kada su u ovom ratu epidemijske bolesti počele kositi naše hrabre vojnike i stanovništvo, naš se Parlament trgao, i iz svoje sredine obrazovao Državni odbor za suzbijanje zaraze. Odmah su tom odboru stavljeni milioni na raspoloženje (sic.) za borbu, dok pre toga načelnik saniteta nije mogao blagovremeno dobiti potrebne sume za lekove, te baš u ovom momentu naši vojnici oskudevaju u lekovima" (13).

Zbog prvenstva koje je imao njegov štab, puk. Genčić ističe: „Državni odbor uzeo je na sebe da posreduje, te da se i one sume koje vojni sanitet traži, što brže i potpunije odobravaju. I zaista, sada sve brže ide, ali je sve to došlo dosta kasno. Mnogobrojne žrtve su već pale, a moralni gubici su još veći. Ustanovljavanje Državnog odbora za suzbijanje zaraza najbolji je argument: da treba stalno da postoji jedno nadleštvo, koje će imati uticaja, vlasti i sredstva, da izvodi sve svoje zdravstvene i higijenske zadatke. Kada se jedared stvori Ministarstvo narodnog zdravlja, onda će lako iz njega organizovati i sanitetsko odeljenje Vrhovne komande za slučaj rata." Ministarstvo narodnog zdravlja, prema projektu koji je predložen u SLD-u, imalo bi, pored civilnog, i veterinarsko i vojno odeljenje, sa jednim višim vojnim lekarom na čelu i ostalim potrebnim osobljem. To vojno telo imalo bi u slučaju mobilizacije zadatak da formira sanitetsko odeljenje pri Vrhovnoj komandi, zbog upravljanja specijalnom sanitetskom službom u operativnoj vojsci, dok bi snabdevanjem i celokupnom sanitetskom službom u zemlji rukovodilo samo Ministarstvo narodnog zdravlja (12).

Genčić nastavlja o gubicima: „Ako je iko strogo kažnjen za propuštene mere, to smo mi kažnjeni; morali smo sa ovim ogromnim žrtvama, što smo ih sada pretrpeli od zaraznih bolesti, da potvrdimo staru istorijsku istinu: da jedna vojska ako nije zdravstveno dobro osigurana, i pored najboljih svojih drugih osobina, ne može da dovrši uspešno i slavno svoj veliki zadatak" (12).

Opredelio se dr Genčić da su potrebni fabrički napravljeni autoklavi i da ih je zato trebalo kupiti u velikom broju.

Interesantno je kakva je situacija u neprijateljskoj vojsci. Ona 1915. nije morala da ima potrebna saznanja o pegavcu. Nijedan zarobljeni lekar nije ponudio predlog kako bi rešavao pitanje spašavanja drugova zarobljenika koji stradaju od pegavca. To je prilično pouzdan dokaz o njenom odnosu prema pegavcu. Neprijateljska vojska, iz država sa nesporno razvijenijom medicinom od one u Srbiji, vrlo izvesno nije imala izgrađen doktrinaran stav s kojim je ušla u rat, kako se rešava epidemija pegavca. Kako je doktrina tek morala da nastane, to je pod znakom pitanja više toga bitnog, kako za izbor strategije tako i taktike. U zabludi su oni koji „nemoć medicine neprijatelja" proglašavaju njihovom namerom vođenja „biološkog rata". Među zarobljenicima, pored nemaštine, mogućeg nemara i sl., kao najpresudniji faktor deluje „nemoć medicine" (naučno nepoznato). Zato, pogotovo u ratu, pegavac deluje nesputano, potpuno prirodno, svom žestinom svoje pogubnosti.

Vlada Austro-Mađarske monarhije sunarodnicima je zarobljenima u Srbiji uputila marta 1915. godine „mast protiv vaši" (16). Ustanovljeno je da je poslata mast bila fenil-metil-etar (17), (2).

Postavlja se pitanje zašto u nevolji koja je 1915. godine moždila svom snagom žitelje Srbije: svoje mišljenje nisu dali, tj. zašto nisu sazivana i konsultovana najviša tela, Glavni sanitetski odbor (Srpskog lekarskog društva) i Vojno-sanitetski komitet? Tako su autoriteti pošteđeni ovog iskušenja, a aktivnost je prepuštena samoinicijativi pojedinca, tipa „javlja se svojevoljno za rad gde bi zapazio da bi njegova pomoć bila od koristi". Neko bi rekao da je

takvim angažovanjem taj sklon „guranju ruke u vatru".

Veliki broj lekara se razboleo od pegavca, jer su bili izoženi napornom i teškom radu [15:36, 42, 72], ali je primećeno i nedovoljno njihovo angažovanje i pored toga što ih je bilo malo. Poželjno je bilo ranjene i obolele što brže transportovati u pozadinu. Potpomaže ambivalentnost takvog (ne) delovanja, da su jedinice morale imati slobodne lekare za slučaj primarnog „svakog trena mogućeg" vojnog angažovanja, pa je tada lekaru koji je i sam u životnoj opasnosti primaran problem ranjavanja, a ne zaraznih bolesti itd. [16:39.; 17:330]. O upotrebi naprava koje su služile za popravljanje higijene nisu mislili, navodno to je bila delatnost drugih. Takav stav nije daleko odmakao od ubeđenja koje deli medicinu na veliku i malu. Onom malom bi se bavili oni koji misle o vašima, higijeni i sl.

Domaći istoričari medicine, koji su istraživali epidemije 1915. godine u Srbiji, poseban značaj pridaju Batutovom članku, u kome je navodno prvi put izneto „da vaš prenosi pegavi tifus". Kako je naknadno ustanovljeno, članak je bio objavljen u „Srpskim novinama" 28.1. 1915. [2, 5]. Ako je za Subbotićev predlog ukopane peći možda bio od značaja Batutov tekst, ipak to ne bi važilo za Genčića (11), koji je znao da vaš prenosi pegavac i pridao joj strategijski značaj pre Batututovog nastupanja 28.1.1915. Naime, on se bar od 15.1. opredelio za taktiku masovnog depedikulacije putem autoklava.

Znači, istog strategijskog i taktičkog opredeljenja bili su Genčić 15.1. и Batut (Slika 2) 28.1.1915. godine.

Slika 2. Biste dr Vojislava Subbotića i dr Milana Jovanovića-Batuta ispred dekanata Medicinskog fakulteta u Beogradu

Stanojević je 1965. smatrao da Odbor nije imao autoritativnog člana koji bi garantovao istinitost stava da bela vaš prenosi pegavac (21). On kasni, baveći se predugo strategijom koju je već apsolvirao organizacioni vrh u suzbijanju epidemija (Genčić i Odbor). Tražio je Stanojević angažovanje Batuta, a on je već dao svoje mišljenje. Tako je pokazao nedovoljnu obaveštenost u vreme rata kao i nakon rata.

Problem je bio 1915. godine, kako uticati na one lekare koji uporno nisu prihvatali da vaš prenosi pegavi tifus i povratnicu. Da li je problem trebalo da bude rešavan naređenjem, kako je zastupao D. Antić? Po njegovom sećanju, nadležni su propustili „da nam u vidu jedne (...) naredbe narede da znamo da pegavi tifus prenose vaške".(22) To ukazuje da naređenje nije realizovano. Istoriografiji je nepoznato da ovakav predlog nije bio usamljen.

Navodno, „Vrhovna komanda" se obratila Odboru sa predlogom, kako bi dobila saglasnost da donese „naređenje: da vaš prenosi pegavi tifus" (dokument nije potpisan i nije datovan) (23). Izvesnije je da je predlog dalo Sanitetsko odeljenje Vrhovne komande ili njegova Komisija za suzbijanje zaraze. U vojsci neizvršenje naređenja ima za posledicu sankciju.

U arhiviranom dokumentu predloga „naređenja" prvo se navode svetski eksperti (iz: Engleske, Francuske, Nemačke i Rusije) koji su garancija tvrđenju da je „apsolutno i nesumnjivo utvrđeno da pegavi tifus prenose samo i jedino bele vaši (ni buve ni stenice ne prenose ovu bolest...)". Potom, sledeje da „(...) svaki lekar koji se ne potčini ovom utvrđenom mišljenju i najsavesnije ne vrši uputstvo o najtačnijem uništavanju belih vašiju čini (...) (prepravljano, križano, GČ) zbog kojeg će, čim se to konstatuje, strogo odgovarati (...)" (23).

U Odboru je dobro urađeno što nije prihvaćen predlog naređenja. Dotadašnje saznanje bilo je na nivou naučne hipoteze, pa je kao takvo služilo izboru strategije delovanja protiv pegavca. Zasnovana je hipoteza na Nikolovim rezultatima, dobijenim na eksperimentalnim životinjama. Nju je dokazao Hanter tek 1919. na osnovu podataka prikupljenih tokom suzbijanja epidemija u Srbiji 1915. (4). Otuda je baš na tom problematičnom mestu u rečenici, kod „definisanja krivice", više puta korigovan tekst i na kraju je ostao nedorečen.

Objašnjava se ispravnost nedavanja saglasnosti trivijalnim: naučna novost se ne sprovodi naredbom, nego usvaja proverom onih kojih se naučna novost tiče. Trebalo je da lekari ili prihvate ili da odbace rezultate Nikolovih ogleda. Visprenost istraživača je da ovo uradi na što očigledniji način, tako da mu se poveruje. (Od lekara se provera očekuje, zato imaju akademsku titulu „dr", takođe, imaju specijalizacije, zvanja primarijusa, naučna zvanja itd.).

Dr Antiću je predlog naređenja bio nepoznat u vreme kada je bio upućen Odboru. Isto tako, nakon ratnih događanja, ostao mu je nepoznat, ali sada sa razlogom da nije istraživana arhivska građa, koja je navodno uništena pri povlačenju (20:330). Dr Stanojević je radio u Sanitetskom odeljenju Đeneralštaba i vodio statističko odeljenje od 1924 do 1929. godine (24). Ono je dobijalo obavezni primerak Službenog vojnog lista. Odnosili su se na arhivu ratnih jedinica i njihovih saniteta 1922. i 1924. godine. Npr. Sl. vojni list 16/1922. s.707.; Sl. vojni list 41/1924, Pregled arhive, posle s. 1816.

Primer „naređenja" ukazuje sa koliko nepokolebljive sigurnosti je Sanitetsko odeljenje Vrhovne komande verovalo Nikolovim ogledima. Otuda se i pomišljalo o unifikaciji sprovođenjem naredbe sa kojom su svi lekari morali da usvoje značaj vašiju za pojavu masovnog obolevanja od pegavog tifusa. Ali tako zahtevano, bilo bi doktrina. Tadašnja saznanja o Nikolovim uspesima nisu mogla da budu na nivou doktrine i kao takva budu sprovedena naredbom i pored podrške ličnosti sa kredibilitetom. Nesporno je i to, da je izvršenim ogledima bilo prilično pouzdano saznanje nauke, na koje je moglo da se pozove u slučaju neuspeha. Ipak, usvajanjem bez provere, postojao je rizik.

Od kakve koristi je u ovoj podfazi pokazano verovanje u Nikolovo saznanje? Ono je bez ikakvog medicinskog značaja ako ne usledi primena, tj. depedikulacija.

Mera je navodno hendikepirana nedostatkom novca. Smatralo se da se može suprotstaviti epidemijama kupovinom velikog broja autoklava. Istorijsko „kako" prethodi problemu i zahteva da se vidi u čemu je problem i kako je rešavan. Novac je mogao da bude odobren, ali na evropskom tržištu zadugo nisu mogli da se kupe fabrički napravljeni aparati. Bili su potrebni vojsci zemalja proizvođača. Otuda se umanjuje značaj ustanovljenog da su već bili skupi, a da je povećana potreba i potražnja za njima uslovila dodatni rast cene. Uz to, osnovna upotreba autoklava je u hirurgiji za suptilnije postupke od depedikulacije (sterilizaciju i dezinfekciju). Znači, zapalo se u situaciju kada „ni pare ne pomažu". Novac je razotkrio suštinski problem, potrebu osmišljavanja sredstava dostupnog velikom broju građana, npr. sa poželjnim svojstivima autoklava (6, 25), a to je naučni problem, poput nedostajućeg leka za neku bolest.

U ovoj podfazi apsolvirana je strategija (da vaši treba uništavati), pa se traga za taktikikom. Dr Genčić je nabrojao mnoge faktore koji su sigurno doprineli da epidemija uzme maha, ali to, slično samooptuživanju, prikriva glavni razlog njenog nastanka, a to je „nemoć medicine", u smislu naučno nepoznatog. Ona, uostalom, u tom trenutku nije ni postojala radi oslanjanja na kupovinu fabričkih autoklava u dalekoj Americi. Uskoro je novi cilj bio da se za ispravno izabranu strategiju (da vaš prenosi pegavac), traži novo taktičko rešenje (sredstvo koje će vaš uništiti umesto autoklava). U Srbiji se pred njim nije zastalo, već je vrlo brzo rešenje ponuđeno putem improvizacija autoklava ili suspenzijom novim sredstvom iste efikasnosti (5). Simultano uvećanje broja obolelih i umrlih ubrzalo je rešavanje nastale naučne nemoći i izrodilo Batutov apel.

Sledeće etape srpske faze traženja rešenja su javna nastupanja, prvo dr Vojislava Subbotića, a potom i dipl. ing. Velisava Vulovića.

Podfaza II – 10.2.1915.

U Srbiji je kao sredstvo za depedikulaciju pre 4.3.1915. godine ponuđena nova namena već postojećih uređaja koje poseduju domaćinstva (sušare za voće, peći za hleb i sl.), tj. zamena vrele pare „suvom vrelinom" (5, 26). Bez preduzimljivosti dr Subbotića (Slika 2), saznanje da vaš prenosi pegavac bilo bi obezvređeno (6, 27). Subbotićeva „ukopana peć" predložena 10.2.1915. je sledećih osobina: pravi se od cigala, beli papir je promenom boje ukazivao na postignutu temperaturu itd. (26). Ali ovo prototipno rešenje bilo je problematično zbog malog kapaciteta razvašljivanja. Ukopana peć nije prevazišla individualna sredstva, već poznata i do tada primenjivana protiv vašiju: živinu mast, peglanje veša itd.

Da bi depedikulacija bila uspešna, morala je da bude masovna, pa tek takva zadobija odliku protivepidemijske mere. Odmah je prepoznato da bi, ako se prototip usavrši u ovom smeru, novi uređaj imao poželjne osobine. Očekivalo se angažovanje čitalaca: „Naši tehničari mogli bi nacrtati nekoliko modela takvih peći, i te crteže u novinama saopštiti" (26).

Predlaganom „naredbom", umnožavanjem saznanja o strategiji kojom bi se mogao suzbiti pe-

gavac, dobilo bi se samo ako nastane naprava kojom će se delujovati „ofanzivno" (masovno i efikasno). Ali kako je naredba namenjena lekarima koji „najsavesnije ne vrše uputstvo o najtačnijem uništavanju belih vašiju" (26), ovaj deo naredbe ne omogućava svojom formulacijom da se utvrdi kojoj ovde podfazi pripada, tj. da li je to bilo pre ili posle novopronađenih komora za masovnu depedikulaciju.

Podfaza III – 24.02 1915.

Desetak dana po objavljivanju članka o „ukopanoj peći", preduzimljivi V. Vulović je sebi postavio zadatak njenog usavršavanja (3, 27).

U „Srpskim novinama" od 24.2. objavljen je članak o pećima namenjenim za masovno razvašljivanje dnevnog kapaciteta obrade 300 - 600 pari odela. Po nacrtima Velisava Vulovića sagrađene komore postale bi značajno protivepidemijsko sredstvo za masovnu depedikulaciju (29, 28).

Odbor je već početkom marta pustio u rad „dezinfekcionu centralu" koja se sastoji iz komore i kupatila. „Centrala" je namenjena većem broju korisnika, bolesnicima i zdravima. Radila je u Nišu, gde su bile skoncentrisane: veliki broj vojnih bolnica, izbeglica, najveći broj zarobljenika, a bila je namenjena i stanovništvu. Dnevni kapacitet joj je bio višestruko veći od zacrtanog 24.02. Kupatilo je moglo dnevno da primi 1200 osoba, ili da obavi razvašljivanje 4000-5000 pari odela (3, 6).

Događaji objavljivanja nacrta peći i puštanja u rad „dezinfekcione centrale" su od izuzetnog značaja i to ne samo za konkretnu situaciju Srbije, već i za medicinu sveta.

Dalje je trebalo depedikulaciju učiniti što dostupnijom korisnicima i to u Nišu i van njega: informisati korisnike o radu „peći na topli suvi vazduh" i izgradnjom ih omasoviti. Dr Genčić je ove aktivnosti Odbora podržao.

Omasovljenje upotrebe sprovedeno je na sledeće načine:

a. Odbor se 01.3.1915. obraća komandantima dokumentom potpisanim od strane predsednika V. Vulovića.

Kritikuje se prenaglašavanje bespomoćnosti: „U borbi protiv epidemije koja u zemlji vlada, neopravdano se preteruje apostrofirajući – kako je velika oskudica u lekarima, u dezinfekcionim sredstvima, u medikamentima, očekujući sav spas od toga, a zaboravljajući od kolikog je presudnog uticaja inicijativa, ustalaštvo i organizacija borbe protivu zla. Ne mislimo time smanjiti imperativnu potrebu za lekarima, medikamentima i dezinfekcionim sredstvima, ali hoćemo da istaknemo značaj inicijative i ličnog ustalaštva kao momenta toliko isto važnog..." (30; 6). Uputan je bio primer preduzimljivog majora Jovana Sretenovića (3). Dati su planovi izgradnje nekoliko tipova komora.

b. Obavešteni su komandanti 12.3.1915. da je za područje Niša puštena u rad „dezinfekciona centrala", te da jedinicama stoji na raspolaganju korišćenje komore i kupatila (6).

Početkom marta 1915. u Srbiji su se sustigla tri događaja, pa se simultano odvijaju pozitivna, ali i ona nepoželjna. Pored opisane preduzimljivosti domaćeg kadra koja se okončava aktiviranjem „dezinfekcione centrale", dolazi Kraljevski vojno-sanitetski korpus oficira pod komandom puk. dr Hantera. Ali i epidemija je napredovala, broj obolelih i umrlih se od januara do marta uvećavao.

Od dolaska misije pukovnika dr Hantera započinje druga faza epidemije.

v. Depedikulacija u osnovnim jedinicama

Obraćanje državnog odbora višoj komandi je realizovano na prvoj liniji fronta. Posle jednog veka, ponudiće se novi argumenti.

Takvim povodom 2017. g. posećeni su Grabovac, Obrenovac. Par razgovora sa starijim građanima nije rezultovao pobuđivanjem sećanja na razgovore sa svojim starijim ukućanima o sušarama, komorama i sl. Sećanje su bila nedovoljna.

U Vojnom arhivu su nađena dokumenta koja govore o aktivnostima u jedinici pukovnika Krste Smiljanića, komandanta Timočke divizije drugog poziva (31), tada lociranoj na području Obrenovca.

Naredbom komandanta od 19.3.1915. zadatak oficira inžinjera, bio je da organizuju dezinfekciju vojničkog odela podizanjem novih ili popravkom već postojećih sušara za voće, peći za hleb, i/ili izgradnjom komora za razvašljivanje (32) (Slika 3).

Ova nepokretna sredstva za depedikulaciju upotrebljavana su u:

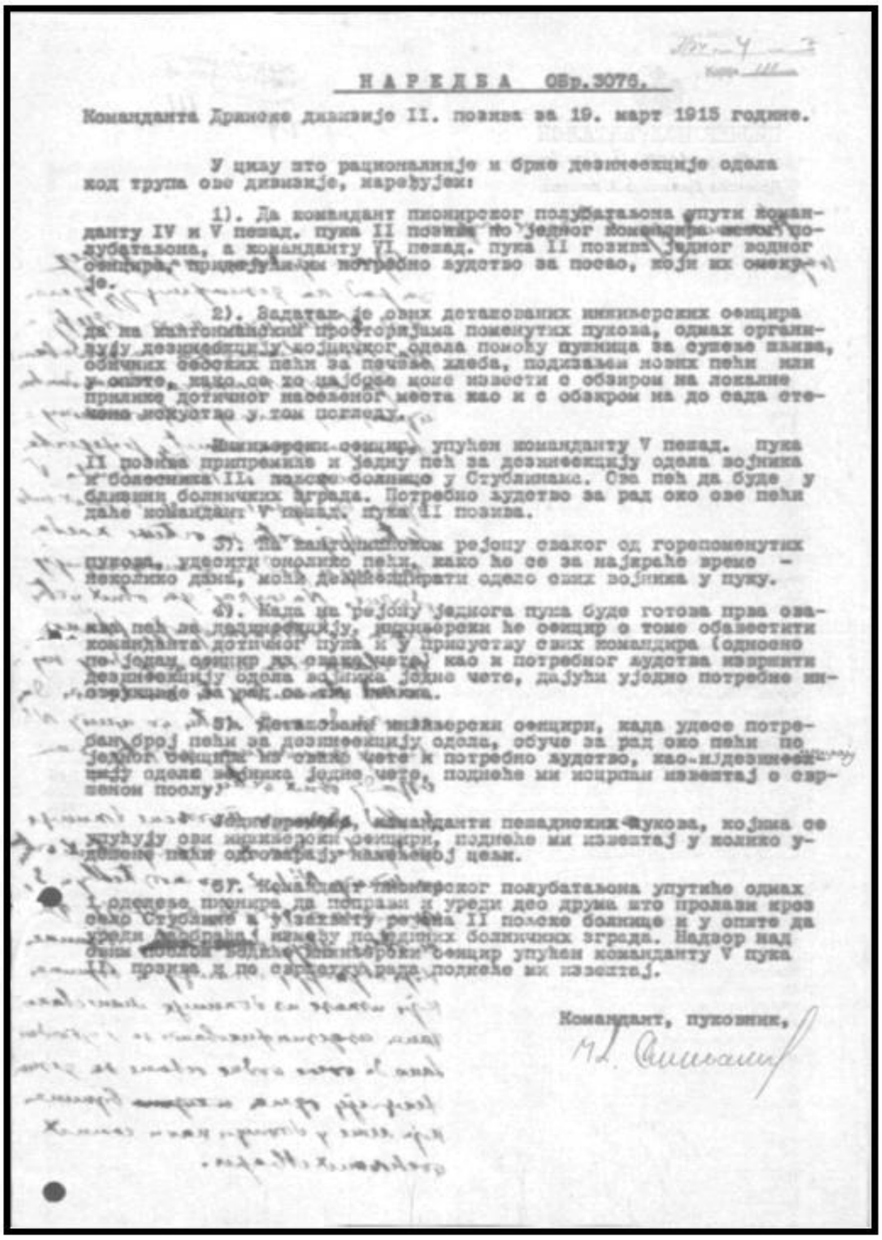
- Selu Stublini, gde je bila smeštena rezervna (poljska) bolnica za lečenje zaraznih bolesti. Ovoj poljskoj bolnici predate su na upotrebu dve „dezinfekcione peći". Po izvršenoj naredbi o tome se obaveštava komandant.
- Selu Grabovcu, gde je za jedinice napravljeno još četiri peći za potrebe zdravih vojnika u pukovima.

Sačuvana je specifikacija potrebnog materijala (greda, eksera, šarki, limova itd.) sa računom za utrošeni materijal. Postojala su bar dva tipa peći. Nisu nađeni opisi izgrađenih peći ili skice.

Po izveštaju, i u Obrenovcu je prepravljena pušnica i sagrađena dezinfekciona peć (33).

Sačuvana je specifikacija potrebnog materijala (greda, eksera, šarki, limova itd) sa računom za utrošeni materijal. Postojala su bar dva tipa peći. Nije nađen opis izgrađenih peći niti skice. Iz dokumenta koji je nastao pri povlačenju srpske vojske prema Albaniji, vidi se da su u toj jedinici, pored peći, korišćena i „Stamersova burad" (metalna „srpska burad") (34).

U Podgorici je 17.12.1915. ustanovljeno razboljevanje od pegavca. Usledila je reakcija komandanta: „U varoši Podgorici mogu se dobaviti burad od zejtina ili od špiritusa, od kojih se vrlo lako može napraviti bure za dezinfekciju odela". Uzgred se ukazuje i na nama ovde bitno iskustvo, u vreme epidemija na području Obrenovca: „Njihova upotreba je dovoljno poznata, jer su se trupe ove divizije služile tim buradima za vreme prošle zaraze nekoliko meseci" (34). Reakcija komandanta bila je brza i adekvatna. Od predavanja predloga 01.03. armijskom vrhu, prispeća naređenja u jedinicu i realizacije izgradnje komora i peći prošlo je oko 5-6 nedelja, više inkubacionih perioda od 12 do 14 dana.



НАРЕДБА ОБр.3076.

Команданта Дринске дивизије II. позива за 19. март 1915 године.

У циљу што рационалније и брже дезинфекције одела код трупа ове дивизије, наређујем:

1). Да командант пионирског полубатаљона упути команданту IV и V пешад. пука II позива по једног командира [illegible] полубатаљона, а команданту VI пешад. пука II позива једног водног официра, придејући им потребно људство за посао, који их очекује.

2). Задатак је ових деташованих инжињерских официра да на квартирским просторијама поменутих пукова, одмах организују дезинфекцију војничког одела помоћу пушница за сушење шљива, обичних сеоских пећи за печење хлеба, подизањем нових пећи или у опште, како се то најбоље може извести с обзиром на локалне прилике дотичног насељеног места као и с обзиром на до сада стечено искуство у том погледу.

Инжињерски официр, упућен команданту V пешад. пука II позива припремиће и једну пећ за дезинфекцију одела војника и болесника II. пољске болнице у Стублинама. Ова пећ да буде у близини болничких зграда. Потребно људство за рад око ове пећи даће командант V пешад. пука II позива.

3). На квартирском рејону сваког од горепоменутих пукова, удесити онолико пећи, како ће се за најкраће време – неколико дана, моћи дезинфицирати одело свих војника у пуку.

4). Када на рејону једнога пука буде готова прва оваква пећ за дезинфекцију, инжињерски ће официр о томе обавестити команданта дотичног пука и у присуству свих командира (односно по један официр из сваке чете) као и потребног људства извршити дезинфекцију одела војника једне чете, дајући уједно потребне инструкције за рад око ових пећи.

5). Деташирани инжињерски официри, када удесе потребан број пећи за дезинфекцију одела, обуче за рад око пећи по једног официра из сваке чете и потребно људство, као и дезинфекцију одела војника једне чете, поднеће ми исцрпан извештај о свршеном послу.

[illegible], команданти пешадиских пукова, којима се упућују ови инжињерски официри, поднеће ми извештај у колико удешене пећи одговарају намењеној цељи.

6). Командант пионирског полубатаљона упутиће одмах 1 одељење пионира да поправи и уреди део друма што пролази кроз село Стублине а у захвату рејона II пољске болнице и у опште да уреди [illegible] између појединих болничких зграда. Надзор над овим послом водиће инжињерски официр упућен команданту V пука II позива и по свршетку рада поднеће ми извештај.

Командант, пуковник,

Slika 3. Naredba od 19.3.1915. o pravljenju više tipova improvizacija: sušara za voće, peći za hleb i dezinfekcionim peći (32)

Različitom brzinom se reagovalo u početku nastajanja epidemija i nekoliko meseci kasnije, kod nove pojave pegavca. U novonastaloj situaciji, to je bilo znatno brže, jer se znalo za „Stamersovo bure" i kako se ono pravi. Sve se događalo ubrzano, reagovalo se pre okončavanja jednog inkubacionog perioda bolesti.

U toku decembra 1914. godine, na početku epidemija, uz ostale faktore, glavni je bio nedostatak naprava za ofanzivno delovanje. Naručeni autoklavi u Americi nisu stigli u Srbiju ni u aprilu (25). Sve to išlo je u prilog brzom rastu epidemija pegavca i rekurensa i doprinelo pogubnom toku.

Kada je 25 godina kasnije, Srpsko lekarsko društvo 1941. donosilo Rezoluciju o pegavcu, u nagoveštavanom ratu ukazano je na sledeće o „opštinskim depedikulacionim središtima" („dezinfekcionoj centrali", GČ): „Ona bi se sastojala od „uređenja primitivnog kupatila za pranje i šišanje i komore za uništavanje vašiju na vešu i odelu. Ukoliko u mestu postoje javna kupatila i dezinfekcioni aparati imaju biti u istim upotrebljeni (...) Jedna bi strana takvog kupatila bila prljava (za svlačenje, šišanje i sapunjanje), u sredini bi se vršilo pranje, a druga strana, čista (za brisanje i oblačenje). Za depedikulaciju vašljivog rublja i odela dolazi u obzir samo uništavanje vašiju vrelim vazduhom u specijalnim komorama ili u adaptiranim za tu potrebu malim sobama, jer je kapacitet parnih dezinfekcionih aparata, a naročito srpskog bureta malen, a i vlažnost odela posle

parne dezinfekcije (...) mnogo bi zadržavala ponovno iskorišćavanje očišćenih stvari, što je za naše prilike, gde ljudi često nemaju drugog rublja i odela, osim onog na sebi, od velike važnosti." Komora bi bila manja soba od 20-30 kubnih metara, u kojoj bi se zagrevanjem mogla postići temperatura od 70 - 80 ^{0}C (35).

Među onima koji su podržali Rezolucije bio je i dr Vladimir Stanojević. Tada je izložio: „U ratu koji se pomalja (...) neman epidemje pegavca će dobijati sve više kako za svoj postanak, tako i za svoje širenje. Ako tome dodamo i naša nedovoljna materijalna sredstva za održavanje telesne čistoće kod širokih slojeva naroda, naša nedovoljna sredstva u rublju, sapunu, perionicama, kupatilima, dezinsekcionim ustanovama na terenu, našu nedovoljnu organizaciju za sprečavanje i suzbijanje epidemije pegavca, onda se u punoj svetlosti prikazuje slika i izgled strahovite opasnosti, kojoj idemo u zimskim danima u susret od pojave i širenja epidemije pegavca kako u narodu, tako i u vojsci (...)" (36). U datom prikazu, prepoznatljiva je stvarnost s početka 1915. godine koju je rešavao i rešio srpski sanitet. Tako su 25 godina kasnije, dr Genčić i Odbor ocenjeni uspešnim, primenom rešenja iz 1915. godine: depedikulacionih središta i dr.

Nova etapa istraživanja događanja iz 1915. godine ostvariva je putem upoznavanja zbivanja istraživanjem arhivske građe i drugih izvora, koji nisu potpuno uništeni pri povlačenju i nalaze se u Kruševcu, kako se tvrdilo u saopštenjima 1925. godine [20:330]. Začetnik takve nove etape medicinskih istraživanja događaja u velikom ratu u Srbiji je dr Aleksandar Nedok, sa svojim brojnim radovima, koji se tiču istorije i organizacije vojnog saniteta.

Zaključak

Vulović je usavršio Subbotićev prototip ukopane komore na topli suvi vazduh. Potom je Odbor objavio nacrte komora. Početkom marta 1915. godine, puštena je u rad „dezinfekciona centrala" u Nišu. Vulović se založio za samoinicijativnost na izradi improvizacija i u primeni drugih mera. Komore manjeg kapaciteta primenjivane su u vojnim jedinicama, npr. na području Obrenovca. Aktivnosti Odbora je podžao načelnik saniteta Vrhovne komande dr Genčić i vojni vrh.

◇ Srpska medicina se izradom i primenom „komora na topli suvi vazduh" svrstava među one koji su naučno doprineli medicini sveta u oblasti suzbijanja i prevencije pegavog tifusa.

◇ Srpski sanitet je realizovao komore na suvi topli vazduh u kratkom vremenu, od svega par nedelja, ali se simultano ispoljilo i negativno delovanje nekoliko talasa obolevanja u trajanju od više inkubacionih perioda za pegavac.

◇ Glavni razlog zašto je nastala epidemija pegavca 1915. godine je „nemoć medicine". Ona se ogleda u početku nastajanja epidemija u odsustvu postojanja pogodnog taktičkog sredstva za masovnu depedikulaciju. Takve naprave za ofanzivno delovanje je tek trebalo smisliti. Tom zadatku je pristupio Srpski sanitet i uspešno ga okončao puštanjem u rad komora, u gradu Nišu i u osnovnim borbenim jedinicama na vojištu.

◇ Usvajanje Nikolovih ogleda je uslov za primenu sredstva, a to je bilo pre 28.1.1915, tj. pre Batutovog članka. Dr Genčić je isto znao bar od 15.1. 1915. godine.

◇ Različita brzina aktiviranja upotrebljavanih sredstava za depedikulaciju u dva događanja takođe govori o prisustvu nemoći medicine. Sredstvo koje nije postojalo, nije moglo ni biti primenjeno u mnogo dramatičnijoj situaciji započinjanja epidemije u Srbiji. Budući da su se stekla saznanja, u novonastaloj situaciji primenjeno je „Stamersovo bure" i to vrlo brzo, u roku kraćem od jednog inkubacionog perioda za pegavac. Sve se to događalo van Srbije, pri povlačenju srpske vojske 1915. u težim uslovima.

◇ Velisav Vulović, predsednik Državnog odbora za suzbijanje zaraza, pokazao je tokom 1915. da je svoje mišljenje sticao na osnovu ozbiljnog i savesnog proučavanja. Branio je ta mišljenja odlučno, dosledno iskreno, spreman da se do kraja založi za odbranu ideja, u čiju je ispravnost verovao. Bio je potpuno predan interesima svoje države i posla.

◇ Zagonetno je zašto komorama na suvi topli vazduh nisu pridali značaj domaći lekari, kao akteri i kao istoričari medicine. Oni nisu zapazili trenutak kojim nastaje „naučni problem", a to je kada je fabrički autoklav trebalo zameniti njegovim improvizacijama. Nova valjana rešenja nastajala su velikom brzinom, „iz dana u dan", pa ih otuda akteri događanja 1915. nisu ni zapazili, a još manje učestvovali u njihovom stvaranju. U posleratnom periodu, u istorijsko-medicinskim razmatranjima, analiza sećanja nije bila od većeg značaja, jer su im ova događanja promakla, a potom saznanja nisu dopunjavana istraživanjem arhivske i dr. građe, koja je danas, sigurno znamo, bar delimično sačuvana.

◇ Rezolucijom SLD pred rat 1941. godine, kod očekivane pojave epidemije pegavog tifusa, predložena su rešenja do kojih se došlo u suzbijanju epidemija 1914/15. godine. Time je lekarski stalež iskazao svoju pozitivnu ocenu o urađenom 1915. godine i tako odao zaluženo priznanje tadašnjem srpskom sanitetu i njegovim čelnicima Lazaru Genčiću i Velisavu Vuloviću, kao najodgovornijim za realizaciju smišljene borbe protiv pegavog tifusa.

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BATUT, 1915: PROSVEĆENI SINOVI, POVEDITE SMIŠLJENU BORBU PROTIV PEGAVOG TIFUSA!

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Prošao je vek od epidemije pegavog tifusa 1914/15. godine, ali nije odgovoreno da li je srpski narod bio poveden u borbu protiv pegavog tifusa i ako jeste, koliko je to bilo uspešno? Da li su lekari ostali nedorečeni ne razmatrajući aktivnost Državnog odbora za suzbijanje zaraznih bolesti (Odbor)? Da li je bilo koraka zbog kojih treba da ga pamte građani Srbije, a pogotovu lekarski stalež? Odbor je odlučivao 1915. godine i to su zvanični stavovi Srbije od kojih treba poći u analizama tog doba, dok su posleratne istorijsko-medicinske ocene sekundarne. Kao predsednik Odbora, Velisav Vulović je bio koordinator ovog tela i utoliko uticajan na rad saniteta Srbije 1915. godine. Nedostatkom dovoljnog broja fabričkih autoklava nastao je pravi naučni problem kako ih zameniti. Vulović je preteča sanitarnih inžinjera usavršavanjem prototipa „komore na topli suvi vazduh". Izgrađena je „dezinfekciona centrala" u Nišu i puštena u rad pre dolaska Hanterove misije. Sa njom se, moglo ofanzivno delovati depedikulacijom u suzbijanju epidemija transmisivnih bolesti. Koristile su je vojska, bolnice, zarobljenici i građanstvo. Uz saglasnost dr Genčića, komora je bila primenjivana i u osnovnim jedinicama na vojištu. Aktivnost srpskog kadra 1915. u suzbijanju epidemija imala je svojih uspeha. Povedena je smišljena borba protiv pegavca i rekurensa i tako zadužen ne samo srpski rod, već i medicina sveta.

Acta Medica Medianae 2018;57(3):145-154.

Ključne reči: *Velisav Vulović, Državni odbor za suzbijanje zaraza, komore na topli suvi vazduh, pegavi tifus, epidemije 1915. godine*

SURVEY OF KNOWLEDGE AND ATTITUDES OF HEAD NURSES REGARDING ORGAN TRANSPLANTATION

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(Vol 56, No 4, December, 2017)

In the paper titled "SURVEY OF KNOWLEDGE AND ATTITUDES OF HEAD NURSES REGARDING ORGAN TRANSPLANTATION" by Željko Vlaisavljević, Slobodan Janković and Ivan Soldatović, published in AMM journal in 2017, number 56 (4), there occurred a technical error on the pages 38-44, with published wrong references for the paper. We hereby apologize to the authors and readers. With authors' approval, we are now publishing the correct reference list.

Acta Medica Medianae 2018;57(3):155-156.

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ISPITIVANJE ZNANJA I STAVOVA GLAVNIH SESTARA O TRANSPLATACIJI ORGANA

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(Vol 56, No 4, December, 2017)

U radu " ISPITIVANJE ZNANJA I STAVOVA GLAVNIH SESTARA O TRANSPLATACIJI ORGANA" autora Željka Vlaisavljevića, Slobodana Jankovića i Ivana Soldatovića, objavljenom u časopisu AMM za 2017. godinu broj 56 (4) na stranama od 38-44, došlo je do tehničke greške, pri kojoj su odštampane pogrešne reference za ovaj rad. Ovom prilikom se izvinjavamo autorima i čitaocima. Uz saglasnost autora, u ovom broju objavljujemo ispravan spisak referenci.

Acta Medica Medianae 2018;57(3):155-156.

JEDINSTVENI KRITERIJUMI ZA OBJAVLJIVANJE NAUČNIH RADOVA U BIOMEDICINSKIM ČASOPISIMA

Ideja o postavljanju jedinstvenih kriterijuma za objavljivanje radova u časopisima za biomedicinske nauke iskristalisana je 1978. godine u Vankuveru. Ovi kriterijumi za rukopise, uključujući pravila za pisanje bibliografije, prvi put su objavljeni 1979. godine. Vankuverska grupa je vremenom prerasla u Međunarodni komitet urednika medicinskih časopisa — International Committee of Medical Journal Editors (ICMJE). Trenutno je na snazi peta revizija kriterijuma za objavljivanje radova u biomedicinskim časopisima, doneta 1997. godine.

Kriterijumi za citiranje i navođenje referenci

Reference se obeležavaju arapskim brojevima u zagradama, pri čemu se reference obele-žavaju brojevima onim redosledom kojim se pojavljuju u tekstu. Reference citirane jedino u tabelama ili legen-di moraju se obeležiti brojem u skladu sa redosledom pojavljivanja u tekstu.

Naslove medicinskih časopisa treba pisati u skraćenom obliku onako kako su navedeni u poglavlju **List of Journals Indexed in Index Medicus**. Lista skraćenih naziva medicinskih časopisa objavljuje se svake godine u januarskom broju ***Index Medicusa***. Ova lista se takođe može naći na adresi **www.nlm.nih.gov**

Izbegavati upotrebu apstrakata kao referenci, već koristiti samo izvorne tekstove (*in extenso* članci). Reference koje se odnose na radove koji su prihvaćeni, ali još nisu odštampani, treba označiti sa "u štampi", pri čemu autor mora imati pismeno odobrenje da citira takve radove i da priloži pismeni dokaz da je citirani rad prihvaćen za štampu. Informacije iz rukopisa koji nisu prihvaćeni za štampanje mogu se citirati u tekstu kao "neobjavljeni rezultati", ali sa pismenom dozvolom autora.

Izbegavati citiranje prethodnih saopštenja (personal communication) ukoliko ona ne obezbeđuju esencijalne rezultate koji još nigde nisu objavljeni. U ovom slučaju, neophodno je u zagradi navesti ime osobe i datum usmenog saopštenja rezultata. Za objavljivanje ovih podataka neophodno je pismeno odobrenje autora.

Kriterijumi za pisanje referenci korišćenih u radu

U ovom pregledu biće obrađena pravila za pi-sanje literaturnih referenci samo za najčešće korišćene tipove publikacija.

Članci u časopisima

1. Standardni članak u časopisu

Navesti prvih šest autora, ukoliko ih je više iza šestog dodati **et al.** ukoliko je referenca na engleskom jeziku ili **i sar.** ukoliko je referenca na srpskom jeziku.

Vega KJ, Pina I, Krevsky B. Heart transplantation is associated with an increased risk for pancreatobiliary disease. Ann Intern Med 1996; 124(11):980-3.

Parkin DM, Clayton D, Black RJ, Masuyer E, Friedl HP, Ivanov E, et al. Childhood–leukaemia in Europe after Chernobyl: 5 year follow-up. Br J Cancer 1996;73:1006-12.

2. Organizacija kao autor

The Cardiac Society of Australia and New Zealand. Clinical exercise stress testing. Safety and performance guidelines. Med J Aust 1996;164:282-4.

3. Članak bez poznatih autora

Cancer in South Africa (editorial). S Afr Med J 1994;84:15.

4. Volumen sa suplementom

Shen HM, Zhang QF. Risk assessment of nickel carcinogenicity and occupational lung cancer. Environ Health Perspect 1994; 102 Suppl 1:275-82.

5. Broj sa suplementom

Payne DK, Sullivan MD, Massie MJ. Women's psychological reactions to breast cancer. Semin Oncol 1996;23(1 Suppl 2):89-97.

6. Volumen sa više delova

Ozben T, Nacitarhan S, Tuncer N. Plasma and urine sialic acid in non-insulin dependent diabetes mellitus. Ann Clin Biochem 1995;32(Pt 3):303-6.

7. Broj sa više delova

Poole GH, Mills SM. One hundred consecutive cases of flap lacerations of the leg in ageing patients. N Z Med J 1994;107(986 Pt 1):377-8.

8. Časopisi sa brojem bez volumena

Turan I, Wredmark T, Fellander-Tsai L. Arthroscopic ankle arthrodesis in rheumatoid arthritis. Clin Orthop 1995;(320):110-4.

9. Časopisi bez volumena i broja

Browell DA, Lennard TW. Immunologic status of the cancer patient and the effects of blood transfusion on antitumor responses. Curr Opin Gen Surg 1993:325-33.

10. Reference u obliku apstrakta ili prethodnih saopštenja

Enzensberger W, Fischer PA. Metronome in Parkinson's disease (letter) Lancet 1996;347:1337.

Clement J, De Bock R. Hematological complica–tions of hantavirus nephropathy (HVN) (abstract). Kidney Int 1992; 42:1285.

Udžbenici i monografije

11. Monografija

Ringsven MK, Bond D. Gerontology and leadership skills for nurses. 2nd ed. Albany (NY): Delmar Publishers; 1996.

12. Autori kao urednici

Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996.

13. Organizacija kao autor i izdavač

Institute of Medicine (US). Looking at the future of the Medicaid program. Washington: The Institute; 1992.

14. Poglavlje u knjizi

Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995. p. 465-78.

15. Conference proceedings

Kimura J, Shibasaki H, editors. Recent advances in clinical neurophysiology. Proceedings of the 10th International Congress of EMG and Clinical Neurophysiology; 1995 Oct 15-19; Kyoto, Japan. Amsterdam: Elsevier; 1996.

16. Conference paper

Bengtsson S, Solheim BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors.

MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sep 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. p. 1561-5.

17. Istraživački ili tehnički izveštaji

Službeni izveštaji (Issued by funding / sponsoring agency):

Smith P, Golladay K. Payment for durable medical equipment billed during skilled nursing facility stays. Final report. Dallas (TX): Dept. of Health and Human Services (US), Office of Evaluation and Inspections; 1994 Oct. Report No.: HHSIGOEI69200860.

Sponzorisani izveštaji (Issued by performing agency)

Field MJ, Tranquada RE, Feasley JC, editors. Health services research: work force and educational issues. Washington: National Academy Press; 1995. Contract No.: AHCPR282942008. Sponsored by the Agency for Health Care Policy and Research.

18. Magistarske i doktorske disertacije

Kaplan SJ. Post-hospital home health care: the elderly's access and utilization [dissertation]. St. Louis (MO): Washington Univ.; 1995.

Druge vrste publikovanog materijala

Neobjavljeni materijal

19. U štampi (In press)

Leshner AI. Molecular mechanisms of cocaine addiction. N Engl J Med. In press 1996.

Elektronski zapisi

20. Internet članak u elektronskom formatu

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar "cited 1996 Jun 5"; 1(1)(24 screens). Available from: URL: http://www.cdc.gov/ncidod/EID/eid.htm

21. Monografija u elektronskom formatu

CDI, clinical dermatology illustrated (monograph on CD-ROM). Reeves JRT, Maibach H. CMEA Multimedia Group, producers. 2nd ed. Version 2.0. San Diego: CMEA; 1995.

22. Kompjuterski podaci

Hemodynamics III: the ups and downs of hemodynamics (computer program). Version 2.2. Orlando (FL): Computerized Educational Systems; 1993.

PROPOZICIJE ZA PISANJE RADOVA U ACTA MEDICA MEDIANAE

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Priloge u vidu teksta, tabela i ilustracija (grafikoni, crteži i dr.) ne unositi u tekst članka, već na kraju teksta, na posebnim stranicama obeleženim u gor-njem levom uglu sa "Tabela, Grafikon, Ilustracija" i arapskim brojem redosledom pojavljivanja u tekstu (npr. Tabela 1, Grafikon 1 i dr.) i svakoj se daje kratak naslov. Kratka objašnjenja i skraćenice daju se u fusnoti. Za fusnotu koristiti sledeće simbole: *, **, ***, #, ##, ###, …itd. Tabele, grafikone i ilustracije treba praviti korišćenjem nekog od programa iz Microsoft Office paketa. Izbegavati upotrebu boja kod izrade grafika.

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