Vol 63, No 3, October, 2024
UDK 61
ISSN 0365-4478 (Printed)
ISSN 1821-2794 (Online)
www.medfak.ni.ac.rs/amm

ACTA MEDICA MEDIANAE

Naučni časopis
Medicinskog fakulteta Univerziteta u Nišu i
Podružnice Srpskog lekarskog društva u Nišu

AMM

Scientific Journal of the University of Nis Faculty of Medicine
and the Department of the Serbian Medical Society in Niš

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Acta Medica Medianae
Vol 63, No 3, October, 2024
UDK 61 ISSN 0365-4478 (Printed version)
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http://www.medfak.ni.ac.rs/amm

Acta Medica Medianae (UDK 61; ISSN 0365-4478 štampana verzija; ISSN 1821-2794 elektronska verzija) je zvanični časopis Medicinskog fakulteta Univerziteta u Nišu i Podružnice Srpskog lekarskog društva u Nišu pod pokroviteljstvom Ministarstva za nauku i tehnološki razvoj Republike Srbije. Časopis izlazi četiri puta godišnje od 1962 godine. Izdavač je Medicinski fakultet Univerziteta u Nišu, Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija. Sadržaj i celokupan tekst časopisa dostupan je na sajtu Medicinskog fakulteta http://www.medfak.ni.ac.rs/amm. Uputstvo autorima se objavljuje u svakom broju, pri čemu je autor dužan da se pridržava navedenih uputstava prilikom predaje rukopisa.
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Acta Medica Medianae zadržava pravo dalje distribucije i štampanja radova.
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Tiraž 200 primeraka. Štampa: "Galaksijanis", Svrljig, Srbija.
Acta Medica Medianae je trenutno indeksirana u *Srpskom citatnom indeksu, DOAJ-u i KOBSON-u.*

Acta Medica Medianae (UDK 61; ISSN 0365-4478 printed version; ISSN 1821-2794 online) is the official Journal of the University of Niš Faculty of Medicine and the Department of the Serbian Medical Society in Niš published with the help of the Ministry of Science and Technological Development of the Republic of Serbia. The Journal has been published four times a year since 1962. The publisher is the University of Niš Faculty of Medicine, Institutional address: dr Zoran Đinđić 81, 18000 Niš, Serbia. Table of contents and full texts of articles are available on the Institutional Home Page at http://www.medfak.ni.ac.rs/amm. Prices are subject to change. All subscriptions start with the first issue of the current year. For payment details contact the Secreteriat at acta@medfak.ni.ac.rs. Instructions for authors appear in every issue. Manuscripts accepted for publication are not returned to the author(s). *Acta Medica Medianae* retains the right for further distribution and printing of the articles.
Editorial correspodence: Journal Acta Medica Medianae, Faculty of Medicine, Dr Zoran Djindjić 81, 18000 Niš, Serbia.
Electronic submission of the papers: acta@medfak.ni.ac.rs, Phone: +381-18-4533001 lok. 113 fax. +381-18-4534336
Printed on acid-free paper; 200 issues. Press: "Galaksijanis", Svrljig,Serbia
Acta Medica Medianae is currently indexed in Serbian Citation Index, DOAJ and KOBSON.

Naučni časopis Medicinskog fakulteta Univerziteta u Nišu i Podružnice Srpskog lekarskog društva u Nišu

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Vol 63, No 3, October, 2024
UDK 61 ISSN 0365-4478 (Printed version)
ISSN 1821-2794 (Online)
http://www.medfak.ni.ac.rs/amm

Publikovanje časopisa Acta Medica Medianae sufinansira Ministarstvo nauke, tehnološkog razvoja i inovacija.

Publishing of the journal Acta Medica Medianae is co-financed by the Ministry of Science, Technological Development and Innovation.

Autor slike na prednjoj stranici:
Nikola Jovanović

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Original article **UDC: 617.7-007.681-07:577.112**
doi: 10.5633/amm.2024.0301

ASSOCIATION BETWEEN GLAUCOMA DAMAGE AND PLASMA CONCENTRATION OF HEAT SHOCK PROTEIN 70

Marija Trenkić[1,2], Tatjana Jevtović–Stoimenov[3], Jelena Bašić[3], Marija Radenković[2], Marija Cvetanović[2], Milan Trenkić[3], Nevena Zlatanović[4]

Heat shock proteins (HSP) or stress proteins are induced in cells. They protect the cell and increase cell survival.

The aim of this research was to determine the plasma concentration of HSP 70 in patients with primary open-angle glaucoma with elevated intraocular pressure (POAG-HTG) and patients with pseudoexfoliative open-angle glaucoma (XFG), and to investigate the relationship between this biomarker and the structural and functional characteristics of glaucoma.

The study included 90 participants divided into three groups: 37 patients with primary open-angle glaucoma with increased intraocular pressure (hypertensive glaucoma, POAG-HTG), 24 patients with pseudoexfoliative open-angle glaucoma (XFG), and 29 participants without systemic diseases and glaucoma, matched by sex and age (control group of subjects, CONT). The concentration of circulating HSP 70 was measured in participants' plasma using the sandwich enzyme-linked immunosorbent assay (ELISA).

Plasma levels of HSP 70 were very similar in all three groups of participants, without any significant differences among the examined patients. A significant negative correlation of the plasma concentration of HSP 70 and RNFL Savg ($p < 0.05$) was found in POAG-HTG patients, whereas the negative correlations of HSP 70 with MD ($p = 0.0538$) and with RNFL Iavg ($p = 0.0584$) were very close to statistical significance.

There was no increase in the plasma concentration of HSP 70 in POAG-HTG and XFG patients. There was an interdependence between the plasma level of HSP 70 and the examined clinical parameters of POAG-HTG patients (MD, RNFL Avg, RNFL Savg and RNFL Iavg). HSP 70 can be a significant biomarker for glaucoma.

Acta Medica Medianae 2024;63(3):5–13.

Key words: *heat shock protein 70, glaucoma, open-angle, hypertension glaucoma, pseudoexfoliation, plasma*

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Introduction

Heat shock proteins (HSPs) are stress proteins and their production in cells protect the cell and increase cell survival. Their concentration increases in physiologically or environmentally stressful conditions. HSPs function as chaperone molecules that prevent aggregation and facilitate the remodelling of deactivated proteins in the cell (1, 2).

HSPs act by the most diverse mechanisms including direct interaction with components of cellular signalling pathways, downstream or upstream regulation of caspase-dependent programmed cell death, or at the mitochondrial level. HSPs can also affect caspase-independent apoptosis, by interacting with apoptogenic factors such as Apoptosis-inducing factor (AIF) or acting at the level of lysosomes. HSP 70 is a guardian of the integrity of the lysosomal membrane. Due to oxidative stress, HSP carboxylation occurs, subsequently causing lysosome rupture. In addition, HSP 70 dysfunction activates nuclear factor-κB (NF-κB) signalling that may also promote neurodegeneration (1–6).

The heat shock protein 70 family (HSP 70 family) includes several members such as the constitutive form HSP 70, inducible HSP 72,

mitochondrial GRP 75, and endoplasmic reticulum GRP 78. These proteins exhibit distinctive neuroprotective effects and function in normal, developmental, and stressful conditions. Although the constitutive form exists under normal conditions, it is more pronounced under stressful conditions (7–13).

HSP 70 is the most structurally and functionally conserved protein in the HSP family. HSP 70 is a ubiquitous class of ATP-dependent chaperone proteins that exert cytoprotective effects. It plays a central role in the cellular control of protein quality. HSP 70 binds to the protein substrate facilitating its unfolding, degradation, transport, regulation, and preventing aggregation (1).

Elevated IOP is one type of mechanical stress that activates HSPs. Several studies have shown that HSPs and anti-HSP antibodies play an important role in glaucoma pathogenesis. Cellular stress and cell death are interrelated events, and HSPs, induced in response to stress, play a role in controlling apoptosis (11). HSPs include anti-apoptotic and proapoptotic proteins that interact with various cellular proteins involved in apoptosis. Their expression level can determine cell fate in response to a stimulus (14).

Increased immunohistochemical staining of HSPs in the glaucomatous retina reflects the function of these proteins in terms of cellular defense and stress response in glaucoma (15, 16).

Optic neuropathy and progression of glaucoma occur due to overexpression of HSP and activation of the auto-stimulatory response (17). Therefore, in patients with glaucoma, an elevated titer of anti-HSP antibodies occurs compared to healthy subjects, which reduces the protective ability of HSP (16). These data indicate that HSPs are essential for RGC survival as molecular chaperones and have pathogenic significance in glaucoma patients (18).

HSPs are involved in multiple stages of apoptosis and their function is to inhibit apoptosis (19). Overexpression of HSP 70 protects mitochondria from the harmful effects of reactive oxygen species (20). HSP 70 inhibits apoptosis by reducing the release of cytochrome-c and increasing the activity of caspase-3 (21). HSP 70 has also been described to inhibit protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (22).

Kim et al. (8) demonstrated that HSP 70 is transferred from cell to cell. However, RGCs are not the sole target of HSP 70-induced neuroprotection. Glial cells also participate in HSP 70 transfer and contribute to neuroprotection.

The aim of our research was to determine the plasma concentration of HSP 70 in patients with primary open-angle glaucoma with elevated intraocular pressure (POAG-HTG) and patients with pseudoexfoliative open-angle glaucoma (XFG), and to investigate the relationship between this biomarker and the structural and functional characteristics of glaucoma disease.

Material and Methods

The research was carried out at the Clinic for Ophthalmology, University Clinical Centre Niš, Department of Biochemistry and Laboratory for Functional Genomics and Proteomics, and Scientific Research Centre for Biomedicine, University of Niš. All participants were informed about the objectives of the research and signed an informed consent to participate, according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the Faculty of Medicine in Niš (Decision number 01-2625-18) and the Ethical Committee of the University Clinical Centre Niš (Decision number 338/43).

The study included 90 participants divided into three groups: 37 patients with primary open-angle glaucoma with increased intraocular pressure (hypertensive glaucoma, POAG-HTG), 24 patients with pseudoexfoliative open-angle glaucoma XFG, and 29 participants patients without systemic diseases and glaucoma, matched by sex and age (control group, CONT).

We performed complete ophthalmic examinations, including a review of the medical history, determination of visual acuity with refractions (Snellen tables), slit-lamp biomicroscopy of the anterior and the posterior segment, gonioscopy using a three-mirror Goldmann gonioscope, Goldmann applanation tonometry (GAT), indirect ophthalmoscopy and determination of the cup size of the optic nerve head (C/D ratio), using a 90 D lens, standard automatic perimetry (Humphrey Visual Field Analyzer, HFA, USA; Carl Zeiss Meditec, Inc., Threshold Test 24-2), and glaucoma OCT scan protocols (OCT, Stratus, Carl Zeiss Meditec, Inc., Dublin, CA) and determination of retinal peripapillary nerve fibre thickness (RNFL). We measured RNFL average (RNFL Avg), in the superior quadrant (RNFL Savg) and in the inferior quadrant (RNFL Iavg).

Inclusion criteria for patients with POAG-HTG were as follows: elevated IOP, characteristic Bjerum's arcuate scotoma, and/or paracentral scotoma, and/or nasal, Rönne's step in Humphrey's computerized visual field, or other relevant defects in the visual field, optic disc cupping, and/or thinning of the nerve fibre layer on OCT, the finding of an open angle, and the absence of a secondary cause of glaucomatous optic neuropathy, such as previous trauma, previous corticosteroid administration, inflammation, or uveitis. Patients with POAG-HTG had intraocular pressure values greater than 21 mmHg upon daily measurement before diagnosis.

Inclusion criteria for patients with XFG were: elevated IOP, changes in the visual field, thinning of the RNFL on OCT, as for POAG, with the presence of pseudoexfoliations on the anterior capsule of the lens and/or along the pupillary margin.

Patients with systemic arterial hypertension, diabetes mellitus, systemic vasculopathies, retinal

disease, eye surgery, trauma and inflammation of the eye were excluded from the study. All control subjects were without glaucoma as confirmed by applying the same diagnostic criteria used for the diagnosis and with no family history of the condition, matched by sex and age. Patients with a history of congenital glaucoma or suspected normotensive (NTG) or hypertensive glaucoma (HTG) were also excluded from further examination.

Collected whole blood samples obtained from participants using EDTA as an anticoagulant, were centrifuged for 10 minutes at 3500 spins, at a temperature of 4 °C. The plasma was separated and frozen at a temperature of -80 °C afterward.

The concentration of circulating HSP 70 was measured by the sandwich enzyme linked immunosorbent assay (ELISA) method in participants' plasma, according to the manufacturer's instructions (Cusabio, CSB-E13463h, P.R. China). The concentration was determined using a standard curve and expressed in ng/ml. Minimum detectable dose (MDD) was 78 pg/ml. In accordance with the manufacturer's instructions, there was no significant cross-reactivity or interference with other proteins.

Statistical analysis

We used the methods of descriptive (absolute numbers, relative numbers, arithmetic mean, standard deviation, median, interval of variation (minimum and maximum values)) and analytical statistics (Mann-Whitney U test, Student's t-test of independent samples, Kruskal-Wallis test, ANOVA, χ^2 test or Fisher's test, Pearson's simple linear correlation coefficient, Spearman's rank correlation coefficient, univariate linear regression analysis). Statistical processing of the results was done with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was used as the threshold for statistical significance.

Research results

Table 1 shows the demographic characteristics of 90 participants (37 POAG-HTG + 24 XFG + 29 CONTROL), basic clinical parameters of glaucoma (IOP, C/D ratio, MD, RNFL Avg, RNFL Savg, and RNFL Iavg), and plasma levels of HSP 70 (Figure 1).

We found no difference in age and sex between the examined groups (Kruskal—Wallis test and Mann—Whitney test). POAG-HTG and XFG were more prevalent in men (54.05%, i.e., 58.33%), whereas, in the control group, women were more prevalent (51.73%), without any significant difference between the groups. XFG patients had the highest IOP, and IOP values in both glaucoma groups were significantly higher compared to the control group ($p < 0.001$). There was no significant difference in the values of the C/D ratio between POAG-HTG and XFG. Although the absolute value of MD was higher in eyes with XFG, it was not significantly different from the value of this parameter in eyes with POAG-HTG. All POAG-HTG and XFG patients were in the second and third groups according to the Hadopp classification, without significant differences in distribution. Average RNFL thickness and RNFL thickness in the superior and inferior quadrants were higher in XFG patients, however, not significantly compared to POAG-HTG patients.

Plasma levels of HSP 70 were very similar in all three groups of subjects. Neither Kruskal—Wallis, nor Mann—Whitney, nor Student's t-test of independent samples, revealed any significant differences between the examined patients.

Spearman's rank correlation coefficient and Pearson's linear correlation coefficient were used to examine the connection between plasma concentration of HSP 70 and the intraocular pressure (IOP), C/D ratio, MD, RNFL Avg, RNFL Savg and RNFL Iavg of the same patient (Table 2). A significant negative correlation of the plasma concentration of HSP 70 and RNFL Savg ($p < 0.05$) was found in POAG-HTG patients, whereas the negative correlations of HSP 70 with MD ($p = 0.0538$) and with RNFL Iavg ($p = 0.0584$) were very close to statistical significance.

Table 1. Demographic and clinical characteristics, and plasma levels of HSP 70 of glaucoma patients and the control group of participants without glaucoma

	POAG-HTG (n = 37)	**XFG** (n = 24)	**CONTROL** (n = 29)	
Age (year) X ± SD (Me) Min–Max	70.95 ± 8.01 70.00 58–87	73.41 ± 6.25 76.00 59–84	71.77 ± 9.38 74.0 51–88	Kruskal–Wallis test Mann–Whitney test
Gender (M/F)	20 (54.05%) /17 (45.94%)	14 (58.33%) /10 (41.67%)	14 (48.27%) /15 (51.73%)	

IOP (mmHg) X ± SD (Me) Min–Max	**21.86 ± 7.43** (20.00) 10–48	**23.58 ± 11.31** (20.50) 10–56	14.76 ± 2.39 (14.00) 8–20	p < 0.001 Kruskal–Wallis and Mann–Whitney test
C/D ratio X ± SD (Me) Min–Max	0.64 ± 0.16 (0.60) 0.4–1	0.63 ± 0.18 (0.55) 0.4–1		Mann–Whitney test
MD (dB) X ± SD (Me) Min–Max	-11.73 ± 9.35 (-8.46) -0.38–-31.27	-12.72 ± 11.21 (-8.62) -0.07–-29.69		Mann–Whitney test
RNFL Avg (µm) X ± SD (Me) Min–Max	78.09 ± 24.48 (80.98) 24.46–143.71	78.62 ± 21.54 (81.63) 45.77–103.69		Mann–Whitney test
RNFL Savg (µm) X ± SD (Me) Min–Max	92.38 ± 34.87 (96.00) 26–180	100.64 ± 37.66 (104.00) 44–161		Mann–Whitney test
RNFL Iavg (µm) X ± SD (Me) Min–Max	94.41 ± 37.95 (101.00) 29–157	96.10 ± 31.11 (91.00) 57–144		Mann–Whitney test
HSP 70 (ng/ml) X ± SD (Me), Min–Max	2.27 ± 1,70 (1.97) 0.18–6.55	2.14 ± 0.96 (2.29) 0.29–3.70	2.20 ± 1.32 (1.93) 0.42–5.46	Kruskal–Wallis and Mann–Whitney test, Student´s t-test

Legend: n—number of participants/eyes and examined plasma samples, POAG-HTG—primary open-angle glaucoma with elevated IOP, hypertensive glaucoma, XFG—pseudoexfoliative glaucoma, CONTROL—control group without glaucoma, IOP—intraocular pressure, C/D—cup/disk ratio, MD—mean deviation, RNFL Avg—average peripapillary retinal nerve fibre layer thickness, RNFL Savg—peripapillary retinal nerve fibre layer thickness in the superior quadrant, RNFL Iavg—peripapillary retinal nerve fibre layer thickness in the inferior quadrant, HSP 70—heat shock protein 70

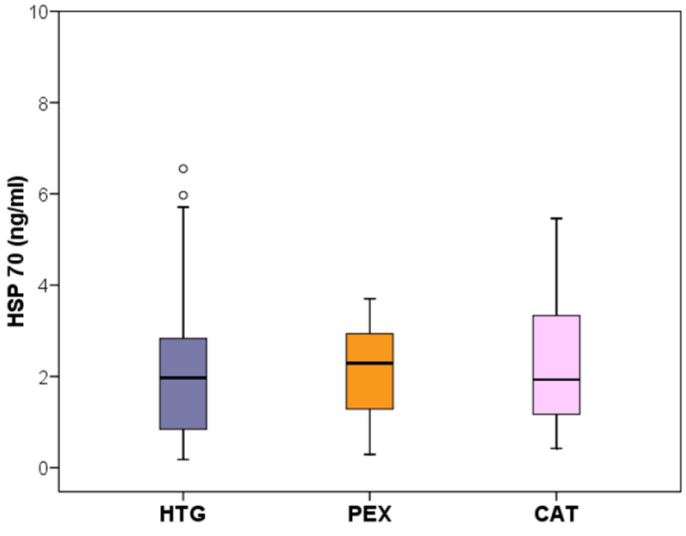

Figure 1. Median, minimum, maximum and 25th and 75th percentile values for HSP 70 in glaucoma and control group

Table 2. Spearman's and Pearson's correlation coefficients of HSP 70 and the examined parameters in glaucoma patients

ρ p (*r*) HSP 70 (ng/ml) and	IOP	C/D ratio	MD	RNFL Avg	RNFL Savg	RNFL Iavg
POAG-HTG	0.12	-0.12	-0.27	-0.26	*-**0.33**	-0.30
XFG	0,07	0,12	-0,46	*0,14*	*-0,05*	*-0,171*

* —p < 0.05, ρ—Spearman rank correlation coefficient
r—Pearson coefficient of linear correlation (values in italics)

Univariate linear regression analysis did not find any effects of HSP 70 on the values of IOP, C/D ratio for POAG-HTG patients or XFG patients (Table 3). This analysis confirmed a significant effect of HSP 70 concentration on MD and RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG patients. An increase in HSP 70 by one measurement unit caused a significant decrease in the MD value by 1.37 dB with a confidence interval of 0.01−2.73, a significant decrease in the value of RNFL Avg by 4.78 with a confidence interval of 0.11−9.45, a significant decrease in the RNFL Savg value of 5.74 with a confidence interval of 0.29−11.20, a significant decrease in the RNFL Iavg value of 7.62 with a confidence interval of 0.89−14.36, in patients with POAG-HTG (Table 3).

Table 3. Results of univariate linear regression analysis and influence evaluation of the HSP 70 on the value of IOP, C/D ratio, MD, RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG and XFG patients

Variable	**POAG-HTG**				**XFG**			
	t	**p**	**B**	**95% CI for B**	**t**	**p**	**B**	**95% CI for B**
IOP (mmHg)	0.50	0.6161	0.30	-0.87—1.47	0.07	0.9446	0.12	-3.28—3.52
C/D	-0.99	0.3255	-0.01	-0.04—0.01	0.51	0.6166	0.03	-0.10—0.16
MD (dB)	**-2.02**	**0.0490**	***-1.37**	**-2.73—-0.01**	-1.17	0.2678	-5.29	-15.27—4.69
RNFL Avg (µm)	**-2.07**	**0.0450**	***-4.78**	**-9.45—-0.11**	-2.07	0.0450	-4.78	-9.45—-0.11
RNFL Sup	**-2.13**	**0.0396**	***-5.74**	**-11.20—0.29**	-0.14	0.8932	-2.90	-50.36—44.56
RNFL Inf	**-2.29**	**0.0276**	***-7.62**	**-14.36—-0.89**	-0.52	0.6174	-8.42	-45.23—28.39

Discussion

Heat shock proteins (HSPs) are ubiquitous intracellular proteins. They are evolutionarily conserved molecules, with the role of chaperone and cytoprotective function (23).

HSP 70 plays an important role in protein metabolism, both under stress and under normal conditions. It facilitates the assembly of newly created proteins, translocation and degradation of damaged proteins, and participates in regulatory processes. In neurons exposed to extremely stressful conditions, such as ischemia and excitotoxicity, the expression of HSP occurs, and it is considered a neuroprotector. In addition, HSPs are highly antigenic, and HSPs or anti-HSP antibodies have a significant pathogenetic effect in glaucoma. The resulting immune response to HSP can have a protective or pathogenic effect. HSP production is up-regulated in RGCs of glaucoma patients. The amount of HSP, as well as HSP 70, is increased in monkeys with experimental glaucoma, and HSP 72 is increased in RGCs in a glaucoma rat model (24−27). However, elevated serum levels of HSP antibodies, detected in glaucoma patients, may activate nerve cell death via apoptosis. Initially, increased HSP expression in the glaucomatous eye may be neuroprotective, protecting against further degeneration and inhibiting apoptosis. Since HSP can also act as an immunostimulatory signal, the breakdown of the immune tolerance and the loss of their protective effect can occur, which results in the loss of regulation of antiapoptotic processes and accelerates and facilitates apoptosis, and finally disease progression occurs. Thus, glaucomatous optic neuropathy may be a consequence of aberrant autoimmunity (28).

HSP expression determines cell fate. Reduced expressivity leads to an ineffective response to stress, and increased expression activates immunodestruction. In both cases, the outcome is cell death, however, through two mechanisms. The first involves oxidative free radicals, and the second involves cytokines as shown by Tezel et al. (16). The presence of HSP in RGCs and glial cells may be important for the stimulation of cytoprotective events at the beginning and then for neurodegenerative changes (16, 25).

Heat shock proteins 70 (HSP 70) are present in the peripheral circulation of healthy individuals (29).

Previously reported data indicate that HSPs are involved in the pathogenesis of glaucoma and the development of glaucomatous neuropathy due to increased IOP. The involvement of HSP in the development of glaucoma is twofold, it can be neuroprotective or degenerative related to the activation of the autoimmune response during the progression of the disease (16, 17, 30).

A study conducted by Lichtenaur et al. indicates that the serum concentration of HSP 70 in healthy subjects was 49 pg/mL ± 22 (31). Another study showed that HSP 70 was detectable only in 77% of the analyzed samples, whereas the serum concentration of this protein decreases with age (400 ng/ml < 40 years; 20 ng/ml > or = 90 years), while the concentrations of anti-HSP 70 antibodies tend to increase with age, but without mutual dependence. These findings suggest that the stress response potential decreases with age (32).

This research found very similar plasma values of HSP 70 in all three groups of participants (POAG-HTG, XFG and CONT 2.20 ± 1.32 ng/ml). Thus, it can be concluded that there is no increase in the plasma concentration of HSP 70 in POAG-HTG, however, not excluding the increase of this protein in the aqueous humour or the RGC nor the increase of antibodies to HSP 70. This partially supports the hypothesis that the decrease in HSP levels leads to a decrease in their protective role in the pathogenesis of glaucoma. Güler M. et al. showed that the level of HSP 70 in the aqueous humour was increased in patients with pseudoexfoliation without glaucoma compared to patients with cataract without pseudoexfoliation (33), thus supporting the neuroprotective role of HSP before the onset of glaucoma and the increase of anti-HSP antibodies.

A significant negative correlation between the serum concentration of HSP 70 and RNFL Sup ($p < 0.05$) was found in patients with POAG-HTG. Hence, plasma concentration of HSP 70 can be a measure of the development and progression of glaucomatous neuropathy in POAG-HTG. Our research has confirmed that HSP 70 concentration has a significant effect on MD and RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG patients. However, it remains unclear why this is not the case with XFG.

Due to a limited number of studies investigating the concentration of HSP 70 in the plasma of glaucoma patients, and almost no research on the correlation between concentration and clinical parameters, we are unable to fully analyze the obtained results. Finally, further research, particularly larger and similarly designed studies should be conducted. Nevertheless, our research has a small contribution to the study of the role of HSP70 in glaucoma and the possibility of using this protein as a biomarker for glaucoma disease.

These results support a mechanism involving the immune response in glaucomatous damage, which may provide a new therapeutic approach to the neuroprotection of glaucomatous optic neuropathy. Understanding genetics and immunity in the pathogenesis of glaucoma is crucial for the development of new treatments.

Conclusion

There was no increase in the plasma concentration of HSP 70 in POAG-HTG and XFG patients. However, there was an interdependence between the plasma level of HSP 70 and the examined clinical parameters of POAG-HTG (MD, RNFL Avg, RNFL Savg and RNFL Iavg). This suggests that HSP 70 can be a significant biomarker for glaucoma.

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Originalni rad **UDC: 617.7-007.681-07:577.112**
doi: 10.5633/amm.2024.0301

POVEZANOST OŠTEĆENJA IZAZVANIH GLAUKOMOM SA KONCENTRACIJOM PROTEINA TOPLOTNOG ŠOKA 70 U PLAZMI

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Proteini toplotnog šoka (engl. *heat shock proteins* – HSP) ili proteini stresa indukuju se u ćelijama. Štite ćeliju i produžavaju opstanak ćelije.

Cilj našeg istraživanja bio je da se utvrdi koncentracija HSP-a 70 u plazmi obolelih od primarnog glaukoma otvorenog ugla sa povišenim intraokularnim pritiskom (POAG-HTG) i obolelih od pseudoeksfolijativnog glaukoma otvorenog ugla (PEXG), kao i da se ispita veza između ovog biomarkera i strukturnih i funkcionalnih karakteristika glaukoma.

U ovu studiju uključili smo 90 ispitanika, koji su podeljeni u tri grupe: 37 obolelih od primarnog glaukoma otvorenog ugla sa povišenim intraokularnim pritiskom (hipertenzivni glaukom, POAG-HTG), 24 obolela od pseudoeksfolijativnog glaukoma otvorenog ugla (PEXG) i 29 ispitanika bez sistemskih bolesti i bez glaukoma, razvrstanih po polu i starosti (kontrolna grupa ispitanika; engl. *control group of subjects* – CONT). Koncentracija cirkulišućeg HSP-a 70 merena je metodom imunoapsorcionog enzimskog testa (engl. *sandwich enzyme-linked immunosorbent assay* – ELISA) u plazmi ispitanika.

Nivoi HSP-a 70 u plazmi bili su veoma slični kod ispitanika iz svih triju grupa, bez značajne razlike. Bolesnici sa POAG-HTG-om imali su značajnu negativnu korelaciju koncentracije HSP-a 70 i RNFL Savg-a u plazmi ($p < 0,05$), a veoma blizu statističke značajnosti bile su negativne korelacije HSP-a 70 i MD-a ($p = 0,0538$) i HSP-a 70 i RNFL Iavg-a ($p = 0,0584$).

Zaključili smo da nema povećanja koncentracije HSP-a 70 u plazmi bolesnika sa POAG-HTG-om i PEXG-om. Postoji međuzavisnost između nivoa HSP-a 70 u plazmi i ispitivanih kliničkih parametara POAG-HTG-a (MD, RNFL Avg, RNFL Savg i RNFL Iavg). HSP 70 može biti značajan biomarker glaukoma.

Acta Medica Medianae 2024; 63(3):5–13.

***Ključne reči**: protein toplotnog šoka 70, glaukom, otvoreni ugao, hipertenzivni glaukom, pseudoeksfolijacija, plazma*

Original article

UDC: 612.753:[616.31:615.46
doi: 10.5633/amm.2024.0302

HISTOLOGICAL EVALUATION OF BONE TISSUE RESPONSE TO SILICON-BASED ENDODONTIC MATERIAL

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Successful endodontic treatment implies that the materials for obturation remain in the tissue, if possible forever. It is therefore essential to know the long-term effects of materials on tissue. This study aimed to evaluate the histological response of bone tissue to the implanted dimethylpolysiloxane-based material in the artificially prepared defect. The sample comprised 20 Wistar rats. The defect was formed in the mandible of rats by sterile stainless steel burs. Dimethylpolysiloxane-based sealer (Roeko Seal) was implanted in the defects of the experimental group while the defects of the control group were left to heal spontaneously. Half of the animals from both groups were put down after thirty days, whereas the other half was euthanized after ninety days. Microscopic preparations were analyzed by light microscope. A fibrous callus and a young bone were observed thirty days after the implantation. Ninety days after the implantation, the bone around the unabsorbed material was completely healed. Roeko Seal does not decelerate the healing of bone tissue, it enables complete healing of tissue around the material.

Acta Medica Medianae 2024;63(3):14–24.

Key words: *sealer, obturation, bone healing*

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Introduction

After the removal of canal contents and treatment of the complete canal system by irrigation, obturation follows as the final stage of endodontic treatment (1). The aim of hermetic obturation is to enable healing processes in the periapical region.

There are a lot of techniques for obturation of canal system, and the majority imply various ways of condensation of gutta-percha in combination with obturation paste (2). The role of the paste in this combination is to make suppressed gutta-percha fill the imperfections of the canal system, fill accessory canals if any, and be the bond between the gutta-percha and the wall of the root canal (3).

The border of canal filling can influence the outcome of endodontic treatment. It is considered that the material should not go over an apical foramen. However, some researchers think that a small amount of a sealer over the apical foramen may have a positive effect on healing processes (3, 4). Obturation material often goes over the apical foramen, given that despite modern achievements, the endodontic procedure is mostly "groping in the dark". Nevertheless, even in cases where the filling was done up to the wanted limit, sealer stays in contact with periapical tissue via apical foramen for a long period (for decades) (5, 6).

This fact stresses the importance of the biological characteristics of obturation materials (7). A great deal of research has shown that most materials in a freshly mixed state show a certain degree of toxicity and cause the reaction of surrounding tissue in which they have been implanted after a short period (1, 8, 9). Successful endodontic treatment means that the obturation material stays incorporated in the tissue, if possible, forever, and that the tooth is functional. It is important to understand how a specific material behaves in the tissue over time and its interaction with the tissue.

Roeko Seal sealer belongs to the group of silicon-based materials. According to the studies published on cell culture silicon-based obturation

materials showed good biological characteristics, which was not the case in sealers of different chemical compositions even in freshly-mixed state (5, 8). According to implantation tests, silicon-based sealers show satisfactory biocompatibility (9, 10, 11). Obturation materials are expected to stay in an organism for a long time, therefore it is of utmost importance to check tissue reaction to them after longer periods.

Aim

The aim of this paper was to investigate tissue reaction to bone implantation of endodontic material Roeko Seal in an artificially prepared defect in the mandible of rats after a long period (30 and 90 days).

Material and Methods

Twenty male Wistar rats with an average weight of 160−180 grams were used for the experimental procedure (the experiment was approved by the Ethic Committee of the Faculty of Medicine in Niš, No. 01 3797). The preparation of experimental animals involved the administration of an anesthetic, namely intraperitoneal injection of ketamine hydrochloride (0.1 ml/100 g). The experimental procedure involved the preparation of bone defect unilaterally (1.4 x 1.6 mm) (left side) between the medial line and the mental foramen using a sterile stainless steel dental burs.

Roeko Seal sealer (Roeko, Germany) was implanted in the formed defects of the experimental group (n = 12) according to the manufacturer's instructions (material composition is shown in Table 1). Prepared defects of the control group (n = 8) were left to heal spontaneously without any implants. One-half of the animals from the experimental (n = 6) and half of the animals from the control group (n = 4) were put down after 30 days, the other half after 90 days. The animals were put down by the excessive administration of the anaesthetic (ketamine hydrochloride).

Samples of tissue were collected by resection of the mandible and consisted of the area of the defect and the surrounding bone. Tissue samples were fixed in 10% buffered formalin, demineralized in 10% formic acid, dehydrated in alcohol and moulded in paraffin wax. Cutting was performed by microtome 2 mm glass knives (Historange). Staining was done by the H&E technique. Microscopic analysis was performed by the light microscope BX50 (Olympus, Japan).

The following parameters were examined: the degree of cell inflammatory response, the degree of fibrovascular proliferation and the reaction of the distal bone. Obtained data were classified according to a modified semiquantitative scale: 0—absence, 1—poorly, 2—moderate and 3—pronounced (12). The obtained results were added to a specially created data base, and were analysed afterwards (Friedman ANOVA and Kruskal−Wallis ANOVA).

Results

Experimental Group

The remainder of the used material for the obturation of the trepanation cavity was observed microscopically within the defects of all the samples of the experimental group. During the process of treating the sampled bone and making histological preparations in the majority of cases, the obturation material fell out or remained in traces, and the experimental defects appeared as empty spaces by the light microscope.

Thirty-Day Findings in the Experimental Group (Roeko Seal)

On the thirtieth day after the implantation, callus and newly formed bone tissue were noticeable. The replacement of fibrous callus with a young immature bone could be observed (Figure 1). The bone distal to the defect had the structure of basophilically prominent border lines of osteon and partially with a greater amount of extracellular matrix. The borders of the osteon were cracked, and partially widened due to the fine-grained or amorphous look of basophilic reaction.

Ninety-Day Findings in the Experimental Group (Roeko Seal)

Ninety days after the implantation a defect could be observed and partially retained material during the completion of preparations. The bone around the unabsorbed material was repaired and completely healed (Figure 2). The boundary of a newly deposited mature bone could be partially observed. Bone mineralization in the area was relatively even, osteons were of smaller diameters, with a small number of concentric lamellae, and cement lines were of prominent basophilic reaction.

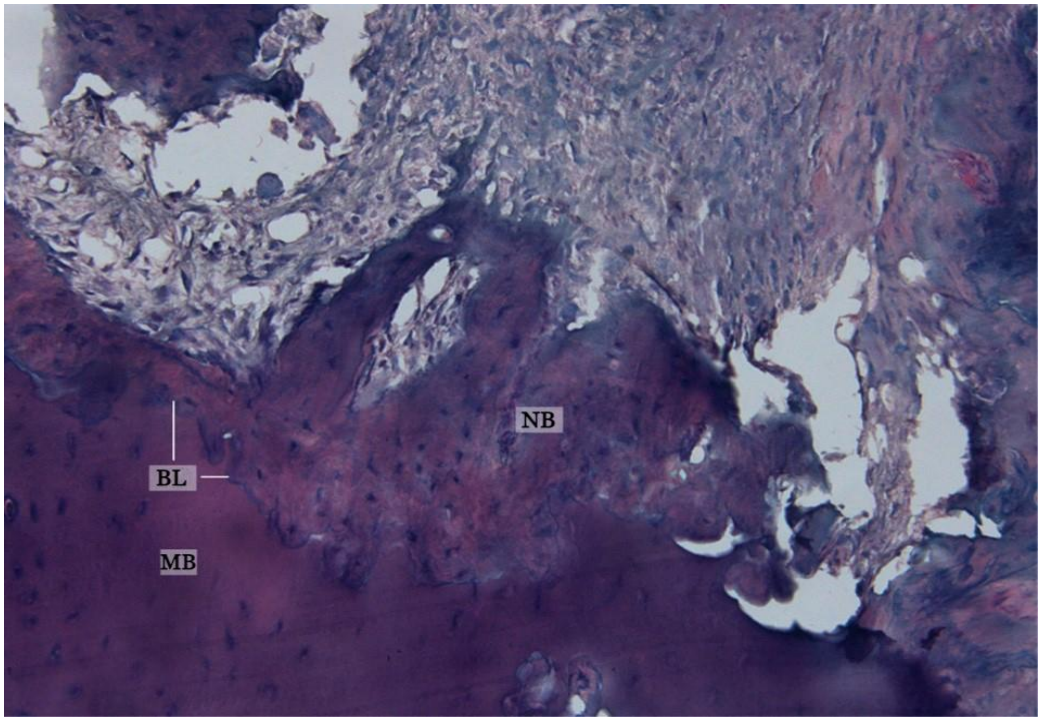

Figure 1. Indicated curved borderline (BL) newly formed hypercellular immature bones (NB) and mature bones (MB) (the degree of fibrovascular proliferation—moderate (2)) (HE, x200)

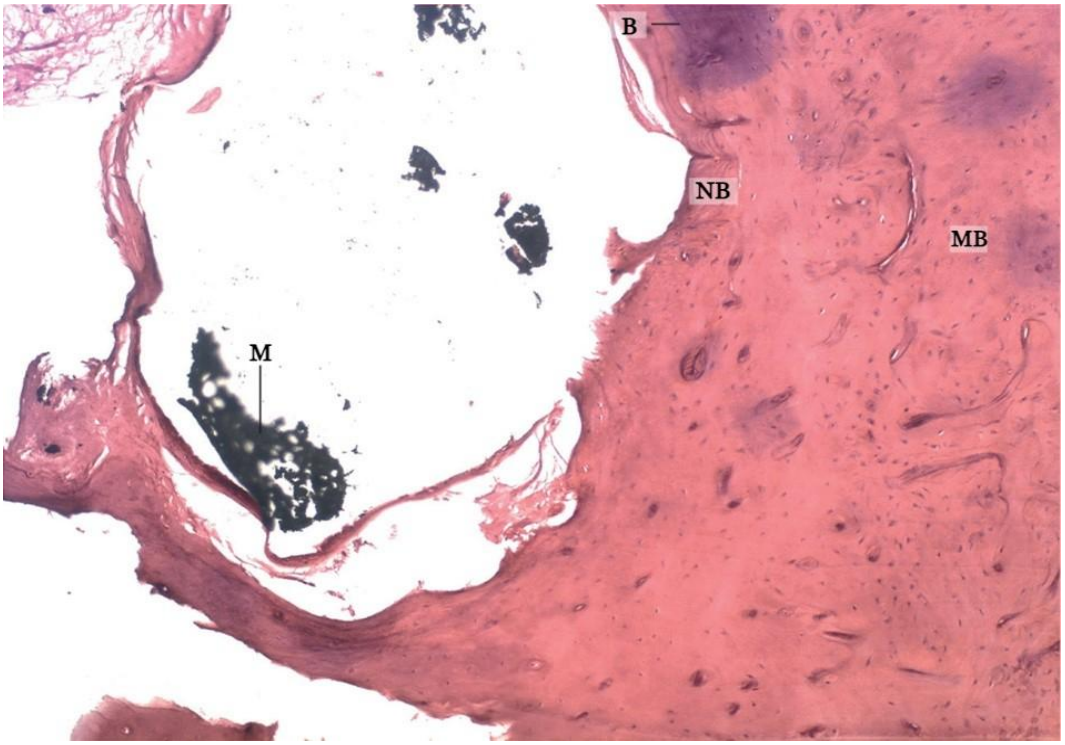

Figure 2. Lamellar bone with formed osteon, trapped scraps of unabsorbed material (M) and partially noticeable boundary (B) of newly formed bone tissue (NB) towards mature bone (MB) (fibrovascular proliferation—absent (0)) (HE, x100)

Control Group

Besides the recorded morphological characteristics of healing on experimental damage, a series of morphological changes could be observed at the maximal distance of 3 mm from the edge of the defect in the control group. These changes depended upon chronological stages of the experiment.

Thirty-Day Findings in the Control Group

On the thirtieth day after the preparation of the defect, the osteosynthetic activity of osteoblasts and the defect filled with newly formed bone tissue could be observed. Endosteal communications were highly developed based on Volkmann and Haversian canal types. Osteocytes were situated in the enlarged lacunae with the rims of intensified basophilia. Changes could be observed on the cement lines in the wider region of the experimental defect in the shape of lacunar enlargement of extracellular matrix between osteons and interstitial lamellae (Figure 3).

Ninety-Day Findings in the Control Group

Ninety days after the preparation of the defect *restitutio ad integrum* was observed, as well as the complete filling of the experimental cavity with bone tissue composed of numerous osteons of smaller diameter, with a certain number of concentric lamellae with the outer boundary characterized by the cement line of intensified basophilic reaction (Figure 4).

Statistical Analysis
(Tables 2—10)

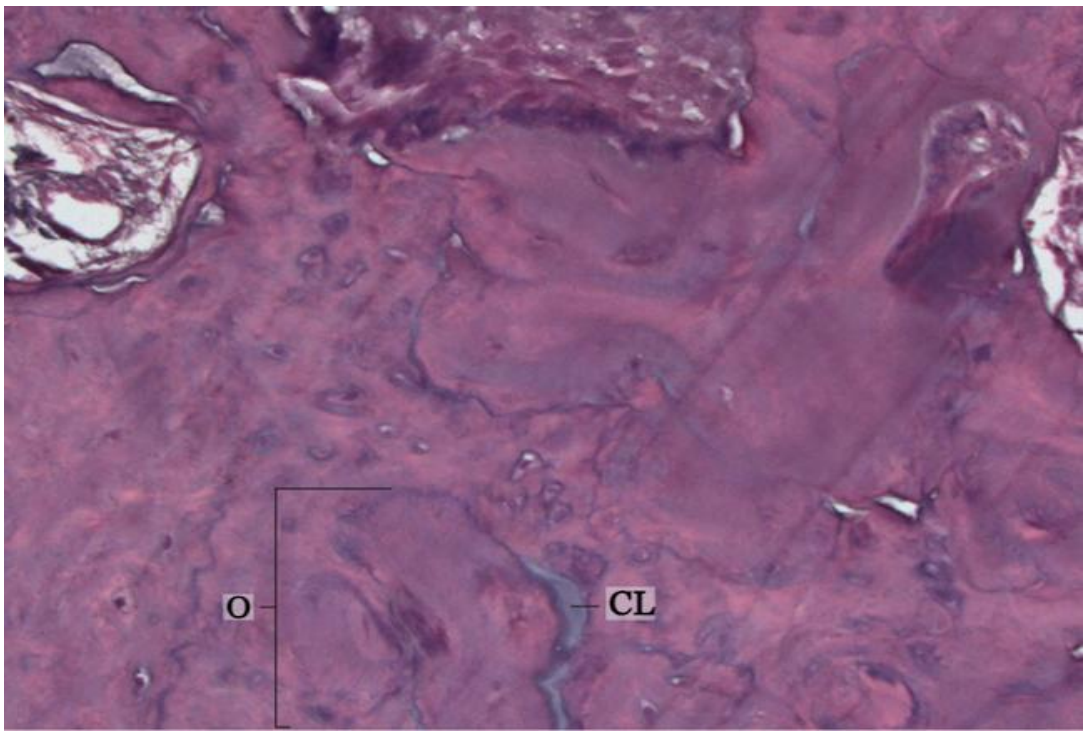

Figure 3. In the wider region relative to the edge of the defect, especially on the edges of osteons (O), cement lines are pronounced (CL), cracked to irregular polygon shape, filled with fine-grained to amorphous material (degree of distal bone reaction—moderate (2)) (HE, x200)

Table 1. Roeko Seal composition

Roeko Seal	
Component A	Component B
Dimethylpolysiloxane Paraffin oil Silicone oil	Zirconium dioxide Hexachloroplatinic acid

Table 2. Differences in INFLAMMATORY RESPONSE between the initial and final state

EXPERIMENTAL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	5	83.33	6	100.0
1	1	16.67	0	0
2	0	0	0	0
3	0	0	0	0

Friedman ANOVA (N = 6, df = 1)

Chi Sqr. = 1.00; p = .317

Table 3. Differences in INFLAMMATORY RESPONSE between the initial and final state

CONTROL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	3	75.0	4	100.0
1	1	25.0	0	0
2	0	0	0	0
3	0	0	0	0

Friedman ANOVA (N = 4, df = 1)

Chi Sqr. = 1.00; p = .317

Table 4. Differences in INFLAMMATORY RESPONSE between the groups (Kruskal—Wallis and ANOVA tests)

Measurings		**Experimental group**	**Control grop**	**H**	**p**
30 days	Σ ranks	32	23	0.09	.760
90 days	Σ ranks	33	22	0.00	1.000

H—values of Kruskal—Wallis and ANOVA tests; p—p-value of probability

Table 5. Differences in the degree of FIBROVASCULAR PROLIFERATION between the initial and final state

EXPERIMENTAL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	0	0	6	100.0
1	0	0	0	0
2	6	100.0	0	0
3	0	0	0	0

Friedman ANOVA (N = 6, df = 3)

Chi Sqr. = 6.00; p = **.014***

* significance at the level of p < 0.05

Table 6. Differences in the degree of FIBROVASCULAR PROLIFERATION between the initial and final state

CONTROL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	0	0	4	100.0
1	1	25.0	0	0
2	3	75.0	0	0
3	0	0	0	0

Friedman ANOVA (N = 4, df = 1)

Chi Sqr. = 4.00; p = **.046***

* significance at the level of p < 0.05

Table 7. Differences in FIBROVASCULAR PROLIFERATION between the groups (Kruskal—Wallis and ANOVA tests)

Measurings		**Experimental group**	**Control group**	**H**	**p**
30 days	Σ ranks	36	19	1.50	.221
90 days	Σ ranks	33	22	0.00	1.000

H—values of Kruskal—Wallis and ANOVA tests; p—p-value of probability

Table 8. Differences in the degree of REMOTE BONE REACTION between the initial and final state

EXPERIMENTAL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	0	0	6	100.0
1	0	0	0	0
2	6	100.0	0	0
3	0	0	0	0

Friedman ANOVA (N = 6, df = 3)

Chi Sqr. = 6.00; p = **.014***

* significance at the level of p < 0.05

Table 9. Differences in the degree of REMOTE BONE REACTION between the initial and final state

CONTROL GROUP

Level of reaction	30 days		90 days	
	Frequency	%	Frequency	%
0	0	0	4	100.0
1	1	25.0	0	0
2	3	75.0	0	0
3	0	0	0	0

Friedman ANOVA (N = 4, df = 1)

Chi Sqr. = 4.00; p = **.046***

* significance at the level of p < 0.05

Table 10. Differences in REMOTE BONE REACTION between the groups (Kruskal—Wallis and ANOVA tests)

Measurings		**Experimental group**	**Control group**	**H**	**p**
30 days	Σ ranks	36	19	1.50	.221
90 days	Σ ranks	33	22	0.00	1.000

H—values of Kruskal—Wallis and ANOVA tests; p—p-value of probability

Discussion

For the investigation into biocompatibility of endodontic materials both *in vitro* (on cell culture) and *in vivo* tests (subcutaneous, intramuscular and intraosseous implantation) can be used (13). Implantation techniques are considered to be superior because of the greater similarity to clinical conditions and the possibility of monitoring the healing process. Materials can be directly injected or implanted via Teflon, silicone or polyethylene tubes into tissues of rats, rabbits, guinea pigs and other experimental animals (14, 15, 16). Subcutaneous implantation is simpler and widely used (17), however, intraosseous implantation can imitate a clinical situation of close contact between an endodontic material and a bone. The implantation test is an unspecific in vivo test of tissue response to materials and as such implies pathohystological analysis after the implantation of tested materials in tissues of different animals. Complete healing of moderate size defect in rats is expected to be completed within 35 days (18), which is why similar time frame was chosen for the first stage of euthanasia.

Inflammatory response of low intensity could be observed in only one experimental animal thirty days after the implantation while it was absent from other animals. Roeko Seal cannot be considered the cause of inflammation in observation periods. The degree of fibrovascular proliferation also decreased in the course of time, which was expected during the process of healing. Thirty days after the implantation of the material, a callus and newly formed bone tissue could be observed. Young bone tissue of lamellar structure filled the space between the material and the unaffected bone tissue until the ninetieth day.

Prepared defect is an extreme stimulus which requires bone remodelling, whereby the bone can repair itself, which leads to the reaction of bone tissue 3 mm from the edge of the defect. Besides the established morphological healing characteristics, a series of morphological changes were observed in osteocytes and their lacunae, cement lines and the existing endosteal canal system, Volkmann and Haversian canals in all the experimental animal groups as well as the control group. Morphological changes in cement lines and endosteal canal system were observed in the thirty-day group, however, their disappearance and return to normal bone morphology were observed later in the ninety-day group.

Roeko Seal did not lead to the extension of the reparation period, nor did it lead to alterations of bone tissue. Discrepancies in histomorphological characteristics in the implanted tissue were slight in comparison to the control group for all the observed parameters (the degree of inflammatory cell response, the degree of fibrovascular proliferation and the reaction of distal bone) for both periods. Roeko Seal proved to be non-biodegradable until the ninetieth day, therefore the defect was not closed as in the control group, however, the bone was repaired and completely healed with the aid of Roeko Seal.

Dimethylpolysiloxane-based material, Roeko Seal can initially cause inflammation after subcutaneous implantation, which is reduced in the course of time and then completely disappears (9, 19). Subcutaneous injection of Roeko Seal into the rat tissue causes a mild to moderate inflammatory reaction within 24 hours and 7 days, but the reaction slows down and becomes chronic by the 30th day with the implant being covered by a fibrous capsule (19).

The reduction in inflammation intensity was also described by Derakhshan et al. who analyzed biocompatibility of Roeko Seal in subcutaneous implantation, in rats that were put down after 7, 14 and 60 days. Roeko Seal showed biocompatibility despite the inflammatory reaction after 7 and 14 days since fibrous capsule was formed which the authors considered to be a good sign because the inflammation was not strong enough to prevent fibroblasts from forming the capsule (9).

The tendency of the degree of inflammatory response to drop was observed in the present study, with a weak inflammatory response present in only one animal on the thirtieth day and absent in other cases.

Other authors have observed that there is a lack of inflammatory response on the fourteenth day after the implantation. Silva-Herzog et al. concluded that Roeko Seal is biocompatible when implanted subcutaneously into the tissue. Fibrous scar tissue with no inflammation was observed on the 14^{th} day (20). In the same study, the spectrophotometric analysis showed that Roeko Seal caused the smallest amount of inflammatory exudate that was significantly different from other investigated materials (AH Plus and Sealapex) and control group (20).

On the other hand, some authors observed inflammation even 30 and 90 days after the implantation with Roeko Seal. Dammaschke et al. noticed the persistence of previously caused inflammation 30 days after the molar filling in rats. They explained the results by the fact that persistent inflammation could be the consequence of the irritable nature of the used sealer (21). Low/moderate inflammatory infiltrate could be detected in Roeko Seal even 90 days after the tooth filling which was regarded as favorable by Tanomaru-Filho et al. Roeko Seal induced periapical reparation with results similar to AH Plus and Resilon/Epiphany which were also tested in this experiment. Positive results were also obtained in the case of the reparation-deposition of mineralized tissue on the apical foramen which covered at least half the surface of the apical aperture (22).

Results obtained in this research do not correspond to the described results since there were no signs of inflammation after 90 days. Roeko Seal showed the qualities of a biocompatible material and therefore the tissue around it gradually recovered and regenerated in

the course of time. Experimental procedure in which teeth of animals were filled was significantly different from bone implantation applied in this experiment which could be the reason why there was a discrepancy between the results.

Ghanaati et al. subcutaneously implanted dimethylpolysiloxane-based material Gutta Flow in rats. Sixty days after the implantation, microscopic analysis showed that the material was well integrated in subcutaneous tissue. Unlike AH Plus based on plastic resin, which was also tested in this research, Gutta Flow did not succumb to biodegradation. Gutta Flow remained encapsulated in subcutaneous tissue as a foreign body. The given data showed that this material induced an inflammatory response which led to its isolation by fibrous capsule within a living organism since the inflammatory cells of a host could not decompose. This may result in the retention of this material in periapical tissue as a foreign body in cases of overfilling. In conclusion, the authors stressed the fact that the use of biodegradable materials reduced the risk of infection and accelerated periapical healing (23). These results are concordant with the results of the present study where silicon-based material was not absorbed within 90 days, even though it did not cause chronic inflammation. The discrepancies in results could be attributed to different experimental models and tissue in which material was implanted.

Roeko Seal is most commonly defined as a nontoxic or low-level toxic sealer even when it comes to in vivo research. It showed high compatibility with L929 and HeLa cells (24). Silicon is considered to be a biocompatible material, therefore these results are expected. Oztan et al. noted the low toxic effect of AH Plus and Roeko Seal sealers on fibroblasts of rats (L929 cells) after experimental periods of 24, 48 and 72 hours (25).

Conclusion

Roeko Seal does not hinder reparatory mechanisms, nor does it impair morphofunctional relationships in bone tissue.

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Originalni rad **UDC: 612.753:[616.31:615.46**
doi: 10.5633/amm.2024.0302

HISTOLOŠKA PROCENA ODGOVORA KOŠTANOG TKIVA NA ENDODONTSKI MATERIJAL NA BAZI SILIKONA

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Uspešan endodontski tretman podrazumeva da materijal za opturaciju ostane u tkivu, zauvek ako je to moguće. Stoga, neophodno je poznavati dugoročne efekte materijala na okolno tkivo. Cilj ove studije bila je histološka procena odgovora koštanog tkiva na materijal na bazi dimetil-polisiloksana implantiran u artificijelni preparirani defekt. Uzorak je obuhvatio 20 *Wistar* pacova. Defekt je formiran u mandibulama pacova sterilnim svrdilima od nerđajućeg čelika. Siler na bazi dimetil--polisiloksana (*Roeko Seal*) implantiran je u defekte pacova iz eksperimentalne grupe, dok su defekti pacova iz kontrolne grupe ostavljeni da spontano zarastu. Jedna polovina životinja iz obeju grupa žrtvovana je nakon 30 dana, a druga nakon 90 dana. Mikroskopski preparati su analizirani na svetlosnom mikroskopu. Fibrozni kalus i mlada kost uočeni su trideset dana nakon implantacije. Devedeset dana nakon implantacije, kost oko neresorbovanog materijala u potpunosti je zacelila. *Roeko Seal* ne usporava zarastanje koštanog tkiva i omogućava potpuno zaceljenje tkiva oko materijala.

Acta Medica Medianae 2024; 63(3):14–24.

***Ključne reči**: siler, opturacija, zarastanje kosti*

Original article

UDC: 616.858-07
doi: 10.5633/amm.2024.0303

DIAGNOSTIC APPLICATION OF COGNITIVE EVENT-RELATED POTENTIALS IN PARKINSON'S DISEASE

Jelena Stamenović[1,2]

Idiopathic Parkinson's disease is a chronic, progressive, neurodegenerative disorder that affects every hundredth person in the population over the age of 60. In addition to the well-known motor signs of the disease (bradykinesia, rigidity and tremor), in the later stages, there is the development of cognitive disorders and the manifestation of the full clinical picture of dementia. An important prerequisite for adequate cognitive functioning is preserved attention. Early recognition of cognitive disorders is very important, not only for medical reasons but also for possible social problems.

The aim of the work was to determine the diagnostic significance of cognitive event-related potentials (ERPs) for the detection of attention disorders in different stages of Parkinson's disease.

Using the neurophysiological method of cognitive ERPs, 45 patients of both sexes suffering from idiopathic Parkinson's disease, aged from 55 to 76 years, were examined.

Although all registered latencies in our study were within physiological values, there is a statistically significant difference in the mean latency values of the N2 and P3 waves between control subjects and Parkinsonian patients. Regardless of the fact that Parkinsonian patients do not have clear clinical signs of dementia, their conscious recognition of the resulting change in a series of stimuli takes longer compared to healthy subjects of the control group.

Based on the results obtained so far, it can be concluded that the mentioned components of ERPs, especially the P3 wave, can be a useful diagnostic tool in the validation of cognitive disorders in non-demented Parkinsonian patients.

Acta Medica Medianae 2024;63(3):25–31.

Key words: *Parkinson's disease, cognitive event-related potentials, P3 wave, attention*

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Introduction

Idiopathic Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder that affects every hundredth person in the population over 60 years old. The prevalence of the disease in Europe and the USA varies from 60–187/100,000 in the general population, while the incidence is 10.1–24/100,000. The pathophysiological basis of the disease is the degeneration of the nigrostriatal dopaminergic neurotransmitter system, with a decrease in the level of dopamine in the striatum. In addition to dopaminergic, other neurotransmitter systems in the central nervous system are also affected—noradrenergic, serotonergic, cholinergic, and GABA-ergic systems. The basic clinical signs, based on which the diagnosis of the disease is made, are bradykinesia, rigidity and tremor, which are joined by postural disorders in the later stages of the disease. Until now, there are no other differential diagnostic procedures, e.g. laboratory or imaging methods, which can confirm the diagnosis of this disease.

In addition to the mentioned motor signs of the disease, in the later stages, there is the development of cognitive disorders and the manifestation of the full clinical picture of dementia. Early recognition of cognitive disorders is very important, not only for medical reasons but also for possible social problems. A prerequisite for cognitive functioning is preserved attention, whose disturbances lead the patient to the further development of cognitive insufficiency. Cognitive event-related potentials (ERPs) with the P3 wave are a very important instrument for examining these disorders.

Aim

The aim of the work was to determine the diagnostic significance of cognitive ERPs for the

detection of attention disorders in different stages of PD.

Material and Methods

Forty-five patients of both sexes suffering from idiopathic PD, aged from 55 to 76 years, were examined. According to the severity of clinical symptoms and signs, patients were classified into stages I–III according to the scale of Hoehn and Yahr. The control group consisted of 35 subjects of the same gender and age.

All patients were taking regularly prescribed antiparkinsonian therapy consisting of dopamine receptor agonists and levodopa, and during cognitive ERPs testing, all were in the "on" phase.

The registration of auditory cognitive ERPs was performed using the standard "oddball" paradigm, which is a task that requires the attention and concentration of the subjects. ERPs are detected by disc silver electrodes attached to the head. Registration was performed over the central, parietal, and temporal regions of the cortex. Two tones of 1000 and 2000 Hz, 80dB, were used as stimuli. The subject counted the target stimulus, i.e. higher 2000 Hz tones, and ignored the standard 1000 Hz tones. The stimulation rate was 1 stimulus in 2 seconds. The analysis time was 1000ms, the frequency range was from 0.1 to 50 Hz, and 32 target stimuli were averaged (1).

The neuropsychological examination was performed using the Mini Mental State Examination (MMSE) test. Statistical processing of the obtained latency values was performed using the standard t-test.

Results

The structure of PD patients according to age and gender is shown in Table 1 and Figure 1. The study included patients who did not have signs of dementia, which was determined using the MMSE test.

Statistically processed latency parameters of registered ERPs—waves N1, P2, N2 and P3 are shown in Table 2 and Figure 2.

Table 1. Presentation of PD patients by stage and gender

STADIUM PB	MALE		FEMALE	
	No.	%	No.	%
I STADIUM	10	22.22	8	17.78
II STADIUM	11	24.44	6	13.33
III STADIUM	7	15.56	3	6.67
TOTAL	28	62.22	17	37.78

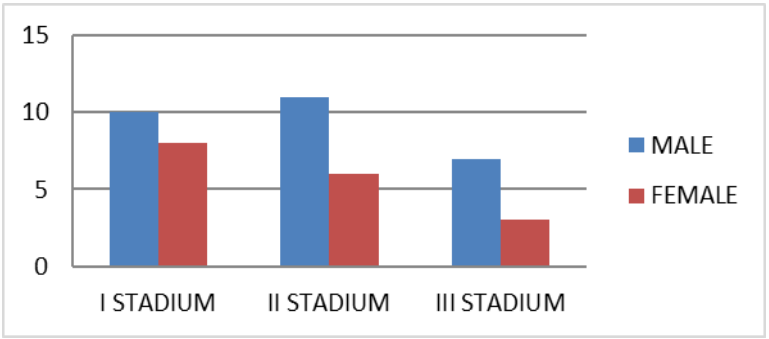

Figure 1. Presentation of PD patients by stage and gender

Table 2. Presentation of the latencies of cognitive evoked potentials in the PD patient group and the control group

LATENCY (ms)	PD patients					CONTROL GROUP					Te
	X1	SD1	CV1	MIN1	MAX1	X2	SD2	CV2	MIN2	MAX2	
N1	103	9.2	0.1	85	128	98.2	7.4	0.04	82	110	1.74
P2	180	14.1	0.1	164	195	174	7.6	0.06	158	186	1.96
N2	262	22.8	0.2	218	294	244	23.1	0.08	202	268	2.18
P3	361	27.4	0.2	312	410	342	26.5	0.04	304	362	2.42

X -middle value
SD -standard deviation
CV -coefficient of variation
MIN -minimum value
MAX -maximum value
Te -empirical value of the t-test

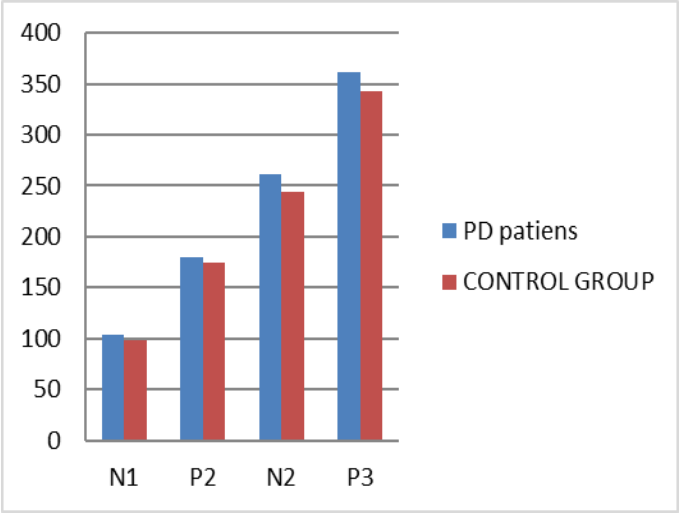

Figure 2. Presentation of the latencies (ms) of cognitive evoked potentials in the PD patient group and the control group

During the examination, the mean values of the latency of the N1 wave in Parkinsonian patients were 103.1 ms, while in healthy control subjects it was 98.2 ms. The mean value of the P2 wave was 180.3 ms in Parkinsonian patients, and 174.2 ms in healthy individuals. The mean latency of the N2 wave was 261.8 ms in PD patients and 244.2 ms in the group of healthy individuals. The mean latency of the P3 wave was 361.4 ms in Parkinsonian patients and 342.2 ms in healthy individuals of the control group.

Discussion

Selective attention refers to the capacity to focus on individual stimuli in the presence of distracting influences from other, external or internal stimuli. Attentional mobility includes the capacity to switch from one stimulus to another. Concentration corresponds to maintaining attention to a certain type of activity. Distractedness and inattention are symptoms of disorders of the mechanisms involved in the organization of attention.

In non-demented Parkinsonian patients, there may be relatively isolated or combined disorders of executive, visuospatial and memory functions. Such disorders can also be detected in patients who have preserved their professional competence and do not necessarily evolve to the clinical picture of dementia.

A risk factor for the onset of dementia can be considered low achievement on the verbal fluency test as well as depression.

Hypofunction of the dopaminergic (primarily in the mesocorticolimbic tract), cholinergic and other neurotransmitter systems is significant in the pathophysiological basis of the described neuropsychological disorders (2).

Auditory cognitive ERPs are thought to be a very good indicator of mental function, as they are highly dependent on cognitive skills, including attention and discrimination. Its application is an effective assessment tool in patients with PD due to its good correlation with other neurocognitive tests that measure key features of the disease (3).

During the recording of the auditory cognitive ERPs, two wave complexes are registered. The first complex is an exogenous long-latency evoked potential that is generated in the primary auditory cortex and is a response to standard stimuli that the subject does not pay attention to. It is represented by a negative N1 and a positive P2 wave. Answers identical to this complex are obtained during registration that does not require conscious and voluntary activity. The N1 wave is the earliest response that can be modulated by psychological processes related to selective attention.

The second complex of waves marked as negative N2 and positive P3 has a more complex structure and is composed of components corresponding to the standard response as well as primarily endogenous components. During the registration of responses to target stimuli, the dominant record is the P3 wave, whose latency correlates with the speed of cognitive processes.

Lei et al. think that rhythmic auditory stimulation may compensate for dysfunctions of the basal ganglia, involves with intrinsic evaluation of temporal intervals and action initiation or continuation. In the cognitive domain, rhythmic auditory stimulation containing periodically presented tones facilitates young healthy participants' attention allocation to anticipated time points, indicated by better performance and larger P3 amplitudes to periodic compared to random stimuli (4).

In the study conducted by Folmer et al., patients with PD showed significantly longer latencies of the P3 and N2 wave components, as well as a lower amplitude of the N2 wave. The latency and amplitude of the N2 component were significantly related to the age of the participants. N2 amplitude was correlated with results from the Rey Auditory Verbal Learning Test of cognitive ability. Latency of the P3 and amplitude of the N2 components were significantly correlated with results from the Spatial Release From Masking behavioural central auditory processing assessment. The mentioned authors believe that the N2 and P3 wave components recorded in this study represent disturbed neural processing in Parkinsonian patients (5).

Using auditory cognitive ERPs - P3 wave, Hunerli et al. examined functional brain changes in patients with PD without cognitive impairment and patients with PD and mild cognitive deficit. Lower amplitudes of P3 waves were registered in Parkinsonian patients without cognitive deficits compared to healthy subjects of the control group. Patients with mild cognitive deficits had lower P3 wave amplitudes compared to patients without cognitive deficits and subjects of the control group. The authors in this study confirm that the amplitude of the P3 wave can be a useful marker for the detection of preclinical changes before the onset of cognitive deterioration in PD (6).

As with other neurodegenerative diseases, the influence of age on the onset and progression of cognitive dysfunction has been investigated in PD. Using neuropsychological tests and auditory cognitive ERPs - P3 wave, Tang et al. examined patients with early- and late-onset PD. The results of this study show that although patients with early-onset PD had a longer disease duration, their cognitive dysfunction progressed more slowly. P3 wave latencies were significantly longer with lower P3 wave amplitudes in the group of patients with later onset of PD. The current findings showed that cognitive dysfunction progressed more slowly in the early-onset PD group. Although patients with later-onset PD showed shorter disease duration, their cognitive abilities, including executive function, visuospatial function, and attention, were impaired (7).

Toda et al. investigated auditory cognitive ERPs - P3 wave in demented and non-demented patients with PD. There were no significant differences neither in the amplitude nor in the latency of the P3 wave between non-demented Parkinsonian patients and healthy subjects of the control group. In demented patients with PD, the latency of the P3 wave was significantly prolonged compared to control subjects. These results suggest that demented Parkinsonian patients have impairments in stimulus evaluation, response selection and execution (8).

The aim of the research carried out by Tokić et al. was to show that patients with Parkinson's disease have a reduced amplitude and prolonged latency of the auditory cognitive ERPs - P3 wave. The testing procedure was conducted and the results were analysed and compared with the reference value for a healthy population. The results showed that Parkinsonian patients have a prolonged latency of the P3 wave, which confirms the presence of cognitive dysfunction in these patients (9).

Impairment of cognitive functions significantly affects the quality of life of PD patients. Although numerous studies have shown that the amplitude and latency of the N2 and P3 waves are correlated with cognitive functions, there are also many controversial findings. Therefore, Xu et al. conducted a meta-analysis of research on N2 and P3 amplitude and latency in PD patients. They concluded that N2 and P3 waves may be potential electrophysiological biomarkers of early cognitive impairment in PD. Due to the simplicity and non-invasiveness of the procedure, they can be a significant support to clinicians in

the diagnosis of early cognitive impairment in patients with PD (10).

Yilmaz et al. investigated whether additional electrophysiological tests help in making a clinical diagnosis of mild cognitive impairment in PD. They assessed changes in the P3 component in non-demented PD patients and analyzed the correlation between cognitive characteristics and changes in the P3 component. In all non-demented patients with PD and the control group, P3 latencies were within physiological limits, while in Parkinsonian patients with mild cognitive deficits, they were prolonged. The results of this study show that the P3 component represents a diagnostic tool for determining PD with a mild cognitive deficit (11).

Changes in cognitive functions are an integral part of the clinical presentation of PD. Prabhakar et al. investigated changes in P3 waves in the early stages of PD and the effect of dopaminergic therapy. By applying auditory cognitive ERPs, they determined that the latency of the P3 wave was not significantly increased in the early stages of PD. This latency was reduced by the introduction of dopaminergic therapy but later increased again. Further consideration of the implications of these data is needed (12).

The conduction time of the depolarization wave within the primary auditory cortex reflects the latencies of the N1 and P2 components, which are correlates of sensory processes in the CNS. The latency of the N2 wave is a correlate of conscious recognition of a change in a series of given stimuli, and it is a reflection of early cognitive processes. The latency of the P3 wave corresponds to the stimulus evaluation time, and it represents the speed of stimulus classification based on the discrimination of two events. Although all registered latencies in our study were within physiological values, there is a statistically significant difference in the mean latency values of the N2 and P3 waves between control subjects and Parkinsonian patients. The mean values of the latencies of the N1 and P2 waves did not differ statistically significantly between these two groups of subjects.

Conclusion

Our study also confirmed the conclusion of previous research that the N2 and P3 components of cognitive auditory ERPs represent a neurophysiological correlate of global cognitive functioning. The difference in the duration of stimulus evaluation between Parkinsonian patients and healthy subjects implies that recognition of changes in a series of uniform stimuli takes longer in non-demented Parkinsonian patients, which indicates possible incipient cognitive impairment. Regardless of the fact that Parkinsonian patients do not have clear clinical signs of dementia, their conscious recognition of the resulting change in a series of stimuli takes longer compared to healthy subjects of the control group. Based on the results so far, it can be concluded that the mentioned components of auditory cognitive ERPs, especially the P3 wave, can be useful diagnostic tools in the validation of cognitive disorders in non-demented Parkinsonian patients.

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Originalni rad **UDC: 616.858-07**
doi: 10.5633/amm.2024.0303

DIJAGNOSTIČKA PRIMENA KOGNITIVNIH EVOCIRANIH POTENCIJALA U PARKINSONOVOJ BOLESTI

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Idiopatska Parkinsonova bolest predstavlja hronični, progresivni, neurodegenerativni poremećaj od kojeg oboleva svaka stota osoba u populaciji starijoj od 60 godina. Osim dobro poznatih motornih znakova bolesti (bradikinezija, rigiditet i tremor), u kasnijim fazama dolazi do razvoja kognitivnih poremećaja i do ispoljavanja pune kliničke slike demencije. Značajan preduslov za adekvatno kognitivno funkcionisanje čini očuvana pažnja. Rano prepoznavanje kognitivnih poremećaja vrlo je značajno, ne samo iz medicinskih razloga već i zbog mogućih socijalnih problema.

Cilj rada je utvrđivanje dijagnostičkog značaja koji kognitivni evocirani potencijali imaju za otkrivanje poremećaja pažnje u različitim stadijumima Parkinsonove bolesti.

Primenom neurofiziološke metode kognitivnih evociranih potencijala ispitano je 45 muškaraca i žena sa idiopatskom Parkinsonovom bolešću, starih od 55 do 76 godina.

Iako su sve registrovane latencije u našoj studiji bile u okvirima fizioloških vrednosti, postoji statistički značajna razlika srednjih vrednosti latencija talasa N2 i P3 između kontrolnih subjekata i ljudi sa Parkisonovom bolešću. Bez obzira što osobe sa Parkinsonovom bolešću nemaju jasne kliničke znake demencije, kod njih svesno prepoznavanje nastale promene u nizu stimulusa traje duže nego kod zdravih subjekata iz kontrolne grupe.

Na osnovu dosadašnjih rezultata može se zaključiti da navedene komponente kognitivnih evociranih potencijala, naročito P3 talas, mogu biti korisno dijagnostičko sredstvo u validaciji kognitivnih poremećaja kod osoba sa Parkinsonovom bolešću koje nisu dementne.

Acta Medica Medianae 2024; 63(3):25–31.

***Ključne reči**: Parkinsonova bolest, kognitivni evocirani potencijali, talas P3, pažnja*

Original article **UDC: 615.322:582.943.15]:543.51**
doi: 10.5633/amm.2024.0304

ORIGANUM VULGARE L.: CHEMICAL PROFILE OF THE EXTRACTED VOLATILE COMPOUNDS AND ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF HYDROLAT

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Hydrolates or floral waters are the outcomes of the hydrodistillation of aromatic plants. The production of hydrolates is simple and affordable because they are byproducts of the essential oil. The composition and biological activities of hydrolates may differ from those of the corresponding essential oils. The main objective of the study was to assess the chemical profile of the volatiles extracted from the hydrolate obtained from the aerial part of *Origanum vulgare* L., but also to evaluate the anti-inflammatory and antioxidant activity of the hydrolate obtained from the aerial part of *Origanum vulgare*. Qualitative and quantitative analyses of the extracted volatiles, performed using gas chromatography/mass spectrometry (GC/MS) and gas chromatography/flame ionization detection (GC/FID), showed that the main components were terpinen-4-ol (36%) and 1-octen-3-ol (33.6%). At all concentrations tested, the hydrolate scavenged 1,1-diphenyl-2 picrylhydrazyl (DPPH) radicals in a way that depended on concentration and showed antioxidant activity in the *β*-carotene/linolenic acid assay. The total antioxidant capacity of oregano hydrolat was calculated using Ferric Reducing Antioxidant Power Assay (FRAP assay), which resulted in a FRAP value of 0.361 ± 0.015 μmol Fe^{2+}/ml. In addition to antioxidant activity, satisfactory anti-inflammatory activity was also observed with the percentage inhibition of BSA denaturation of 71.2 ± 0.006%. Demonstrated antioxidant and anti-inflammatory properties of *O. vulgare* hydrolate may be crucial to its future and use in many industrial fields.

Acta Medica Medianae 2024;63(3):32–41.

Key words: *gas chromatography/mass spectrometry, gas chromatography/flame ionization detection, oregano, hydrosol, terpinen-4-ol, 1-octen-3-ol*

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Introduction

Hydrolates or floral waters are acquired during the essential oil extraction procedure from aromatic plants. According to an international definition, a hydrolate is the distilled aromatic water that is left over after the essential oil has been separated and hydro-distilled or steam-distilled (1). They are made up of volatile oil components that are hydrophilic, polar and oxygenated, and form hydrogen bonds with water and condensed water during the distillation process (2). During the distillation of fragrant plants, it was found that some components of the essential oils were lost to the water (3).

Hydrolates are used as flavorings and refreshing drinks in traditional medicine in Mediterranean countries. Compared to essential oils, hydrolates are simpler and less expensive to make, and they seem to be less harmful to human health (4). Hydrolates produced in the early and late stages of distillation have different chemical compositions and olfactory notes. This can be attributed to the presence of terpenoids with high and low boiling points in them. In addition, the aromatic profile of hydrolates can differ significantly from that of the corresponding essential oils, as they lack hydrophobic, water-insoluble isoprenoid molecules (hydrocarbons). The production of hydrolates is simple and affordable since hydrolates are byproducts of the essential oil industry. The composition and therapeutic capabilities of hydrolates made from the same plant parts in various countries or areas within a country, during various seasons, at various development stages, or under various management approaches, may vary (5).

Origanum vulgare L., oregano, is a perennial herbaceous plant from the family Lamiaceae. It has spikes of white, purple, or pink flowers and dark oval, aromatic leaves. *O. vulgare* is often referred to as the "prince of herbs" and is a well-known aromatic and medicinal plant (6). The name means "joy of the mountains" and is derived from the Greek terms for mountain (oros) and joy (ganos). The ancient Greek goddess Aphrodite treasured oregano, which was once thought to bring good luck. Oregano was used in ancient Egypt as an antidote and as a preservative. The Greeks utilized the aerial part of *O. vulgare* both topically and orally to treat dropsy, convulsions, and skin irritations and infections. It was also a highly effective remedy against poisons (7). The medicinal parts are the aerial part of plant, harvested during the flowering season, dried, the fresh flowering herb, and the essential oil extracted from fresh or dried leaves (8). *O. vulgare* is used in folk medicine to treat a variety of conditions, including rheumatoid arthritis, dyspepsia, painful menstruation, coughing, irritation of the bronchial mucous membranes, urinary tract infections, and diaphoresis (9). The essential oil of *O. vulgare* is also widely used. It is made up of a combination of terpenoid components with antioxidant, antibacterial, antifungal, antiviral, antihyperglycemic, anti-inflammatory and antimutagenic properties (10, 11).

Among the scientific community, hydrolates have long been considered waste materials from hydro- or steam-distillation. Given the sustainability and added value of this by-product of the essential oil industry, there is growing interest in hydrolates, particularly in their composition and biological properties.

Aim

The aim of this study was: (1) determination of the chemical profile of the extracted volatiles from the hydrolate remaining after hydrodistillation of the aerial parts of *O. vulgare*, (2) determination of the antioxidant capacity of the hydrolate of the areal parts of *O. vulgare* using three different assays: DPPH assay, *β*-carotene bleaching assay and FRAP assay, and (3) determination of the anti-inflammatory activity of the hydrolate from the areal parts of *O. vulgare* by *in vitro* protein denaturation assay.

Material and Methods

Plant Material and Chemicals

The hydrolate of the aerial part of *O. vulgare* was obtained through industrial production by the company "PROMONTIS Production", Vilandrica, Gadžin Han. After the isolation of the hydrolate by industrial hydrodistillation, the isolation of volatile compounds from the hydrolate followed the procedure reported by Maciąg and Kalemba by liquid-liquid extraction with diethyl ether (12). The qualitative and quantitative composition of isolated volatiles was examined using GC/MS and GC/FID.

All chemicals used were obtained from Sigma Aldrich (USA), or Zorka Pharma (Šabac, Serbia). All solvents and chemicals were of analytical grade.

Determination of the Chemical Profile of Extracted Volatiles of Oregano Hydrolate

Qualitative and quantitative analyses of the extracted volatiles of oregano hydrolate were performed using GC/MS and GC/FID. An Agilent Technologies 7890B gas chromatograph, fitted with a non-polar silica capillary column for HP-5MS (5% diphenyl and 95% dimethyl polysiloxane, 30 m × 0.25 mm, 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, USA), was used to perform the GC/MS analysis of the extracted volatiles of oregano hydrolate. The column was coupled to an inert, selective 5977 A mass detector manufactured by the same company. The flow rate of the carrier gas, helium, was 1 cm^3/min. A split inlet set to 250 °C in 10:1 split mode was used to introduce one microliter of the prepared diethyl ether solution into the column. The mass spectra were obtained in the 25–550 m/z region in EI mode (70 eV). For the GC/FID analysis, identical analytical parameters were utilized. The corresponding fluxes for the fuel gas (H_2), oxidizing gas (Air), make-up gas (N_2), and carrier gas (He) were 1, 25, 30, and 400 cm^3/min. The flame-ionization detector (FID) had its temperature adjusted to 300 °C.

The MSD ChemStation, AMDIS_32, and MassHunter Qualitative Analysis software (Agilent Technologies, USA) were utilized for data processing. Using a homologous series of n-alkanes from C8–C20 as standards, the retention indices of the constituents from the investigated sample were experimentally calculated. The process of identifying each component involved comparing retention times, their retention indices (RIexp) with literature-available values (13), and their EI mass spectra with authentic standards and mass spectra libraries from RTLPEST 3, NIST 2011, and Willey 6.

Determination of Antioxidant Capacity

DPPH assay

The antioxidant activity of the hydrolate of the aerial part of *O. vulgare* was assessed using 1,1-diphenyl-2 picrylhydrazyl (DPPH) free radical scavenging assay. The color changed from violet to yellow when DPPH was reduced to 2,2-diphenyl-1-picrylhydrazine (DPPHH), and an ELISA microplate reader was used to measure it at 540 nm (14). The assay was performed according to Pavlović et al. by incubating different concentrations of hydrolate (20−70% v/v) with DPPH in 96% (v/v) ethanol solution for 30 minutes at room temperature and in the dark (15). The

distilled water is present in the blank sample. As a control, 96% (v/v) ethanol containing DPPH was used. Synthetic antioxidants BHT and BHA were used as the reference compounds. The following formula was used to determine the percentage of DPPH free radical inhibition: % DPPH= (A_c – A_s) /A_c x 100 where A_c is the absorbance of the control, and As is the absorbance of the sample.

β-carotene Bleaching Assay

The *β*-carotene bleaching method assesses the capacity of various components to impede the process of lipid peroxidation. Radicals generated by the oxidation of linoleic acid in the assay oxidize *β*-carotene, leading to the loss of the chromophore of the system and distinctive orange color, which is measured spectrophotometrically at 450 nm (16). By Pavlović et al., 200 mg of Tween-20 and 25 µl of linoleic acid were combined with 1 ml of *β*-carotene solution in chloroform (1 mg/5 ml), and that mixture was allowed to evaporate under vacuum at a temperature as high as 40 °C. An emulsion formed as a result of shaking the mixture after 50 ml of distilled water was added. A freshly made *β*-carotene linoleic acid emulsion was added to the sample on a 96-well microtitration plate. Three duplicates of each concentration (1.11–11.1% v/v) were made for testing. The plate was read in a microplate reader immediately (t = 0 min) and after 120 minutes of incubation at 55 °C (t = 120 min) (17). Formula (18) was utilized to determine the percentage (%) inhibition of samples against *β*-carotene bleaching: % inhibition = 100 - (A_{120} / A_0) x 100, where A_{120} is the absorbance of the sample at t = 120 min and A_0 is the absorbance of the sample at t = 0 min. Synthetic antioxidants BHT and BHA were used as the reference compounds.

FRAP Assay

The ability of the test sample to reduce iron(III) tripyridyltriazine (Fe^{3+}-TPTZ) at low pH to an intense blue colored iron(II) tripyridyltriazine complex (Fe^{2+}-TPTZ) is the basis for the FRAP method used to estimate the total reduction potential of the hydrolate of the aerial part of *O. vulgare* (19). According to Pellegrini et al., the FRAP reagent was freshly prepared and consisted of the following ingredients: 10 mmol/l TPTZ in 40 mmol/l HCl, sodium acetate buffer (300 mmol/l, pH 3.6) and $FeCl_3$ x $6H_2O$ solution (20 mmol/l), each in a ratio of 10:1:1 (v/v/v). After adding 3000 µl FRAP reagent to 100 µl hydrolate, the absorbance was measured at 593 nm and compared after 5 minutes with the blank sample which consisted of 100 µl distilled water and 3000 µl FRAP reagent (20). For the construction of the calibration curve, six concentrations of $FeSO_4$ x $7H_2O$ (100, 200, 400, 600, 800 and 1000 mmol/l) were used. The resulting FRAP value is presented as µmol ferric iron reduced per ml of sample.

Anti-Inflammatory Activity

The protein denaturation assay was performed with a 5% w/v aqueous solution of BSA (bovine serum albumin) according to Lavanya et al. (21). The test solution is an aqueous solution of hydrolate and bovine serum albumin with a weight percentage of 5% w/v. An aqueous solution of distilled water and bovine serum albumin at a concentration of 5% w/v served as the control. The 5% w/v aqueous solution of diclofenac sodium and bovine serum albumin served as the standard solution against which the findings were evaluated. Using 1N HCl, the pH of each of the aforementioned solutions was brought to 6.3. The samples underwent a 20-minute incubation period at 37 °C, after which they were heated to 57 °C for three minutes. Phosphate buffer was added to the aforementioned solutions after chilling. An ELISA microplate reader was used to measure the absorbance at 340 nm. The following formula was used to determine the inhibition percentage of protein denaturation:

Protein denaturation (%) = 100 - ((optical density of test solution—optical density of product)/optical density of test control) x 100). The control represents 100% protein denaturation. The results were compared with diclofenac (100 µg/ml).

Results

Determination of the Chemical Profile of Extracted Volatiles of Oregano Hydrolate

The percentage composition of the extracted volatiles obtained as well as the main classes of the identified constituents is shown in Figure 1 and Table 1. Sixteen compounds were identified in the extracted volatiles of oregano hydrolate. Terpenes represented the most abundant compound class (59.4%): terpinen-4-ol, 1,8-cineole, *α*-terpinene, γ-terpinene, terpinolene, *trans*-linalool oxide (furanoid), linalool, cis-linalool oxide (furanoid) and *α*-terpineol. According to the analysis, alcohols (3-methyl-1-butanol, 2-methyl-1-butanol, 3-(*cis*)-hexenol, 1-octen-3-ol, and 3-octanol) make up 40.6% of the total. Traces of aromatic components were detected.

In the extracted volatiles of oregano hydrolate isolated from aerial parts, the main components were terpinen-4-ol (36%) and 1-octen-3-ol (33.6%).

Determination of the Ability to Neutralize Free Radicals by the DPPH Test

According to DPPH test, although the tested sample possesses anti-radical activity, none of the tested concentrations of oregano hydrolate failed to reach the IC_{50}, Table 2. The range of free radical neutralizing ability was from 27.81 ± 0.002% (at lowest concentration) to 35.82 ± 0.002% (at high concentration). To compare

antiradical activity, the ability of commercial synthetic antioxidants, BHT and BHA, to remove free radicals was also studied. Under the same conditions under which the different concentrations of hydrolate were tested, IC_{50} values for BHT and BHA were: 22.82 ± 2.07 μg/ml and 2.44 ± 0.09 μg/ml, respectively.

Table 1. Chemical composition of the extracted volatiles of oregano hydrolate

No.	t_{ret}, min	Compound	RI^{exp}	RI^{lit}	Method of identification	Relatieve amount,%
1.	4.88	3-Methyl-1-butanol	732	731	RI, MS	tr
2.	4.95	2-Methyl-1-butanol	734	724	RI, MS	tr
3.	7.53	3-(*cis*)-Hexenol	849	850	RI, MS	4.5
4.	**10.45**	**1-Octen-3-ol**	**976**	**974**	**RI, MS**	**33.6**
5.	10.78	3-Octanol	990	988	RI, MS	2.5
6.	11.13	*α*-Terpinene	1006	1014	RI, MS	tr
7.	11.37	*p*-Cymene	1016	1020	RI, MS	tr
8.	11.45	1,8-Cineole	1020	1026	RI, MS	7.8
9.	11.92	Phenylacetaldehyde	1041	1036	RI, MS	tr
10.	12.11	*γ*-Terpinene	1049	1054	RI, MS	tr
11.	12.48	*cis*-Linalool oxide (furanoid)	1066	1067	RI, MS	tr
12.	12.76	Terpinolene	1079	1086	RI, MS	tr
13.	12.83	*trans*-Linalool oxide (furanoid)	1082	1084	RI, MS	tr
14.	13.16	Linalool	1096	1095	RI, MS	7.6
15.	**14.91**	**Terpinen-4-ol**	**1180**	**1174**	**RI, MS**	**36.0**
16.	15.26	*α*-Terpineol	1196	1186	RI, MS	8.0
Grouped compounds (%)				Total identified		100
Alcohols (1−5)				40.6		
Terpenes (6, 8, 10−16)				59.4		
Aromatic compounds (7, 9)				tr		

[1] t^{ret}: Retention time; RIlit: Retention indices from literature (Adams, 2007); RI^{exp}: Experimentally determined retention indices using a homologous series of n-alkanes (C8-C20) on the HP-5MS column; MS: constituent identified by mass-spectra comparison; RI: constituent identified by retention index matching; tr: trace amount (< 0.05%).

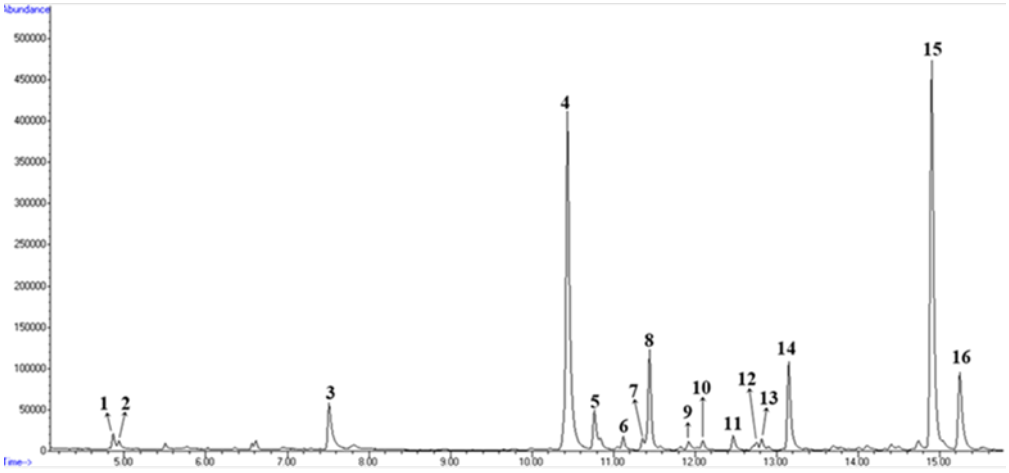

Figure 1. GC/FID chromatogram of the extracted volatiles of oregano hydrolate

Table 2. *In vitro* antioxidant and anti-inflammatory activity of *O. vulgare* hydrolate

Concentration of hydrolate in DPPH assay (% v/v)	Results of DPPH assay (%)	Concentration of hydrolate in β-carotene bleaching assay (% v/v)	Results of β-carotene bleaching assay (%)	Concentration of hydrolate in FRAP assay (% v/v)	Result of FRAP assay (µmol Fe^{2+}/ml)	Concentration of hydrolate in BSA assay (% v/v)	Result of BSA assay (%)
70	35.82 ± 0.002	11.1	46.62 ± 0.023	100	361 ± 0.015	100	71.2±0.006
60	32.2 ± 0.008	8.3	38.67 ± 0.026				
50	31.37 ± 0.015	5.56	36.72 ± 0.045				
30	30.12 ± 0.001	2.78	17.06 ± 0.03				
20	27.81 ± 0.002	1.11	6.46 ± 0.07				
BHT (IC_{50})	22.82 ± 2.07 µg/ml	BHT (IC_{50})	0.03 ± 0.00 µg/ml	/	/	Diclofenac	95.6±0.001
BHA (IC_{50})	2.44 ± 0.09 µg/ml	BHA (IC_{50})	0.04 ± 0.01 µg/ml	/	/		

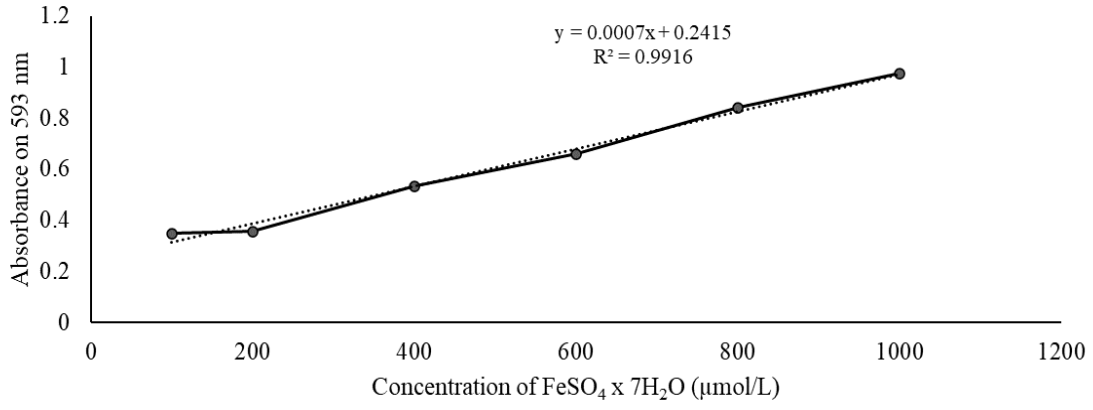

Figure 2. The standard curve obtained using ferrous sulfate solutions (100−1000 µmol/l)

Determination of the Ability to Inhibit β-carotene Bleaching

The results of the determination of *β*-carotene discoloration in the *β*-carotene/linoleic acid system as a function of hydrolate concentration expressed in percentage inhibition are presented in Table 2. In the current study hydrolate of *O. vulgare* showed mean inhibition of protein denaturation of 46.62 ± 0.023, 38.67 ± 0.026, 36.72 ± 0.045, 17.06 ± 0.03 and 6.46 ± 0.07% for doses of 11.1, 8.3, 5.56, 2.78 and 1.11% (v/v), respectively. Under the same conditions under which the different concentrations of hydrolate were tested, synthetic antioxidants showed activity in disrupting the chain reaction of lipid peroxidation: IC_{50} values were 0.03 ± 0.00 μg/ml (BHT) and 0.04 ± 0.01 μg/ml (BHA).

Determination of the Total Antioxidant Potential by the FRAP Method

The standard curve constructed by using ferrous sulfate solutions of known concentrations (Fe^{2+} of 100−1000 mmol/l) (Figure 2) was used to calculate the antioxidant potential: $y = 0.0007x + 0.2415$; $R^2 = 0.9916$.

The results determining the total antioxidant potential of oregano hydrolate showed that the FRAP value was 0.361 ± 0.015 μmol Fe^{2+}/ml (Table 2).

Anti-Inflammatory Activity

The percentage of BSA denaturation inhibition of 71.2 ± 0.006% (Table 2), which was lower than the standard value for diclofenac (95.6 ± 0.001%), indicated a considerable anti-inflammatory effect from *O. vulgare* hydrolate.

Discussion

Based on GC/MS and GC/FID, the main compounds of the volatiles extracted from the hydrolates obtained from the aerial part of *O. vulgare* are terpenes (59.4%) and alcohols (40.6%). The most abundant terpenes among the extracted volatile compounds from the hydrolate were terpinen-4-ol (36%), 1,8-cineole (7.8%) and linalool (7.6%); while the most abundant alcohols were 1-octen-3-ol (33.6%), 3-(*cis*)-hexenol (4.5%) and 3-octanol (2.5%). In addition, terpinen-4-ol and 1-octen-3-ol were the most abundant volatiles extracted from the oregano hydrolate. The primary bioactive ingredient in a range of aromatic plants is terpinen-4-ol, a naturally occurring monoterpene (22). Khan et al. found similar results after analyzing the volatile components of hydrolate *O. vulgare* from Saudi Arabia and determining that the primary chemicals were terpinen-4-ol and carvacrol (23). According to the results of the current study, 1-octen-3-ol was also one of the primary components of the volatiles extracted from the hydrolates of *O. vulgare*, accounting for 33.6%. Known as mushroom alcohol, 1-octen-3-ol was isolated from a variety of plants and fungi (24).

As far as we know, there has not been much information on antioxidant properties of *O. vulgare* hydrolate published in the literature. Three complimentary test systems—DPPH free radical scavenging, lipid peroxidation inhibition, and total antioxidant capacity (FRAP) were used to measure antioxidant activity. In the concentration range we tested, *O. vulgare* hydrolate was able to scavenge DPPH radicals and showed concentration-dependent antioxidant activity in the *β*-carotene/linolenic acid assay. Additionally, a reducing effect on iron(III) ions was observed. It is assumed that terpinen-4-ol is responsible for the observed antioxidant effect, which, at 36%, is the most abundant among the extracted volatile compounds of oregano hydrolates isolated from aerial parts. In the study conducted by Aslam et al., terpinen-4–ol exhibited a DPPH radical scavenging potential of 48.7 ± 0.87% in comparison to BHA which was 44.2 ± 0.08%, and also demonstrated a reducing power at the highest dose of 60 mg/kg in the FRAP assay 72.68% (25).

Protein denaturation occurs via an unexpected mechanism involving changes in hydrophobic, disulfide, and electrostatic hydrogen bonds (26). Protein denaturation results in the creation of autoantigens in inflammatory illnesses such as cancer, diabetes, and rheumatoid arthritis. (27). Therefore, it is possible to reduce inflammatory activity by inhibiting protein denaturation. A nonsteroidal anti-inflammatory drug (NSAID), diclofenac, was used as the reference drug in this study. By inhibiting the activity of the enzyme cyclooxygenase, NSAIDs have an anti-inflammatory effect. On the other hand, ulceration, bleeding, perforation, and constipation are negative effects of these drugs (28). In comparison to the denaturation process with bovine serum albumin, the hydrolate of *O. vulgare* showed an anti-inflammatory effect (Table 2). The functional groups which might influence the anti-inflammatory activity observed herein, are terpene and alcohol compounds (29). The anti-inflammatory activity of terpenes is determined by the presence of methylene groups and phenolic O-H. Studies have shown that these substances block the signaling pathways for mitogen-activated protein kinase and nuclear transcription factor-*β* (29). Of the volatiles extracted from oregano hydrolate, terpinen-4-ol is the main constituent whose analysis and anti-inflammatory activities have been demonstrated in previous studies (30, 31). Increased intracellular inflammatory factors are the result of activation of nuclear factor kappa *β* (NF-κ*β*) in response to LPS lipopolysaccharide-triggered cells. By significantly preventing NF-κ*β* activation, terpinen-4-ol can reduce the inflammatory response (30). Hydrolate of the aerial part of *O. vulgare* has attracted considerable interest due to its biological activity, including antioxidant and anti-inflammatory

effects (32). In the analysis of the volatile compounds, we were only able to detect a part of the compounds present in the hydrolate; a part remained in the aqueous phase after extraction with ether and could be responsible for the activities we investigated, which is why further investigations are required.

Conclusion

Terpinen-4-ol (36%) and 1-octen-3-ol (33.6%) were found to be the main volatiles extracted from the hydrolate obtained after distillation of the essential oil isolated from the aerial part of the plant *O. vulgare*. GC/MS and GC/FID analyses were performed to determine the chemical composition of the volatile compounds extracted from the hydrolate. The hydrolate sample affected the neutralization of DPPH radicals and *β*-caroten bleaching to some extent at all concentrations tested. The total reducing power in the FRAP assay was 0.361 ± 0.015 μmol Fe^{2+}/ml hydrolate. Additionally, the anti-inflammatory activity was shown in the BSA denaturation inhibition test. Based on the promising results we have presented, hydrolate obtained from the aerial part of *O. vulgare* might have potential application as a natural additive as the result of antioxidant and anti-inflammatory activity.

Acknowlegments

This research was supported by the Ministry of Education and Science of the Republic of Serbia (Grant No. 451-03-65/2024-03/200113 and 451-03-66/2024-03/200113) and the Faculty of Medicine University of Niš Internal Scientific Project No. 15.

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Originalni rad **UDC: 615.322:582.943.15]:543.51**
doi: 10.5633/amm.2024.0304

ORIGANUM VULGARE L.: HEMIJSKI PROFIL EKSTRAHOVANIH ISPARLJIVIH KOMPONENATA I ANTIOKSIDATIVNA I ANTIINFLAMATORNA AKTIVNOST HIDROLATA

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Hidrolati ili cvetne vode proizvodi su hidrodestilacije aromatičnih biljaka. Predstavljaju nusproizvode etarskog ulja, pa je njihova proizvodnja jednostavna i pristupačna. Hemijski sastav i biološka aktivnost hidrolata i odgovarajućih etarskih ulja mogu se razlikovati. Osnovni cilj ove studije bio je da se ispita hemijski profil ekstrahovanih isparljivih komponenata hidrolata dobijenih iz nadzemnog dela biljne vrste *Origanum vulgare* L., ali i da se ispita antiinflamatorna i antioksidativna aktivnost hidrolata. Kvalitativna i kvantitativna analiza ekstahovanih isparljivih komponenata hidrolata izvršena pomoću gasne hematografije i masene spektomerije (engl. *gas chromatography/mass spectrometry* – GC/MS), kao i pomoću gasne hematografije / detekcije plamene jonizacije (engl. *gas chromatography-flame ionization detection* – GC/FID) pokazala je da su glavne komponente terpinen-4-ol (36%) i 1-okten-3-ol (33,6%). Sve ispitivane koncentracije hidrolata pokazale su sposobnost uklanjanja slobodnih DPPH radikala na način zavisan od koncentracije, kao i aktivnost u *β*-karoten / linolna kiselina testu. Ukupni antioksidativni kapacitet origana procenjen je korišćenjem testa kojim se ispituje antioksidativna moć redukcijom gvožđa (engl. *ferric reducing ability of plasma* – FRAP test), čija je vrednost iznosila 0,361 µmol Fe^{2+}/ml ± 0,015 µmol Fe^{2+}/ml. Pored antioksidativne aktivnosti, zabeležena je i zadovoljavajuća antiinflamatorna aktivnost sa procentom inhibicije denaturacije BSA od 71,2% ± 0,006%. Pokazana antioksidativna i antiinflamatorna aktivnost hidrolata *O. vulgare* mogu biti važne za njegovu buduću upotrebu u mnogim industrijskim oblastima.

Acta Medica Medianae 2024; 63(3):32–41.

***Ključne reči**: gasna hematografija / masena spektomerija, gasna hematografija / detekcija plamene jonizacije, divlji origano, hidrosol, terpinen-4-ol, 1-okten-3-ol*

Original article

UDC: 615.2/.3.099-055.2]:616.98:578.834
doi: 10.5633/amm.2024.0305

THERAPEUTIC DRUG INTOXICATION PATTERN IN THE FEMALE POPULATION DURING THE COVID-19 PANDEMIC IN THE SOUTHEAST SERBIA

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Drugs are the second cause of human intoxication-related mortality and the first in intoxication records. About 1 in 4 individuals around the world will develop mental illness at some point in their lifetimes. Viral outbreaks, such as the coronavirus disease (COVID-19) pandemic, are associated with short-term and long-term psychological and societal distress. The current review of poisoning patterns in southeast Serbia is imperative. A retrospective cross-sectional study was carried out for two years, from March 2020 to March 2022, to evaluate the prevalence and trends of pharmaceutical drug poisonings. Retrospective data on poisoning cases was collected from the medical records section of the Toxicological Laboratory of the Institute of Forensic Medicine in Niš. Of the 310 cases, 59.35% of intoxications were observed in the female population. The most significant female predominance was observed in the age category 12–19 years (75%). In all age groups over 51, there are more female intoxications than male intoxications by a factor of more than 2.17. In age category 12–19, the most commonly detected drug class was sedatives, followed by analgesics and antiepileptics. In the population over 51, the most frequently detected drug classes in intoxication were sedatives (32.67%), followed by drugs for cardiovascular diseases (15.84%), antiepileptics (13.86%), and antidepressants (11.88%). Women over 51 and in the adolescent age category should be the target group for education and raising awareness about mental health. The prescription of sedatives should always be carefully considered, as this class of drugs is the most common in poisoning.

Acta Medica Medianae 2024;63(3):42–47.

Key words: *drugs, intoxication, COVID-19, female*

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Introduction

Drugs are the second cause of human intoxication-related mortality and the first in intoxication records. Additionally, the years of life that could be lost due to drug intoxication point to significant social and financial costs. National public health data raise awareness the rate of drug poisoning mortality is increasing in many countries (Japan, Australia, England etc.) (1–3).

Drug intoxication can be classified according to intention into intentional (self-harm, suicidal) and unintentional (accidental) intoxications. Intentional intoxication can be a consequence of poor mental health. Drugs are mostly available substances, which is why they are high on the list of substances for self-harm. On the other hand, improper drug storage, a lack of awareness of drug toxicity, and drug interactions are common reasons for unintentional poisoning (4).

About 1 in 4 individuals around the world will develop mental illness at some point in their lifetimes. Women are impacted at a higher rate than men, with 1 in 5 women experiencing a prevalent mental illness (e.g., anxiety, depression) versus only 1 in 8 men (5, 6).

More than 1 in 5 women in the United States experienced a mental health condition in the past year, such as depression or anxiety. Many mental health conditions, such as depression and bipolar disorder, affect more women than men or affect women in different ways than men (7).

Three-quarters (75%) of mental health issues are established before the age of 24, and

young women have emerged as the highest-risk group for mental ill health (8).

Viral outbreaks, such as the COVID-19 pandemic, are associated with short-term and long-term psychological and societal distress (9).

The COVID-19 pandemic has resulted in an unprecedented societal burden, from a burden on health systems, inevitable deterioration of the economy, higher rates of unemployment, global social restrictions, lockdowns, and disturbing news stories that impose considerable stress. These factors could trigger a new onset of mental health disorders or worsen previously developed mental health disorders (10).

Among many studies conducted worldwide, Kiang et al. observed an increase of 44% in the period between January 2020 and December 2020 over the same period of the previous year in California (11).

Recent research estimates that 1 in 8 children and young people experience mental health problems in England, and this record went up to 1 in 6 during the COVID-19 pandemic (12).

Epidemiological data on this important health issue are, however, scarce in Serbia. Therefore, the need for a current review of poisoning patterns in southeast Serbia is imperative. This study sought to characterize poisoning in the female population and common pharmaceutical drugs as toxic agents.

Material and Methods

The study was conducted in Niš, city in southeast Serbia which has a total population of about 2 million inhabitants. To assess the prevalence and patterns of poisonings with pharmaceutical drugs, a retrospective cross-sectional study was conducted for two years, from March 2020 to March 2022. Retrospective data on poisoning cases was collected from the medical records section of the Toxicological Laboratory of the Institute of Forensic Medicine in Niš. All cases of poisoning available in the medical records departments were included in the study. The cases were reviewed for gender, age, and identified substances. Results were statistically evaluated using Microsoft Excel.

Results

Number of Poisoning Cases

Among 510 samples sent to the toxicological laboratory of the Institute of Forensic Medicine from the University Clinical Centre of Niš, intoxications with different agents were proven in 435 cases (85.30%). Prescribed and over-the-counter (OTC) drug poisonings were reported in 323 cases (74.25%).

Age Categorization

Of 323 cases, 13 had no information on age or gender. Of the 310 cases, 59.35% of intoxications were observed in the female population, with a female-over-male ratio of 1:1.5. All cases were categorized into age and gender categories, which are presented in Figure 1.

An increased incidence of female cases was reported in all age categories, except in categories 28–35 and 36–43 years, but the most significant female predominance was observed in the age category 12–19 years (75%). In all age categories over 51 years, the ratio of intoxications in the female category and the male category is more than 2.17.

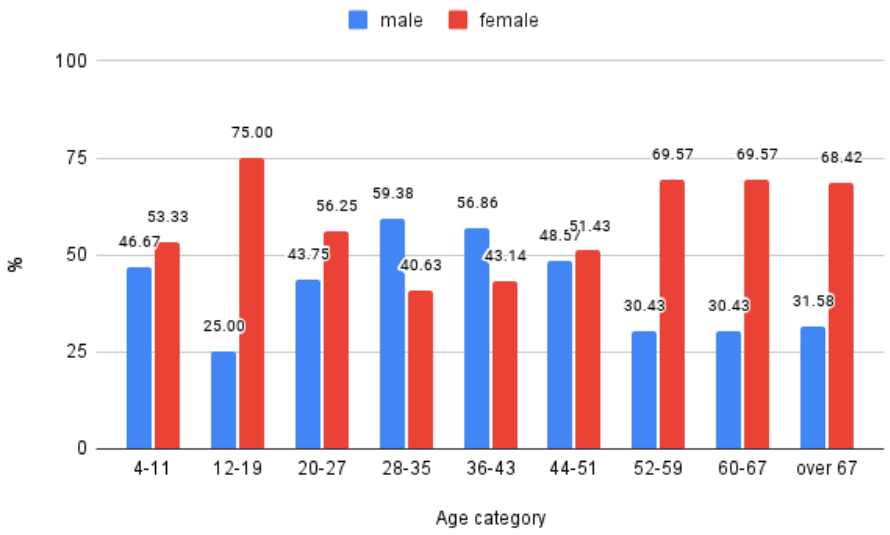

Figure 1. Distribution of intoxications among age and gender categories

Adolescent category

In age category 12–19, the most commonly detected drug class was sedatives, followed by analgesics and antiepileptics. It is presented in Figure 2. Among antidepressants, sertraline (77.78%) was proven in the highest number of cases, while in the class of sedatives, diazepam and bromazepam predominance was observed, as expected.

Multi-drug poisoning was reported in 56.67% of cases. Alcohol was detected in 4 patients, in 2 of whom it was combined with sedatives.

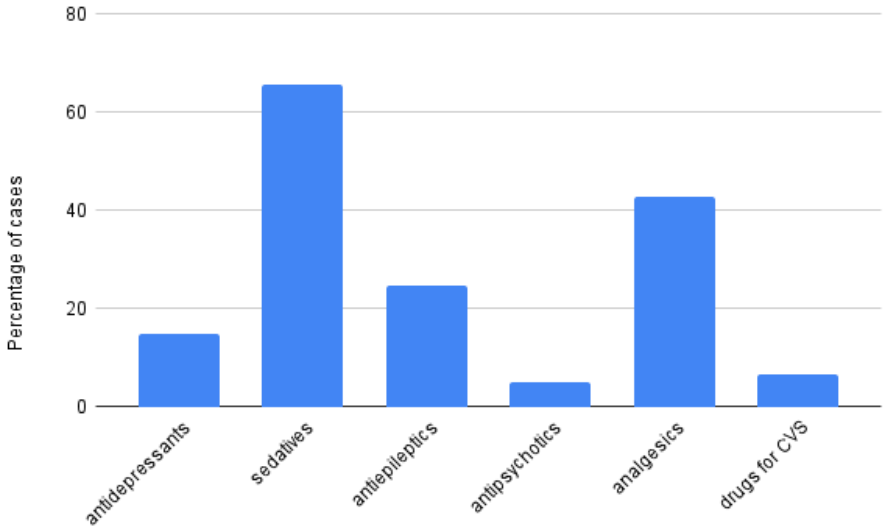

Figure 2. Distribution of drug classes in intoxications among female adolescents

Females over 51 years

Twenty point ninety-six percent of drug intoxications were observed in females older than 51 (Figure 3). The largest incidence (42.22%) was seen in age groups between 51 and 60, followed by those between 61 and 70 (33.33%). Multidrug intoxications were reported in 80% of cases, with polypsychotic drug intoxications in 37.78% of these cases. Regarding therapeutic classes, the most frequently detected drug classes in intoxication were sedatives (32.67%), followed by drugs for cardiovascular diseases (15.84%), antiepileptics (13.86%), and antidepressants (11.88%). Bromazepam and diazepam were

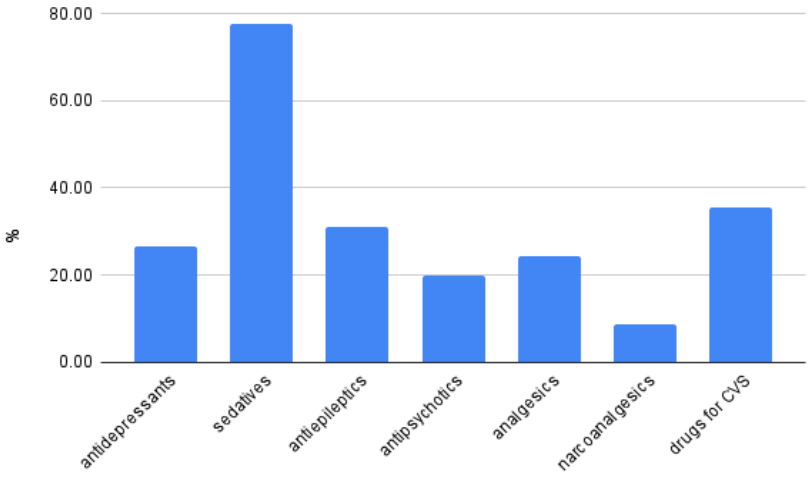

Figure 3. Distribution of drug classes in intoxications among women over 51 years

detected in 13.86% and 10.89% of all cases, respectively. Traditionally, paracetamol and ibuprofen were the most frequently detected from the group of analgesics. In all cases of ethanol intoxication, benzodiazepines were also identified.

Discussion

Many studies on this important topic are conducted worldwide. Increased incidences in the female population in age categories 12–19 and older than 51 years show an urgent situation regarding female mental health to health professionals. Drug overdoses can affect people of any age. Most childhood poisonings are accidental while poisoning in adolescents is mainly intentional (self-harm) (13).

The highest prevalence in the category 12–19 years can possibly be explained by the COVID-19 pandemic, when pupils and students were not allowed to go to school and they used to finish all their obligations online. The negative effect of COVID-19 on social life and, consequently, on the mental health of the teenage population is obvious, according to the results of this study. In a study conducted in Belgium before COVID-19, the highest prevalence of poisonings was observed in the age group 21–40 years (43.0%). These results are different from the ones that we have obtained, which can be explained by the previously mentioned COVID-19 lockdown (14).

In a study regarding acute intentional poisonings in children in Romania during the COVID-19 pandemic, a higher frequency of acute intentional poisonings was noticed in girls (65.49%) as compared to boys (34.50%), as well as in our research (15).

A high frequency of sedatives detected in samples indicates a high prevalence of usage of sedatives in Serbia. The reason for this has to be carefully considered, as this class of drugs is very often abused. Prescription and availability of sedatives should be evaluated to avoid misuse and abuse. It is essential to raise awareness about the rational prescription of sedatives in our country. The second most commonly detected group is antiepileptics. Similar results were published by the National Center for Poisoning Control at the Military Medical Academy in Belgrade: 61.7% of sedative intoxications and 19.5% of antiepileptic intoxications, compared to 30.63% of sedative intoxications and 19.97% of antiepileptic intoxications in our study. The percentage of sedative intoxications in our study is much lower than the percentage of intoxications in the evaluation of the National Poisoning Center.

Analgesics are high on the list of drugs most commonly abused, as expected, with paracetamol and ibuprofen predominating in both observed categories. It is expected, as this is an OTC drug class that is available to obtain without a prescription. Also, these drugs are present in almost all homes, so it was expected that they would be abused by adolescents. Adults have to keep drugs away from children and adolescents.

A low number of intoxications with drugs for cardiovascular diseases can be explained by the high compliance and adherence of these patients. These patients are possibly better educated about proper drug use.

In the adolescent population, fewer drug classes are noted than in the overall population. The most commonly detected drug class was also sedatives. In the study conducted by Jonassen et al. in Norway, OTC analgesics in adolescents were evaluated. They concluded that depression and anxiety are the strongest psychological predictors of weekly OTC analgesic use. Higher symptom levels and being female increase the strength of this association. Depression and anxiety also predict weekly OTC analgesic use after controlling for physiological pain (16).

In England, studies have shown in recent years, that medically attended poisonings among preschool children have reduced, but those among adolescents appear to have increased (17, 18).

Althobaiti et al. have monitored intoxication in children during the COVID-19 pandemic. They have mostly documented carbamazepine, methanol, risperidone, propranolol, and olanzapine intoxications. Benzodiazepines were the most commonly used drug (18%), which is in accordance with our results (19).

Polysubstance intoxications are very difficult for clinicians, as symptoms can be altered due to substance-drug interactions (20). An especially dangerous combination is alcohol and psychopharmaceuticals with sedative effects, which can lead to depression of the CNS and consequently be lethal (21). Also, polysubstance poisoning is demanding and time-consuming for toxicology analysts (22).

Conclusion

Our results draw attention to women's mental health. Women over 51 and in the adolescent age category should be the target group for education and raising awareness about mental health. The prescription of sedatives should always be carefully considered, as this class of drugs is the most common in poisoning. The general population must be informed about drug interactions with other substances, such as other drugs, ethanol, and illicit drugs. An important concern should be raised about the availability of prescription medications within the reach of children. This research can be used as a basis for future research and comparison to evaluate changes in patterns of intoxication in southeast Serbia.

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Originalni rad **UDC: 615.2/.3.099-055.2]:616.98:578.834**
doi: 10.5633/amm.2024.0305

TROVANJA ŽENA LEKOVIMA U TOKU PANDEMIJE COVID-19 U JUGOISTOČNOJ SRBIJI

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Lekovi su drugi na listi uzročnika smrtnosti povezane sa trovanjima i prvi na listi uzročnika trovanja. U proseku, jedna od četiri osobe na svetu razvije neki oblik mentalne bolesti u toku života. Pandemije, poput pandemije virusa korona 2019 (COVID-19), imaju kratkoročne, ali i dugoročne efekte na mentalno zdravlje i važan uticaj na društveni život. Trenutno nema podataka o trovanjima lekovima u toku pandemije COVID-19 na jugoistoku Srbije, pa je analiza ovog fenomena bila neophodna. U te svrhe sprovedena je retrospektivna studija preseka za period od marta 2020. do marta 2022. godine. Korišćeni su podaci toksikološke laboratorije Zavoda za sudsku medicinu u Nišu. Od 310 slučajeva intoksikacije bilo je 59,35% zabeleženih u ženskoj populaciji. Najveći broj slučajeva u ženskoj populaciji primećen je u kategoriji žena starih od 12 do 19 godina (75%). Među onima koji su imali više od 51 godine broj trovanja bio je 2,17 puta veći kod žena. Prilikom posmatranja kategorije osoba starih od 12 do 19 godina primećen je najveći broj trovanja sedativima, analgeticima i antiepilepticima. Najčešći uzročnici trovanja u populaciji koja ima više od 51 godine bili su sedativi, lekovi za lečenje kardiovaskularnih bolesti, antiepileptici i antidepresivi. S obzirom na ove rezultate, može se reći da žene u periodu adolescencije i žene starije od 51 godine treba da budu ciljna grupa prilikom edukacija i podizanja svesti o mentalnom zdravlju.

Propisivanje sedativa uvek treba pažljivo razmotriti budući da se ova grupa lekova najčešće koristi za trovanje.

Acta Medica Medianae 2024; 63(3):42–47.

***Ključne reči**: lekovi, trovanje, COVID-19, žene*

Original article

UDC: 616.379-008.64:613
doi: 10.5633/amm.2024.0306

SELF-CARE ACTIVITIES AS PREDICTORS OF GOOD GLYCAEMIC CONTROL IN DIABETES: DIFFERENCES BETWEEN TYPE 1 AND TYPE 2 DIABETES

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Diabetes mellitus is a chronic disease that is mostly controlled by the affected individual nowadays. Activities in self-control of diabetes include self-monitoring of blood glucose, eating a healthy diet, being physically active, taking the recommended medication, and consulting health care professionals. Different studies have shown that educational and psychosocial interventions can have a significant effect on improving diabetes self-control and reducing complications. The aim of this study was to examine the differences in self-care activities between patients with Type 1 and Type 2 diabetes as well as the role of these activities in predicting good glycaemic control. Our results suggest that in Serbia people with Type 1 diabetes have a much harder task in achieving good glycaemic control than people with Type 2 diabetes, even when there is no difference between these groups in practicing the majority of self-care activities. In future, education for people with Type 1 diabetes should emphasise monitoring blood glucose. For both types of diabetes, a healthy diet should be addressed.

Acta Medica Medianae 2024;63(3):48–54.

Key words: *diabetes, self-care in diabetes, glycaemic control, diabetes education*

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Introduction

Diabetes mellitus is a chronic disease that is mostly controlled by the affected individual nowadays (1). This self-control involves health behaviours in accordance with medical recommendations that affect various aspects of everyday life, such as self-monitoring of blood glucose, eating a healthy diet, being physically active, taking the recommended medication, and consulting health care professionals (2). This shift of responsibility from healthcare professionals to individuals with diabetes places a high burden on patients (3). Most people with diabetes perceive their treatment regimen as challenging (4). Failure to successfully manage diabetes, leads to poor health outcomes, regardless of the progress in diabetes treatment technology (5). Unfortunately, inadequate management of diabetes is much more common than expected with up to 74% of people with diabetes having bad blood glucose control (6). This can lead to different complications associated with diabetes such as cardiovascular diseases, neuropathy, limb amputation, and retinopathy (7), as well as higher risks for developing depression and anxiety (8), resulting from distress associated with disease management known as diabetes distress (9). This puts an additional burden on people with diabetes as they fear the risk of developing various complications as a consequence of inadequate diabetes regulation. To avoid the complications associated with diabetes, active, problem-oriented behaviour is necessary—people with diabetes must take full responsibility for the daily control of the disease, in various situations, over a long period of time (5).

Certain studies show that more than half of people with Type 2 diabetes lack knowledge about their disease and its regulation (10). Research (11) has shown that educational and psychosocial interventions have a significant effect on improving diabetes self-control and reducing complications. These benefits associated with self-management education and lifestyle change for people with diabetes have demonstrated the cost-effectiveness of interventions at an economic level as they exceed the costs associated with the intervention (12).

Because it reflects average glycaemia over a long period and has been shown to be a good predictor of later complications of diabetes (13), the HbA1c parameter has often been used in research as a measure of successful diabetes control (14, 15). We can simply define it as an average glucose level in the last 3 months. According to the recommendations of the American Diabetes Association, the target values of HbA1c for people with diabetes should be below 7% (7). Research has shown that up to 84% of people with Type 1 diabetes fail to maintain an HbA1c level below 7% (6).

Research (16) has shown that blood sugar control in people with Type 1 diabetes is much more sensitive to variations in self-care behaviours and monitoring than in people with Type 2 diabetes, where the emphasis is placed on lifestyle changes (diet and physical activity) in order to improve sugar control. In people with Type 2 diabetes, in most cases the body produces a certain amount of insulin on its own, so therapy for this type of diabetes may or may not include insulin, in which case the burden of self-management is much smaller. Regardless of the type of therapy, most people with diabetes perceive the therapy regimen as challenging (4).

In 2021, with a prevalence rate of 9.1%, Serbia ranked fifth in Europe in the number of people with diabetes (17). It is predicted that by 2045 the rate will increase to 10.9%, indicating a very worrying trend. This global epidemic of diabetes calls for attention to developing better education and support systems for successful control of the disease. In order to successfully define goals and develop effective educational and support programs for people with both Type 1 and Type 2 diabetes, differences in the effects of self-care activities on health outcomes between these two types of diabetes need to be addressed.

Aim

The aim of this study was to examine the differences in self-care activities between patients with Type 1 and Type 2 diabetes as well as the role of these activities in predicting good glycemic control.

Material and Methods

This research was conducted online, during June and July of 2023, by distributing online forms of questionnaires on groups and pages on social media dedicated to people with diabetes in Serbia. At the beginning of the questionnaire, the aim and purpose of the research were explained, and the respondents gave their consent for participating and data processing before accessing the questionnaire itself.

The research sample included 285 participants, out of which 52 (18.2%) were men and 233 (81.8%) were women. The participants' age ranged from 17 to 73 years with an average age of 43.35 years. The majority of the respondents, i.e., 235 (82.5%), reported that they lived in the city, while 50 (17.5%) lived in a village. The highest percentage of respondents had completed college (39.6%) or high school (38.6%), and 66.7% of them were employed. The majority of the respondents (58.9%) described their financial situation as satisfactory. In terms of marital status, 55.1% were married, 22.5% were single, 11.2% were living with a partner but unmarried, 7.7% were divorced, and 3.5% were widowed. Out of the total respondents, 163 (57.2%) suffered from Type 1 diabetes, 114 (40%) suffered from Type 2 diabetes and 8 (2.8%) suffered from other types of diabetes.

Summary of Diabetes Self-Care Activities (SDSCA) (Toobert et al., 2000) scale was used as a measure of self-care behaviour in diabetes. For this study, the scale was translated into Serbian language using the double-blind method. The scale consists of 11 questions that measure the frequency of diabetes self-care activities in the past 7 days in the 6 following domains: general diet, specific diet, foot care, blood sugar measurement, physical activity, and smoking. A higher score on each of the dimensions indicates better self-care behaviours, except for the scale of smoking where a higher score indicates a higher number of cigarettes consumed in a day for smokers. In this research, the dimension of a specific diet was divided into fruit and vegetable consumption dimensions where a higher score indicates better self-care behaviour, and high-fat food intake where a higher score indicates worse self-care behaviour.

Respondents were asked to report the last *level of HbA1c* they measured, and this was used as a measurement of good glycaemic control and an indicator of health outcomes in diabetes.

Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS) version 20. T-test was used to identify intergroup differences and linear regression for creating prediction models. The correlations were tested using Pearson's correlation coefficient.

Results

Descriptive statistics and reliability for dimensions of the SDSCA scale are shown in Table 1. Based on the values of Skewness and Kurtosis we can conclude the normal distribution of the variables (Curran et al., 1996). All dimensions of SDSCA showed good reliability, measured by Chronbach alpha, except for dimension-specific diet. Based on recommendations by the original authors of the SDSCA scale, this dimension was divided into two singular-item questions, and each of them was treated as a separate dimension of the scale.

The average HbA1c level in the sample was 7.11%, which is slightly above the average recommended level for optimal control of diabetes of 7% (ADA, 2013). The years of life with diabetes

in the sample ranged from 1 to 48 years, with an average of 12.35 years. After checking for differences in age and correlations with gender across all analyzed dimensions, no significant results were found prior to further analysis.

Mean values of the SDSCA dimension varied between 2.75 and 4.99. A generally healthy diet was followed 4.35 days in a week on average, fruits and vegetables were consumed 3.78 days a week, high-fat food consumption occurred 3.51 days a week, and physical activity and exercise were performed 4.35 days per week. With blood glucose testing done 4.99 days a week on average and foot care-related activities carried out 2.54 days a week on average, we can conclude that activities related to blood glucose monitoring were followed for most of the days on average and foot care for least of the days on average out of all self-care related activities in diabetes.

Table 1. Descriptive statistics

	N	Min	Max	M	SD	Skewness	Kurtosis	α
HbA1c	260	3	14	7.11	1.43	1.41	4.32	/
Diabetes duration	282	1	48	12.35	10.43	1.16	1.01	/
General diet	285	0	7	4.35	2.10	-.61	-.64	.88
Specific diet								
Fruits and vegetables	285	0	7	3.78	2.43	-.26	-1.18	/
High-fat food	285	0	7	3.51	1.99	.08	-.84	/
Exercise	285	0	7	4.35	2.10	-.61	-.64	.84
Blood glucose testing	285	0	7	4.99	2.54	-.86	-.80	.85
Foot care	285	0	7	2.75	2.54	.46	-1.19	.74
Cigarettes per day	142	0	70	17.47	14.49	1.46	2.58	/

* N = sample size; M = mean; SD = standard deviation; α = Cronbach alpha

Table 2. Pearson´s correlations

	1	2	3	4	5	6	7	8	9
HbA1c (1)	1	.163**	-.172**	-.187**	.037	-.069	-.037	-.035	-.006
Diabetes YoL (2)	.163**	1	-.041	-.066	-.066	-.082	.115	.174**	-.062
General diet (3)	-.172**	-.041	1	.385**	-.057	.380**	.430**	.224**	-.077
Fruits and vegetables (4)	-.187**	-.066	.385**	1	.119*	.311**	.159**	.066	.004
High-fat food (5)	.037	-.066	-.057	.119*	1	.078	.020	-.022	.052
Exercise (6)	-.069	-.082	.380**	.311**	.078	1	.161**	.165**	.112
Blood glucose testing (7)	-.037	.115	.430**	.159**	.020	.161**	1	.247**	-.051
Foot care (8)	-.035	.174**	.224**	.066	-.022	.165**	.247**	1	-.058
Cigarettes per day (9)	-.006	-.062	-.077	.004	.052	.112	-.051	-.058	1

* **p > 0.01; * p > 0.05

Intercorrelation coefficients for all variables are shown in Table 2. HbA1c level was positively correlated with years of life with diabetes, and negatively correlated with general diet and fruit and vegetable consumption, as dimensions of SDSCA. Dimensions of SDSCA were correlated positively with each other, specifically general diet with fruit and vegetable consumption, exercise, blood glucose testing and foot care, fruit and vegetable consumption with high-fat food intake, exercise, and blood glucose testing, exercise with blood glucose testing and foot care and foot care with blood glucose testing and years of life with diabetes.

To examine differences in self-care activities and health outcomes between type 1 and type 2 diabetes, t-test for each dimension of SDSCA and HbA1c was done. Results are shown in Table 3. There was a significant difference between Type 1 and Type 2 diabetes in levels of HbA1c, with Type 2 (M = 6.82; SD = 1.43) diabetes scoring lower levels of HbA1c on average compared to Type 1

(M = 7.25; SD = 1.34). There was a significant difference between Type 1 and Type 2 diabetes regarding blood glucose testing, with Type 1 diabetes patients (M = 6.09; SD = 1.71) measuring blood glucose daily more times on average compared to Type 2 (M = 3.60; SD = 2.69). To test whether HbA1c level can be predicted by SDSCA dimensions for Type 1 and Type 2 diabetes individually, linear regression analysis for each subsample was conducted (Table 4). For Type 1 diabetes, a regression model was borderline significant (R = .28, F (6.149) = 2.12, p = .054), explaining 7.9% variance of HbA1c, with general diet (β = -.20, p = .04) and blood glucose testing being (β = -.19, p = .02) significantly associated with lower levels of HbA1c. For Type 2 diabetes, regression model was significant (R = .40, F (6.90) = 2.93, p = .012), explaining 16.3% variance of HbA1c, with fruit and vegetable consumption (β = -.37, p = .00) being significantly associated with lower levels of HbA1c.

Table 3. T-test

	Type 1 M (SD)	Type 2 M (SD)	t	p
HbA1c	7.25 (1.34)	6.82 (1.43)	**2.42**	**.02**
General diet	4.51 (2.07)	4.17 (2.1)	1.36	.18
Fruits and vegetables	3.88 (2.4)	3.61 (2.49)	.88	.38
High-fat food	3.58 (1.99)	3.41 (2.01)	.67	.50
Exercise	4.04 (2.28)	3.7 (2.37)	1.18	.24
Blood glucose testing	6.09 (1.71)	3.6 (2.69)	**8.74**	**.00**
Foot care	2.86 (2.48)	2.64 (2.63)	.71	.48
Cigarettes per day	17.66 (14.88)	17.39 (14.38)	.11	.92

*M = mean; SD = standard deviation; p = p level

Table 4. Determinants of HbA1c for Type 1 and Type 2 diabetes

Type 1	B	β	p	Type 2	B	β	p
General diet	-.13	-.20	**.04**	General diet	-.01	-.01	.92
Fruits and vegetables	.03	.06	.52	Fruits and vegetables	-.21	-.37	**.00**
High-fat food	.02	.03	.75	High-fat food	.07	.10	.31
Exercise	.03	.05	.54	Exercise	-.05	-.08	.49
Blood glucose testing	-.16	-.19	**.02**	Blood glucose testing	.04	.07	.55
Foot care	.02	.03	.71	Foot care	.00	.00	.98

Discussion

The aim of this study was to examine the differences in self-care activities between patients with Type 1 and Type 2 diabetes as well as the role of these activities in predicting good glycaemic control.

The average level of HbA1c of 7.11% in this study, suggests that glycaemic control is not so satisfactory among people with diabetes in Serbia. Results also suggest that there is a difference between Type 1 and Type 2 diabetes in achieving good glycaemic control, with Type 2 diabetes patients achieving better levels of HbA1c. This is in accordance with previous studies which suggest that achieving blood sugar control for people with Type 1 diabetes is much more challenging compared to Type 2 (16). We can conclude that people with Type 1 diabetes need more support regarding self-control in diabetes since the burden of the disease is much greater for them. Besides differences in glycaemic control, our results show that people with Type 1 diabetes check their blood glucose much more frequently than people with Type 2. This is in accordance with general guidelines for therapy of Type 1 diabetes, where regular blood glucose measuring is emphasized (7).

Placing importance on this kind of activity proved to be justified once more, based on our results showing that in people with Type 1 diabetes, the higher frequency of measuring sugar as well as a practising more healthy eating plan, in general, are associated with lower levels of HBA1c. The relationship between blood glucose measurement and glycaemic control was not found to be significant in people with Type 2 diabetes, but regular consumption of vegetables and fruits was, which is in line with the general recommendations for diabetes control for this type

of diabetes where emphasis is put on changing lifestyle (18).

In general, our results suggest that the level of HbA1c rises as the number of years living with diabetes increases. This could be explained by the probable development of certain complications among patients, influencing the burden of the disease, quality of life, and self-care activities, in turn reducing the success of glycaemic control.

What was interesting, the consumption of red meat and full dairy products was not associated with glycaemic control outcomes or other self-care activities. It seems that people with diabetes in Serbia may not be familiar with the recommendations to avoid high-fat foods. Additionally, there may be a trend of using the keto diet which mostly consists of meat and vegetable consumption. It's important to investigate how these dietary habits affect the health outcomes of those with diabetes in Serbia.

There are some limitations of the study that need to be addressed. Firstly, the sampling process was biased towards individuals with diabetes who were active on Facebook groups for people with diabetes. This means that the sample may not be representative of the wider population of individuals with diabetes. Additionally, the sample wasn't balanced in terms of the proportion of Type 1 and Type 2 diabetes. There were more people with Type 1 diabetes in the study, which suggests that they are more proactive in seeking advice and information on such groups. Finally, the process of collecting information about the level of HbA1c wasn't reliable enough. Nevertheless, we think that the results of this study can significantly contribute to understanding and creating better practices in the treatment and education of people with diabetes.

Conclusion

Based on our findings, it can be concluded that in Serbia, achieving good glycaemic control is more challenging for people with Type 1 diabetes than for people with Type 2 diabetes, despite no significant difference in their compliance with most self-care activities. Individuals with Type 1 diabetes should receive the education that emphasizes monitoring blood sugar levels and adopting a healthy lifestyle. Meanwhile, for people with Type 2 diabetes, the focus should be on improving their daily habits, particularly by increasing their consumption of fruits and vegetables.

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Originalni rad **UDC: 616.379-008.64:613**
doi: 10.5633/amm.2024.0306

BRIGA O SEBI KAO PREDIKTOR DOBRE GLIKEMIJSKE KONTROLE U DIJABETESU: RAZLIKE IZMEĐU DIJABETESA TIPA 1 I TIPA 2

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Dijabetes melitus je hronična bolest koju danas kontroliše prvenstveno sama obolela osoba. Aktivnosti samokontrole dijabetesa uključuju redovnu kontrolu glukoze u krvi, zdravu ishranu, redovnu fizičku aktivnost, uzimanje preporučenih lekova i redovne konsultacije sa zdravstvenim radnicima. Različite studije su pokazale da edukativne i psihosocijalne intervencije mogu imati značajan uticaj na poboljšanje samokontrole dijabetesa i na smanjenje komplikacija. Cilj ove studije bio je da se ispitaju razlike u aktivnostima u vezi sa brigom o sebi između obolelih od dijabetesa melitusa tipa 1 i obolelih od dijabetesa melitusa tipa 2, kao i da se ispita uloga ovih aktivnosti u predviđanju dobre glikemijske kontrole. Naši rezultati ukazuju na to da u Srbiji osobe sa dijabetesom melitusom tipa 1 imaju mnogo teži zadatak kada je reč o sprovođenju dobre kontrole glikemije nego osobe sa dijabetesom melitusom tipa 2, čak i kada ne postoji razlika između ovih bolesnika u pogledu brige o sebi. U budućim programima edukacije za osobe sa dijabetesom melitusom tipa 1 naglasak treba staviti na kontinuirano praćenje glukoze u krvi. I kod jednog i kod drugog tipa dijabetesa melitusa treba obratiti pažnju na edukovanje o zdravoj ishrani.

Acta Medica Medianae 2024; 63(3):48–54.

***Ključne reči**: dijabetes, briga o sebi tokom dijabetesa, kontrola glikemije, edukacija o dijabetesu*

Original article **UDC: 616.711-009:[616.98:578.834**
doi: 10.5633/amm.2024.0307

CERVICOBRACHIAL SYNDROME: PREVALENCE AND CLINICAL CORRELATION WITH CORONAVIRUS 2019 DISEASE AMONG HOSPITALIZED PATIENTS

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There are currently emerging concerns about the multisystem involvement of the coronavirus disease 2019 (COVID-19) infection as well as potential long-term complications. The aim of our study was to examine the prevalence and clinical characteristics of cervicobrachial syndrome among hospitalized patients with COVID-19. The research was conducted as a prospective study on 147 patients with previously confirmed COVID-19 infection by using a Real-Time Polymerase Chain Reaction assay for SARS-Cov-2. After neurological assessment and meticulous patient history-taking, the patients were divided into a group with and a group without a previous history of cervicobrachialgia. The pain intensity was self-assessed by patients using a numerical scale ranging from 0 to 10, while routine laboratory analyzes related to COVID-19 were performed from venous blood samples. Our data demonstrate a statistically significant association between previous history and recurrence of cervicobrachialgia (LR = 28.655; p = 0.000). Moreover, the pain intensity assessment in patients with a previous history of cervicobrachialgia statistically significantly correlates with the current degree of cervicobrachialgia during hospitalization (p = 0.000). Furthermore, a weak positive correlation (r = 0.168; p = 0.046) was noted between the current degree of cervicobrachiialgia and the neutrophil to lymphocyte ratio. The present study demonstrates a statistically significant association between the previous history of cervicobrachial syndrome and its recurrence, as well as pain intensity assessment in hospitalized patients due to COVID-19. The neutrophil-to-lymphocyte ratio positively correlates with the degree of cervicobrachialgia and may indicate an increase in the local inflammatory response in cervicobrachial syndrome.

Acta Medica Medianae 2024;63(3):55–63.

Key words: *coronavirus disease 2019, cervical pain, cervicobrachial neuralgia, coronavirus disease 2019 vaccines, neutrophil-to-lymphocyte ratio*

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Introduction

The coronavirus disease 2019 (COVID-19) pandemic was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and there are currently emerging concerns about the multisystem involvement of the COVID-19 infection as well as potential long-term complications (1, 2). The pandemic has indisputably exerted a profound influence on the health domain, notably in terms of both morbidity and mortality outcomes, since the numerous diagnostic and therapeutic measures for other diseases were postponed (1).

Individuals infected with COVID-19 exhibit a wide spectrum of clinical manifestations, while the most frequently encountered symptoms include fever, myalgia, fatigue, non-productive cough, productive cough, pharyngitis, headache, ageusia, anosmia, nausea, fever, dyspnea, diarrhoea, vomiting (3). Moreover, neurological symptomatology can encompass not only headache, anosmia and ageusia, but there is also a growing body of evidence to suggest that infection can induce the emergence of stroke, Guillain–Barré syndrome, syncope and convulsions, and in the most severe cases, cause toxic metabolic encephalopathy (4).

Cervicobrachial syndrome represents pain in the neck and arms, which may be caused by cervical radiculopathy due to disc herniation and is clinically manifested as discomfort, numbness or

tingling in the arm and accompanying headache (5). Some other etiologic factors that may cause cervicobrachialgia in patients without neurological deficits could encompass facet joint pain, fibromyalgia with associated trigger points and inflamed nerve tissue, while there are also potential non-somatic referral patterns (5, 6).

Considering the profound impact of the COVID-19 pandemic, there has been a significant reduction in the scope of diagnostic and therapeutic procedures on the cervical spine (7, 8). Furthermore, there is no data in the relevant scientific literature about cervicobrachial syndrome in hospitalized COVID-19 patients. Therefore, the aim of our study was to examine the prevalence and characteristics of cervicobrachial syndrome in hospitalized COVID-19 patients.

Patients and methods

The authors conducted this prospective study on patients during the treatment in a COVID-19 hospital in the period from April 2021 to May 2022, after obtaining the consent of the referent ethics committee. The study followed the principles of the Declaration of Helsinki from 2013 (seventh revision) while the ethics committee approved the study (Number 9882/5 on April 2, 2021). Upon obtaining the informed consent as well, the research was conducted on 147 patients with a previously confirmed COVID-19 infection with a Real-Time Polymerase Chain Reaction assay for SARS-Cov-2 by using the Bioer LineGene 9600 Plus Real Time Thermalcycler PCR Systems (Biosynex, Illkirch- Graffenstaden, France).

Furthermore, all the participants involved were individuals, older than 18 years, with a mild clinical presentation and absence of significant comorbidities (their SpO2 value exceeded 90%). Our patients were hospitalized for a minimum of 7 days and were under constant supervision by medical staff, which included regular assessment of body temperature, oxygen saturation, breathing rate and diuresis. Vaccination status was determined based on the vaccination certificate from the state medical database. On the basis of vaccination status, patients were divided into the unvaccinated group, the group vaccinated with one dose, and the group vaccinated with 2 or more doses, while the patients previously had the option to be vaccinated with the vaccines of their choice.

The confirmation of the diagnosis of cervicobrachial syndrome was achieved through anamnestic data, as well as by neurological assessment of the patients with manual muscle testing, upper limb reflex evaluation, Spurling's Test, shoulder abduction test and upper limb tension tests.

According to the presence of cervicobrachialgia, the patients were categorized into group I, which was comprised of patients with a documented history of cervicobrachial syndrome, as well as Group II, which encompassed patients experiencing cervicobrachialgia for the first time, without any prior occurrences.

The intensity of cervicobrachial pain was self-evaluated by the patients with a numerical scale ranging from 0 (indicating no pain) to 10 (representing the most severe pain imaginable). Throughout the patient's hospitalization, cervicobrachialgia was treated with conservative therapy that included only paracetamol and dexamethasone, since other nonsteroidal anti-inflammatory drugs and depot corticosteroids were not recommended by the National guidelines for the treatment of COVID-19 infection, while a small number of patients underwent kinesitherapy. Additional diagnostic and therapeutic procedures for cervicobrachial syndrome were postponed until patients had recovered from the COVID-19 infection.

Statistical data processing

Data entry and statistical processing were performed using the SPSS software package (IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). Among the basic descriptive statistical parameters, standard statistical methods were used for qualitative and quantitative assessment of the results: absolute numbers, relative numbers (%), arithmetic mean ($\bar{x}$), standard deviation (SD), and minimum and maximum values. The normality of the distribution of individual values was examined by Kolmogorov–Smirnov test. The χ^2 test was used to test the statistical significance and interpret the relationship between two categorical variables. The statistical hypothesis was tested at the level of significance for the risk of $\alpha = 0.05$, i.e. the difference between the samples was considered significant if $p < 0.05$.

We applied the Mann–Whitney U test as a nonparametric test to compare outcomes between two independent groups. One-way ANOVA (Analysis of Variance) was employed as a statistical test to analyze the difference between the means of more than two groups. Binary logistic regression analysis was used to estimate the probability or likelihood of the cervicobrachialgia occurring based on the values of the predictor variables, as a regression model where the target variable is binary.

Results

There was no statistically significant association between the incidence of cervicobrachial syndrome among genders (Chi-square statistic was 0.848 and $p = 0.357$). There was no statistically significant difference in the age of the patients (Table 1) regarding the presence of cervicobrachialgia (Z-score was -0.803 and $p = 0.422$). The average age of the patients in our study was 66.35 years (min 29, max 89), while the gender structure consisted of 88 (59.86%)

male and 59 female (40.13%) patients. Out of a total of 147 patients from our study, 127 patients (86.39%) were not vaccinated, 14 patients (9.52%) were vaccinated with two doses of vaccine, while 6 patients (4.08%) were vaccinated with one dose of vaccine (Table 2).

The results of our research indicate that a total of 27 patients (64.28%) with a previous history of cervicobrachialgia had a recurrence of this syndrome (Table 3). However, 19 patients (18.09%) who had never experienced cervicobrachialgia before suffered from this syndrome for the first time during the hospitalization. Our data demonstrated a statistically significant association between previous history and recurrence of cervicobrachialgia (LR = 28.655; p = 0.000) (Table 3). Moreover, the pain intensity assessment in patients with a previous history of cervicobrachialgia statistically significantly correlated with the current degree of cervicobrachialgia during hospitalization (p = 0.000) (Table 4).

Furthermore, a weak positive correlation (r = 0.168; p = 0.046) was noted between the current degree of cervicobrachialgia and the neutrophil-to-lymphocyte ratio (Table 4). Additionally, no statistically significant correlation was found between the inflammatory parameters and the degree of pain (Table 4). Moreover, the vaccination status of the patients was also not statistically significantly associated (F = 0.328; p = 0.895) with the cervicobrachial syndrome (Table 4.).

Various other symptoms and signs of COVID-19 infection, which we considered during our research did not show statistically significant associations with the occurrence of cervicobrachialgia (Table 5).

Table 1. Demographic characteristics of the examined patients

Variable	Patients with cervicobrachial syndrome	Patients without cervicobrachial syndrome	p‡
	Number (Percentage)	Number (Percentage)	
Gender			
Male	25 (54.35%)	63 (62.37%)	
Female	21 (45.65%)	38 (37.62%)	p = 0.357
Age (years) †	65.60 ± 14.03	66.34 ± 13.93	p = 0.422

† Mean ± standard deviation, ‡ Chi−squared test, ‖ Mann−Whitney U-test

Table 2. Vaccination status of the hospitalized patients

Variable	Frequency	Percent
No vaccines	127	86.39
Pfizer−BioNTech COVID-19 Vaccine 1 dose	1	0.68
Sinopharm [Vero Cell]- Inactivated 1 dose	2	1.36
Sinopharm [Vero Cell]- Inactivated 2 doses	14	9.52
The Oxford/AstraZeneca (ChAdOx1-S [recombinant] vaccine) COVID-19 vaccine—1 dose	3	2.04
Total	147	100

Table 3. The presence of cervicobrachial syndrome in comparison to the patient's prior medical history

Variable	**Patients with cervicobrachial syndrome**	**Patients without cervicobrachial syndrome**	**p‡**
	Number (Percentage)	Number (Percentage)	
Previous history of cervicobrachial syndrome	27 (64.28%)	15 (35.71%)	
The patient has never had cervicobrachial syndrome	19 (18.09%)	86 (81.91%)	**p = 0.000**

‡Chi-squared test

Table 4. Relationship between the degree of cervicobrachialgia, vaccination status, hospitalization and laboratory parameters

Variables	Current degree of cervicobrachialgia	
	Pearson Correlation	p
Hospitalization in patients with previous history of cervicobrachialgia	0.579	**0.000**
Fever	-0.085	0.306
Creatine kinase	0.080	0.768
Interleukin 6	-0.150	0.465
Lymphocyte count	-0.045	0.601
Lymphocyte percentage	0.101	0.336
Neutrophil count	0.043	0.627
Neutrophil percentage	-0.005	0.964
Neutrophil to lymphocyte ratio	0.168	**0.046**
Vaccination status	0.328†	0.895
D-dimer	-0.116	0.450
C-reactive protein	-0.064	0.442

†F—a value on the F distribution in the ANOVA test

Table 5. The influence of the tested parameters on the development of cervicobrachial syndrome (Binary logistic regression)

Variable	OR	95% CI	p
Myalgia	0.446	0.146–1.364	0.157
Fatigue	0.975	0.231–4.117	0.973
Nonproductive cough	1.254	0.413–3.812	0.690
Productive cough	0.373	0.100–1.384	0.140
Pharyngitis	1.440	0.321–6.456	0.690
Headache	1.241	0.341–4.514	0.743
Dysgeusia	7.645	0.008–7295.427	0.561
Anosmia	0.495	0.001–441.843	0.839
Nausea	1.817	0.491–6.718	0.371
Fever	0.665	0.170–2.601	0.557
Dyspnea	0.476	0.114–1.990	0.309
Diarrhoea	0.455	0.077–2.698	0.386
Vomiting	2.487	0.214–28.859	0.466

Discussion

Based on the available relevant scientific data and empirical evidence from referent databases (MEDLINE, PubMed, Embase, ClinicalTrials.gov, Cochrane databases, EBSCO, Redalyc) and to the best of our knowledge, this is the only study that has investigated and reported the frequency and characteristics of cervicobrachialgia in hospitalized COVID-19 patients.

With the implementation of immunization strategies against COVID-19, the pandemic trajectory was reversed, but on the other hand, numerous side effects were recorded. One study conducted in Mexico showed that the most common neurological side effects after vaccination were headache, transient sensory symptoms and weakness, while epileptic seizures, Guillain–Barré syndrome and transverse myelitis were documented solely in a couple of cases (9). On the other hand, Göbel et al. recorded that although the headache occurred often, it was transient in duration (18.0 ± 27.0 h after vaccination and lasted 14.2 ± 21.3 h) (10). It has been assumed that the vaccination against SARS-CoV-2 can lead to the development of transverse myelitis as a serious complication due to inflammatory and immune reactions, and be clinically manifested by motor, sensory and autonomic dysfunctions, which has been recorded in extremely rare cases (11, 12, 13). The results of our research indicate that there was no statistically significant association between the vaccination status of the patients ($F = 0.328$; $p = 0.895$) and the cervicobrachial syndrome. Among numerous studies examining the effects of vaccination against COVID-19, no association between vaccination status and the frequency of cervicobrachialgia has been examined and reported.

The findings derived from our research demonstrated that in 64.28% of patients with a previous history of cervicobrachialgia a recurrence occurred, while 18.09% of patients encountered cervicobrachial pain for the first time in their lifetime during hospitalization. A potential explanation for the exacerbation of chronic pain such as cervicobrachial syndrome is that its multifactorial and multidimensional nature may be caused by both psychosocial factors and central sensitization, which we could associate with hospital treatment during the COVID-19 pandemic (5). Moreover, our results indicate a statistically significant association between previous history and recurrence of cervicobrachialgia ($LR = 28.655$; $p = 0.000$) while the degree of pain in patients with a previous history of cervicobrachialgia statistically significantly correlates with the current degree of cervicobrachialgia during the hospital treatment ($p = 0.000$). The findings obtained from one research have shown that degenerative spine diseases with

radicular pain distribution, such as lumbago and sciatica are statistically significantly more prone to relapse in hospitalized patients treated for COVID-19 (14). Moreover, the results we obtained could be explained by the fact that the locomotor system in hospitalized COVID-19 patients is minimally active for a significant time, and applied to all bed-rest-related illnesses, while the patients in our study were hospitalized for a minimum of 7 days. It is widely acknowledged that a low level of physical activity is a risk factor for worsening of degenerative spine disorders (14, 15).

Numerous hypotheses attempt to clarify the mechanism by which SARS-CoV-2 exerts its neurotropic effects on the central and peripheral nervous system. The effects on the nervous system are widely postulated to result from direct viral invasion of nervous tissue or indirectly through systemic hyperinflammation. Furthermore, proinflammatory mediators are released by excessive activation of the innate immune system, which serves as a distinctive hallmark of COVID-19 (16, 17).

The radicular distribution of pain in disc herniation and compression on the nerve root is attributed to mechanical pressure, as well as a local inflammatory response. Furthermore, in patients with disc herniation, a local increase in the concentration of inflammatory cytokines, such as interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), interleukin 21 (IL-21), interleukin 17 (IL-17) and cyclooxygenase-2 (COX-2), has been observed, while the high-sensitivity CRP increases in proportion to the extent of the herniation (18). As a result, the local concentration of chemokines around the compressed nerve root and disc herniation increases, and the cellular concentration and activation of macrophages, mast cells, T and B lymphocytes becomes greater (18).

Additionally, concentrations of IL-6 and IL-8 have been demonstrated to be significantly higher in polydiscopathy compared to single-segment disc disease (19). On the other hand, pro-inflammatory cytokines such as Interleukin 1 (IL-1α and IL-1β) increase following the occurrence of COVID-19 infection and damage to the respiratory tract epithelium, after which IL-6, TNF-α and IFN-γ increase their concentration in the blood, correlating with the degree of inflammation (20). Moreover, a comprehensive understanding of the underlying pathophysiological mechanisms of how the above-mentioned inflammatory mediators increase both in COVID-19 infection and cervical disc herniation remains elusive, and additional research is needed to provide a comprehensive explanation between the connection between cervicobrachial syndrome and COVID-19.

On the other hand, the results of our research showed that inflammation markers, apart from neutrophil-to-lymphocyte ratio (NLR), were not statistically significantly elevated in patients with cervicobrachialgia (Table 4). Furthermore, NLR can be determined by dividing the neutrophil and lymphocyte counts and has been recognized as a marker of different inflammatory diseases. The clinical condition and disease status in patients with inflammatory connective tissue diseases, rheumatoid arthritis and other rheumatic diseases, ulcerative colitis, infectious conditions as well as autoimmune diseases could be observed by an increase in NLR, as suggested by some authors (21, 22). Additionally, there is an increasing body of research that indicates that NLR could be the widely available and more economical replacement for markers of systemic inflammation, oncological diseases, as well as various neurological disorders (23, 24). On the other hand, Gelibter et al. obtained results indicating that NLR was not a reliable marker neither for the activity of demyelinating diseases nor a determinant for predicting disability in these patients (25). Parthasarathi et al. demonstrated in a systematic review that NLR could be useful in estimating the morbidity and mortality associated with COVID-19, while NLR values > 6.5 were correlated with a higher morbidity and worse disease outcome (26). In addition, the results of our research indicate a weak positive correlation ($r = 0.168$; $p = 0.046$) between the degree of cervicobrachial syndrome and the NLR values (Table 4). Although the data about the correlation between NLR and cervicobrachial syndrome remains scarce, we identify a potential explanation for our results in the fact that correlations between NLR and different neurological, inflammatory and rheumatic diseases have been shown in some previous research, while cervicobrachial syndrome comprises all of these conditions (21–26).

Several noteworthy neuroprotective factors have been examined as potential predictors for peripheral and central nervous system damage caused by COVID-19 and include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4 and NT-5, NT-6, NT-7 and neurofilament light chain (NFLC) in the blood. Additionally, some of the functions of the previously mentioned neurotropic factors include roles in survival, homeostasis and development of neurons, while alterations in their values are associated with inflammatory, musculoskeletal and pain-sensitive diseases (27, 28). The authors of one study showed an association between lower levels of BDNF and neurological decline of the COVID patients (29). Moreover, according to the results of one research, the concentration of NFLC in the plasma of patients infected with COVID-19 was elevated during the first few days of hospitalization, and therefore NFLC has been proposed as a potential marker for the assessment of neurological involvement during the pandemic (28, 30). Therefore, further investigation is needed to examine the neurological consequences of the SARS-CoV-2 virus and biochemical markers, which would help in the early detection and prevention of neurological complications.

Conclusion

The present study demonstrates a statistically significant association between the previous history of cervicobrachial syndrome and its recurrence, as well as pain intensity assessment in hospitalized patients due to COVID-19. The neutrophil-to-lymphocyte ratio positively correlates with the degree of cervicobrachialgia and may indicate an increase in the local inflammatory response in cervicobrachial syndrome. Furthermore, the results have the potential to facilitate a more holistic approach towards managing the complications of COVID-19.

Acknowledgements

The authors would like to acknowledge the invaluable assistance provided by all research assistants involved in the meticulous data collection process. The authors also extend their sincere appreciation to all study participants for their invaluable contributions.

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Originalni rad **UDC: 616.711-009:[616.98:578.834**
doi: 10.5633/amm.2024.0307

CERVIKOBRAHIJALNI SINDROM: PREVALENCIJA I KLINIČKA KORELACIJA SA KORONAVIRUSNOM BOLEŠĆU 2019 KOD HOSPITALIZOVANIH BOLESNIKA

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Trenutno je prisutna zabrinutost zbog multisistemskih implikacija infekcije koronavirusnom bolešću 2019 (COVID-19), kao i zbog njenih potencijalnih dugoročnih komplikacija. Cilj našeg istraživanja bilo je ispitivanje prevalencije i kliničkih karakteristika cervikobrahijalnog sindroma kod hospitalizovanih bolesnika sa oboljenjem COVID-19. Prospektivnom studijom obuhvaćeno je 147 bolesnika kojima je, korišćenjem testa lančane reakcije polimeraze u realnom vremenu za SARS-Cov-2, prethodno potvrđena infekcija COVID-19. Nakon neurološke procene i pažljivog uzimanja anamneze, bolesnici su razvrstani u grupu bolesnika sa prethodnom istorijom cervikobrahialgije i grupu bolesnika bez prethodne istorije bolesti. Intenzitet bola procenjivali su sami bolesnici koristeći numeričku skalu u rasponu od 0 do 10. Rutinske laboratorijske analize u vezi sa koronavirusnom bolešću 2019 rađene su na osnovu uzoraka venske krvi. Naši podaci pokazuju statistički značajnu povezanost prethodne istorije cervikobrahialgije sa njenim recidivom (LR = 28,655; p = 0,000). Štaviše, procena intenziteta bola kod bolesnika sa prethodnom istorijom cervikobrahialgije statistički značajno korelira sa trenutnim stepenom cervikobrahialgije tokom hospitalizacije (p = 0,000). Takođe, zabeležena je slaba pozitivna korelacija (r = 0,168; p = 0,046) između trenutnog stepena cervikobrahialgije i odnosa neutrofila i limfocita. U ovoj studiji prikazane su statistički značajna povezanost prethodne istorije cervikobrahijalnog sindroma sa njegovim ponavljanjem i procena intenziteta bola kod hospitalizovanih bolesnika zbog COVID-19 infekcije. Odnos neutrofila i limfocita pozitivno korelira sa stepenom cervikobrahialgije i može ukazivati na povećanje lokalnog inflamatornog odgovora kod cervikobrahijalnog sindroma.

Acta Medica Medianae 2024; 63(3):55–63.

***Ključne reči**: koronavirusna bolest 2019, bol u vratu, cervikobrahijalna neuralgija, vakcine protiv koronavirusne bolesti 2019, odnos neutrofila i limfocita*

Original article

UDC: 616.342-076
doi: 10.5633/amm.2024.0308

MORPHOMETRIC ANALYSIS OF DUODENAL BIOPSIES IN PATIENTS WITH SUSPECTED COELIAC DISEASE

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Coeliac disease (CD) is an immune-mediated systemic disorder mostly presented in the form of small intestine enteropathy caused by gluten and related prolamins intake, from cereals such as wheat, barley, and rye. The diagnosis of CD is currently based on clinical presentation, pathohistological evaluation of the small intestine biopsies and positive serology. The aim of our study was to investigate histological abnormalities in the villous architecture of the duodenal bulb and postbulb segment in patients diagnosed with CD and in those biopsies sent for examination but the diagnosis was not confirmed. Morphometric analysis was performed on 35 duodenal samples obtained from patients with the initial clinical diagnosis of CD while some patients had dyspepsia as a primary diagnosis. The obtained data of villus width measured in the bulbar and postbulbar part of the duodenum were found to be statistically significantly different ($p = 0.0226$). The width of the duodenal villi in the bulbar part was significantly thicker than the one in the postbulbar part, while the value of the villous height at the examined places was not statistically significant. Also, none of the cases in this study showed any extensive abnormalities in villous architecture. Besides pathohistological examination which remains the gold standard in diagnosing, morphometric analysis may also be helpful in the detection of the latent forms of this entity. Having in mind that the chronic persistence of this disease may indicate various systemic dysfunction, long-term follow-up of these patients is necessary.

Acta Medica Medianae 2024;63(3):64–70.

Key words: *morphometry, duodenum, duodenal biopsies, coeliac disease*

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Introduction

Coeliac disease (CD) is an immune-mediated systemic disorder mostly presented in the form of small intestine enteropathy caused by gluten and related prolamins intake, from cereals such as wheat, barley, and rye (1). Clinical presentation of CD varies, but it is mostly characterized by a combination of gastrointestinal symptoms, such as malabsorption, persistent diarrhoea, abdominal discomfort, pain, and extra-intestinal manifestations, which include dermatitis herpetiformis, nutritional deficiency, anaemia, osteoporosis, endocrine and neurologic disorders (2). However, some patients may be asymptomatic or have discrete signs of the disease (3). The pathogenesis of this intestine injury is presented as an interaction between inflammatory cells (IELs) from the lamina propria and gliadin from food sources (4). The diagnosis of CD is currently based on clinical presentation, pathohistological evaluation of the small intestine biopsies and positive serology. In some clinical cases, the diagnostic criteria can be ambiguous, so a precise evaluation of the laboratory and histopathological results is necessary (5).

Mostly, this autoimmune disease primarily affects the superficial mucosa of the small intestine, while deeper layers are rarely implicated (5, 6). Thus, the histologic examination of mucosal changes might be considered the gold standard for CD diagnosis, since it is present in patients both with/without clinical symptoms or signs (7). The

most characteristic histological features of CD are abnormalities in villous architecture with a reduction in villus height (Vh), crypt hyperplasia with an increase in its depth (Cd), and inflammatory cell infiltration, which mainly comprises IELs (7, 8). It is also known as a condition characterized by a normal villous structure with a discreet increase in the number of inflammatory cells and crypt hyperplasia, defined as "microscopic enteritis" (9). The pathohistological diagnosis of CD is mainly based on the Marsh–Oberhuber semiquantitative classification which grades the small intestine changes into four categories, with several subgrades, depending on the specific changes (10). The disturbances in the normal villous architecture are found to be the features of the types 3 and 4 presented as a different degree of villous blunting, flattening, or a hypoplastic lesion, while types 1 and 2 show alterations only in the number of the IELs, without any histological abnormalities (6, 11).

As a result of the higher levels of acid in the duodenal lumen, mucosal morphology is characterized by short or broad villi, sometimes branching, while in the lamina propria, a greater number of inflammatory cells are present (12, 13). On the other hand, patients with active and untreated CD often have various changes in the mucosal architecture, such as villous atrophy (VA), crypt elongation, flattening of the surface epithelium, decrease in the number of Goblet cells and increase of the lymphocytes and plasmocytes in the epithelium of the villi and crypt, and also in the lamina propria (13, 14). Interestingly, these histological abnormalities are not usually present only in patients with CD, but also could be found in a variety of disorders including inflammatory bowel disease (Crohn's disease), autoimmune or immunodeficiency infection, nutritional and medication-related disorders (15).

Mucosal changes in patients with suspected CD mostly present in the duodenal bulb, and the biopsy samples taken from there may be useful in diagnosing this disorder (14, 16). Also, histological examination of the differences between the biopsy obtained from the duodenal bulb and the second part of the duodenum may help in the interpretation of the intestinal abnormalities in this specific entity (13, 14).

The aim of our study was to investigate histological abnormalities in the villous architecture of the duodenal bulb and postbulb segment in patients with suspected CD.

Material and Methods

The morphometric analysis was performed on 35 duodenal samples obtained from patients aged from 18 to 30, by routine endoscopic procedure. Analyzed duodenal specimens are part of the collection database of the Centre for Pathology and Pathological Anatomy, University Clinical Centre Niš, Serbia. Duodenal samples were routinely processed and stained with hematoxylin and eosin (H&E) following the standard protocol. Biopsies were examined using a light microscope Olympus BX50 (Olympus, Japan) connected with a digital camera Leica DFC 295 (Leica Microsystems, Germany) at the Morphometric Laboratory, Department of Anatomy, Faculty of Medicine, University of Niš.

In most cases, the initial clinical diagnose was CD, while some patients had dyspepsia as a primary diagnose. From each patient, the duodenal mucosa sample was obtained from both duodenal bulb and postbulbar segment of the duodenum. Five high magnification fields (×200) from each specimen were photographed, and non-processed images analyzed in the ImageJ (http://rsb.info.nih.gov/ij/) software. Examined morphometric parameters included villus length and width of the bulbar and postbulbar duodenum part expressed in μm. Villus height was measured from the base of the villi to its basal lamina, not taking the epithelial surface into account. In the case of the villous width, it was expressed as the mean value obtained after the measurement of width in the base, middle and apical part of the villous (Figure 1).

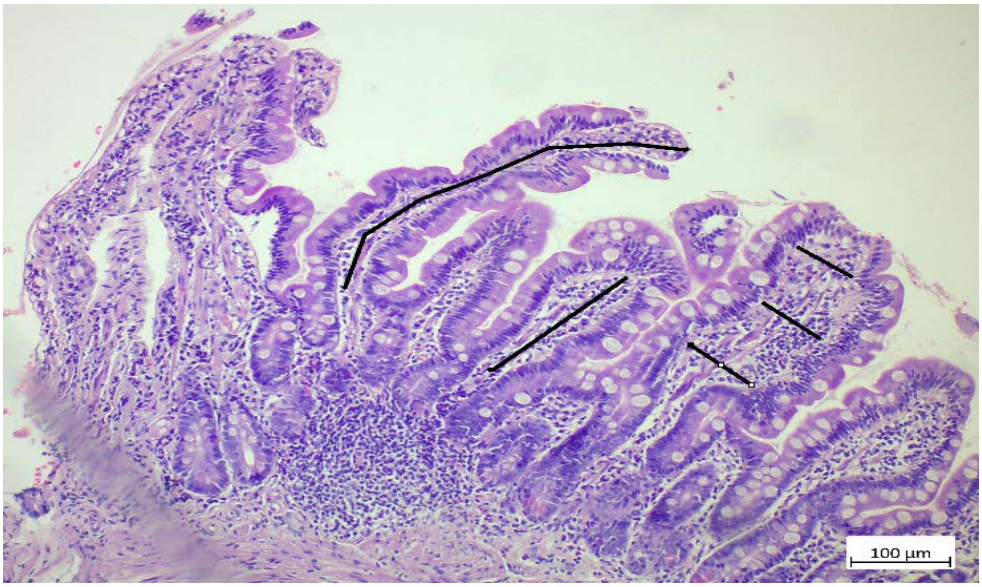

Figure 1. Example of morphometric measurement of the villus height and width in the duodenal bulb (H&E, magnification ×100)

Statistical analysis

The obtained data are given as mean ± SD and further compared using Student's t-test (GraphPad Prism, 8.0). Probability values (p) ≤ 0.05 were considered to be statistically significant.

Results

In 35 examined cases, the value of villus height obtained from bulbar part of the duodenum ranged from 145 to 365 µm (Figure 1). On the other hand, the same morphometric parameter measured in the second part of the duodenum (postbulbar) showed values ranging from 166 to 322 µm. When the villus height of the two measured parts was compared, no statistically significant differences were found (Figure 2).

The obtained data of villus width measured in the bulbar and postbulbar part of the duodenum were found to be statistically significantly different (p = 0.0226) (Figure 3). Duodenal villi width in the bulbar part was significantly thicker (mean value 47.6 µm) than the one in the postbulbar part (mean value 43.7 µm).

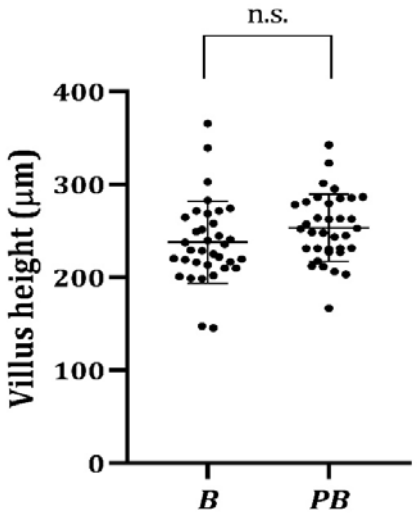

Figure 2. Villus height in the bulbar and postbulbar part of the duodenum, ns—no statistically significant difference was found using the Student's t-test

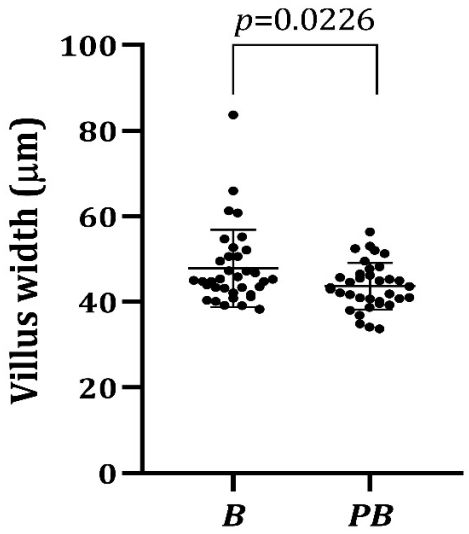

Figure 3. Villus width in the bulbar and postbulbar part of the duodenum with a statistically significant difference of p = 0.0226 was found using the Student's t-test

None of the examined cases in this study showed any extensive abnormalities in villous architecture. In most cases, normal villous morphology, without destructive lesions was observed (Figure 4). Based on these findings, our patients could be categorized as lower grades according to the Marsh−Oberhuber classification.

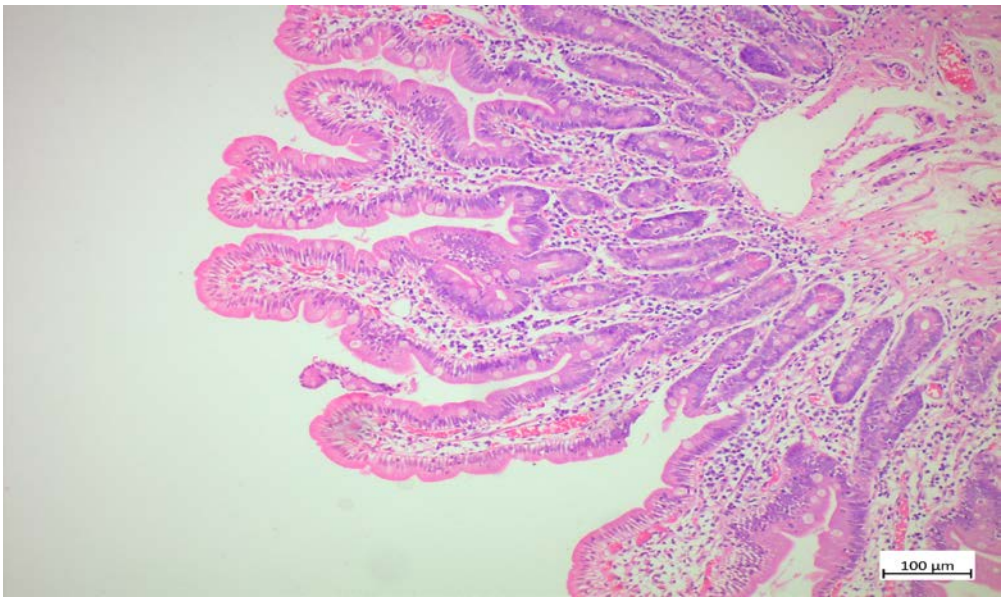

Figure 4. Pathohistological examination of a biopsy obtained from the duodenal bulb of the patient suspected of coeliac disease, showed normal villous architecture with a discreet increase in the number of IELs (H&E, magnification ×100)

Discussion

The diagnosis of CD, a complex autoimmune disorder, is based on clinical and histological findings, as well as on positive serology (16). Knowing that higher levels of transglutaminase antibodies may suggest the presence of CD, in the case of seronegative patients with evident clinical signs, most clinicians assert the necessity of the histological examination (16, 17). The most

frequent clinical symptom seen in patients with CD is bloating which is often accompanied with either diarrhoea, constipation, heartburn or nutritional deficiency (8, 18). All studied patients presented with similar gastrointestinal symptoms, however, no additional information was given about some extraintestinal disorders. In routine clinical practice, some disorders may imitate CD such as Helicobacter pylori infection, giardiasis, autoimmune enteropathy, eosinophilic gastroenteritis, drug-induced enteropathy, intestinal lymphoma, Crohn's disease, tropical sprue, etc. (18, 19). Moreover, the diagnosis of CD should be clearly separated from that of gastroduodenal inflammation (gastroduodenitis), which has almost identical clinical symptomatology, but with no significant mucosal disturbances (20).

Distal duodenum and proximal jejunum represent the best sites for detecting villous abnormalities which are seen in CD (21). In most patients, the degree of VA was present especially in the distal part of the duodenum, while some of them did not have any abnormalities at other examined locations (19). Thus, it is suggested that the most representative sampling sites in patients suspected of CD are the duodenal bulb and distal duodenum, from where two and four biopsies, respectively, should be taken and compared (9). The design of this study overlaps with a previous one (11), where the comparison of the two duodenal segments was shown to have a significant rationale. Some authors suggest that besides the adequate number of biopsies, the orientation of a sample, in position 9 and 12 o'clock, is necessary for precise evaluation of the degree of VA (12, 16, 22). Furthermore, it is desirable to cut biopsy samples at the right angle, where mucosa and crypt must be cut longitudinally in order to obtain a better image(s) for morphometric measurements (22, 23).

Duodenal biopsies obtained from patients suspected of having CD, atypical, asymptomatic or subclinical manifestation, may exert various grades of VA, often with typical endoscopic features such as "mosaic", "scalloping" or flattening of duodenal folds and emphasized vascular patterns (6, 18). Also, the characteristic mucosal changes in patients with CD are mostly presented with abnormalities in villous architecture and a reduction in Vh, crypt hyperplasia with an increase in its Cd, and inflammatory cell infiltration, especially of the IELs (9, 10, 15). Furthermore, a study conducted by Chaudhari et al. suggested various forms of villous lesions from flattening to atrophy, with a moderate density of inflammatory cells and duodenal metaplasia (24).

In this study, investigated biopsies were taken following the mentioned recommendations and the results implied significantly larger villous width in the bulbar part of the duodenum, than in the post-bulbar (Figure 3). These findings are in accordance with some previous ones (9), however, no significant deviation in villus height was noted as stated elsewhere (11, 14). Furthermore, examination of the duodenal bulb villi showed a possibility of their shortening, blunting and sometimes the absence of Brunner's glands and lymphoid aggregates, which can be the result of higher secretion of gastric acid (23, 25).

Compared to the normal intestinal samples, inflamed duodenal mucosa shows broader villi above Brunner's glands while a significant difference in villus length was not confirmed by our investigation, which is consistent with other studies (23). Significant villous width may be explained by the dilatation of Brunner's glands, extensive inflammation and lymph-plasmocyte infiltration of the lamina muscularis mucosae and sometimes gastric metaplasia of the duodenal epithelium (11).

Interestingly, in some cases, mucosal changes may be absent or minimal, besides representative clinical symptoms and positive serology (25). A similar observation was noticed in many here-studied cases. Some authors suggest that measurement of the morphometric parameter defined as the ratio between villus height and crypt depth (Vh : CrD ratio) can be helpful in detecting latent and minimal mucosal lesions, with a potential of taking the second duodenal biopsy for long-term follow-up of these patients (21, 26, 27). It is worth mentioning that pathohistological examination of the biopsy samples of patients undergoing gluten-free diet also represents a significant challenge for pathologists because in that case, mucosal changes may disappear (28).

Conclusion

Coeliac disease, as a complex inflammatory condition that affects multiple organ systems, provides a possibility for many nonmalignant and malignant complications. Since the diagnosis is based on the correlation between clinical presentation, histologic features and positive serology, pathohistological examination of the small intestine remains the gold standard. Detailed morphometric analysis of the mucosal changes could help detect latent forms of this gluten-mediated disorder. Based on the findings of our study, villi width was significantly higher in the duodenal bulb than in the postbulbar part, while the villous height was unaltered, suggesting that slight changes occurred in some borderline cases. These results could be obtained only if several biopsies taken from two anatomical sites were analyzed, which implies that it should be a routine practice in the diagnosis of coeliac disease.

Acknowlegments

The authors dedicate this paper to the late Professor Ivan Jovanović (MD, PhD) in gratitude for his unreserved support, motivation, and selfless sharing of knowledge.

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Originalni rad **UDC: 616.342-076**
doi: 10.5633/amm.2024.0308

MORFOMETRIJSKA ANALIZA BIOPSIJA DUODENUMA KOD BOLESNIKA KOD KOJIH POSTOJI SUMNJA NA POSTOJANJE CELIJAČNE BOLESTI

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Celijačna bolest je imunološki posredovano sistemsko oboljenje koje se najčešće prezentuje u vidu enteropatije tankog creva izazvane unošenjem glutena i njemu sličnih prolamina iz žitarica poput pšenice, ječma i raži. Dijagnoza celijačne bolesti je trenutno zasnovana na kliničkoj prezentaciji, patohistološkoj analizi biopsija tankog creva i pozitivnoj serologiji. Cilj našeg rada bio je da utvrdimo histološke promene u strukturi resica bulbusa i postbulbarnog dela duodenuma kod bolesnika sa dijagnozom celijačne bolesti i kod osoba kod kojih ona nije utvrđena. Morfometrijska analiza sprovedena je na 35 duodenalnih uzoraka dobijenih od bolesnika kod kojih postoji sumnja na postojanje celijakije, dok su neki od bolesnika imali dispepsiju kao primarnu dijagnozu. Dobijeni rezultati o širini resica merenih u bulbusu i postbulbarnom delu bili su statistički značajni ($p = 0,226$). Širina resica u bulbusu duodenuma bila je značajno veća od širine resica u postbulbarnom delu, dok vrednost visine resica na ispitivanim mestima nije bila statistički značajna. Takođe, nijedan slučaj u ovoj studiji nije pokazao značajne promene u građi vilusa. Pored patohistološke analize, koja predstavlja zlatni standard u dijagnostici, morfometrijska analiza takođe može biti od pomoći u otkrivanju latentnih formi ove pojave. S obzirom na to da hronično perzistiranje ove bolesti može usloviti brojne sistemske poremećaje, neophodno je dugoročno praćenje ovih bolesnika.

Acta Medica Medianae 2024; 63(3): 64–70.

***Ključne reči**: morfometrija, duodenum, biopsije duodenuma, celijačna bolest*

Review article **UDC: 615.835:796.056.1**
doi: 10.5633/amm.2024.0309

EFFECTS OF HYPERBARIC OXYGEN THERAPY ON RECOVERY AND PHYSICAL PERFORMANCE: A SYSTEMATIC REVIEW

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The potential mechanisms underlying the improvement in physical performance with hyperbaric oxygen therapy (HBOT) are multifaceted and not yet fully understood. Therefore, this systematic review aimed to identify and summarize the relevant literature on the influence of HBOT on recovery and performance. To identify potential studies, a comprehensive search was performed in two electronic databases: PubMed and MEDLINE. We identified 13 relevant studies with a total of 271 participants, of which 249 were males, while 22 were females. The studies on post-exercise recovery suggest that hyperbaric oxygen therapy may positively affect various recovery parameters which include reducing lactate concentration, improving heart rate recovery, enhancing antioxidant capacity, and accelerating recuperation. The studies on HBOT effects on physical performance provide some intriguing insights. While most studies indicate the potential of hyperbaric oxygen therapy to influence physical performance positively, it is crucial to consider that the effectiveness of HBOT can vary based on factors like exercise type, intensity, and individual athlete characteristics. The use of HBOT for recovery and performance is a promising field, but further research is required to establish standardized protocols and to better understand the specific conditions under which hyperbaric oxygen therapy can be most beneficial.

Acta Medica Medianae 2024;63(3):71–79.

Key words: *recovery, performance, oxygen, athletes*

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Introduction

Hyperbaric oxygen therapy (HBOT) is a treatment in which 100% oxygen is supplied under elevated pressure. Such treatment increases dissolved oxygen levels in the blood and results in high partial pressure of oxygen in peripheral tissues, which is beneficial for conditions associated with low O_2 environments, such as carbon monoxide poisoning, decompression sickness, arterial gas embolism, and tissue oxygen deprivation due to radiation-induced tissue damage (1, 2). HBOT is also a safe, effective, and non-invasive method for treating various conditions (3).

Athlete recovery after training is a constant concern for coaches, as inadequate recovery can lead to chronic fatigue, decreased performance, and increased risk of injury (4). In sports injuries, soft tissues, including muscles, ligaments, and tendons, are often damaged by oxygen deprivation due to oedema or haemorrhage in the injured tissue. Similarly, HBOT has become a recommended treatment for healing injuries in non-athletes (5, 6). In this context, the main function of this treatment is to accelerate the healing of soft tissues by reducing local hypoxia, inflammation, and oedema (7), and it may be beneficial for healing knee or ankle ligament injuries, joint sprains, or muscle injuries (8). HBOT has been reported to accelerate cell regeneration and tissue repair, which should help eliminate fatigue and restore endurance. It has gained considerable attention among sports medicine practitioners as a supportive therapy to accelerate recovery from muscle injury in athletes, but its exact efficacy remains unclear (9–12).

There are fewer studies in the literature on the use of HBOT in high performance athletes. Ishii (10) reported the use of HBOT as a recovery method for muscle fatigue during the Nagano Winter Olympics. It was found that all athletes benefited from HBOT treatment and recovered faster. In addition, Haapaniemi (13) and Fischer

(14) indicated that lactic acid and ammonia were cleared more rapidly by HBOT treatment, resulting in a shorter recovery time. Staples (12) investigated whether intermittent HBOT treatment improved recovery from delayed-onset muscle soreness (DOMS) of the quadriceps in 66 untrained men. The results suggest that treatment with HBOT can improve recovery, but the aforementioned study had a complex protocol and the experimental design was not entirely clear (the exclusion of some participants and group assignment was not clarified), making interpretation very difficult. Therefore, the aim of this systematic review was to identify and summarize the relevant literature on the influence of hyperbaric chamber therapy on recovery and performance.

Material and Methods

This systematic review was conducted according to the Preferred Reporting in Systematic Reviews and Meta-Analyses (PRISMA) guidelines (15).

Eligibility Criteria

The inclusion criteria were published studies in English, male or female adults, and the HBOT was conducted as an experimental program. In addition, no inclusion criteria were applied regarding participants' baseline fitness level or sports experience.

Search Strategy

To identify potential studies, a comprehensive search was performed in two electronic databases: PubMed and MEDLINE (Ovid), limiting the search to the following keywords: “oxygen”, ‟treatment”, ‟hyperbaric therapy”, “hyperbaric oxygenation”, ‟recovery”, “performance” and ‟muscle injury”. When available, we used Medical Subject Headings (MeSH). We used the Boolean operators ‟OR” and ‟AND” to combine terms. A descriptive method was used to analyse the data obtained. All titles and abstracts were reviewed for potential papers to be included in the systematic review. In addition, we performed a backward and forward search by searching the references and citations of the included studies. Reference lists of previous reviews were also reviewed. Relevant studies were selected after a thorough review if they met the inclusion criteria. Figure 1 shows the flow diagram.

Study Selection and Data Collection

A duplicate review was performed using EndNote X9, and all articles were screened using the Rayyan app (16). Two reviewers (GD and NT) independently reviewed titles and abstracts using the aforementioned app. All included articles were subjected to quality assessment.

Risk of Bias Assessment

The quality of the included studies was assessed using the Cochrane Collaboration's revised risk-of-bias tool (17). The following biases were assessed: bias due to the randomization process, bias due to deviations from planned interventions, bias due to missing outcome data, bias in outcome measurement, bias in the selection of reported outcomes, and overall bias. The risk of bias was rated as low, somewhat concerning, or high for each domain and overall bias.

Results

Study Selection

A detailed overview of the process of collecting adequate studies is shown in Figure 1. The automation tools used in the databases were language (English) and type of publication (academic journals). Duplicate entries were removed after combining the results of the search strategy. Titles and abstracts were screened to identify studies that used hyperbaric therapy to promote muscle recovery or muscle performance. The full texts of these articles were read to determine if the inclusion criteria were fully met. All studies that met the inclusion criteria were rescreened to determine if they were eligible for the systematic literature review.

After a general search of the database, 97 relevant studies were identified. Based on titles and duplicates, 80 studies were excluded, along with the additional 4 studies written in a language other than English. A total of 13 studies met the defined criteria and were included in the systematic review.

A total of 13 relevant studies were included in the systematic review. These studies met the pre-defined criteria, such as being published in the English language in the period from 1999 to 2021 and involving adult male and female participants. The primary focus of the final analysis was to examine the results obtained in hyperbaric chamber treatment conducted as an experimental program.

Characteristic of the Included Studies

We identified 13 relevant studies, with the total of 271 participants. There were 249 male participants and 22 female participants. The highest number of participants was 60 (18) and the lowest was 10 (19, 20). Only Babul (21) had female participants, while the rest of the studies had male participants, while mixed gender studies were only in one research (22). The atmosphere of absolute pressure was in most of the studies 2.5 ATA (22–24, 20, 18, 25–27). The rest of the studies used 2.0 ATA (21, 28) and 1.3 ATA (19, 29). Also, the longest time spent in the chamber

was 100 minutes (26), while the shortest was 30 minutes (18, 19).

Table 1. Studies included for analysis

Study	Characteristics of the sample of the participants				Intervention protocol	Intervention characteristics		Outcomes
	Sample size, n (F)	Groups	Age (y)	Sport		EG	CON	
Mc Gavok et al. (1999)	12 (6 F)	/	M - 29.5±4.0 F - 23.5±3.5	Trained runners	90 min treadmill run at 75–80% VO_2max	95% O_2 at 2.5 ATA for 90 minutes	NN for 90 minutes	VO_2max ↔
Mekjavic et al. (2000)	24	EG - 12 CON - 12	20-35	Healthy participants	Maximal isometric strength	100 O_2 concentration at 2.5 ATA for 60 minutes	NN at 0.2 ATA for 60 minutes	DOMS ↔ Elbow flexor max iso strength ↔
Webster et al. (2002)	12	EG – 6 CON - 6	24.2±3.2	Healthy participants	Strenuous eccentric exercise	100% O_2 concentration at 2.5 ATA for 60 minutes	NN at 1.3 ATA for 60 minutes	IMPT ↔PIT ↑* (HBOT) Pain sensation and unpleasantness ↑* (HBOT)
Hodges et al. (2002)	10	/	25.7±5.5	Moderately trained participants	Maximal incremental test for assessing VO_2max	95% O_2 at 2.5 ATA for 90 minutes	/	BLA ↔ VO_2max ↔ Run time (min) ↔ HRmax ↔
Sueblinvong et al. (2004)	60	RR – 20 HBOT – 20 OR – 20	21+2 21+2 21+2	Naval cadets, fitness levels similar to professional athletes	Incremental test on the cycle ergometer	100% O_2 concentration at 2.5 ATA for 30 minutes	RR – passive rest OR - O_2 inhalation	BLA ↓* (HBOT)
Babul et al. (2003)	16 (F)	EG – 8 CON - 8	25.25±4.1 25.49±4.24	Sedentary female	300 eccentric contractions of the non-dominant leg	100% O_2 concentration at 2.0 ATA for 60 minutes	21% O_2 at 1.2 ATA for 60 minutes	PMS ↔ IS ↔ CK ↔
Shimonda et al. (2015)	20	EG – 10 CON - 10	22.0±1.1 21.9±0.7	Healthy participants	Maximal isometric plantar flexion intermittently – a 2-second contraction followed by a 2-second rest x 50	100% O_2 concentration at 2.5 ATA for 60 minutes	NN at 1.2 ATA for 70 minutes	Force production ↑* (EG)
Branco et al. (2016)	11	/	29.7±6.6	Jiu-jitsu athletes	Jiu-jitsu intense training sessions	100% O_2 concentration at 2.39 ATA for 89 minutes	NN for 90 minutes	BLA ↔ RPE ↔ RPR ↑* Cortisol ↔ Testosterone ↔ CK ↔ ALT ↔ AST ↔ LDH ↔
Park et al. (2018)	10	PRT PST CON	21.10±1.25	Amateur football players	Maximal exercise load test on the treadmill (Bruce protocol)	30% O_2 concetration at 1.3 ATA for 30 min	/	BLA ↓* (POT) HR (bpm) ↓* (POT) BAP ↔
Chen et al. (2019)	41	EG -20 CON - 21	23.9±5.1 26.3±5.6	Professional baseball athletes	Intensive training sessions	100 O_2 concentration at 2.5 ATA for 100 minutes	NN at 1.3 ATA for 100 minutes	CK ↓* (EG) MB ↓* (EG) GOT ↓* (EG) Pain intensity and interference ↓* (EG)
Woo et al. (2020)	12	EG – 6 CON – 6			Maximal incremental test, Bruce protocol	100% O_2 concentration at 2.5 ATA for 60 minutes	NN for 60 minutes	BAP CK ↓* (EG) LDH ↓* (EG)
Hadanny et al. (2022)	31	EG – 16 CON 15	40-50	Master athletes	Maximal incremental test on the cycle ergometer	100 O_2 concentration at 2.0 ATA for 60 minutes	1.02 ATA for 60 minutes	VO_2max ↑* (EG) VO_2at ↑* (EG)
Mihailović et al. (2023)	12	/	NA	Professional cyclists	Fatiguing exercise for 10 minutes (consisting of two steps of 5 minutes at 80% and 90% of MAP) and 5-minute maximal cycling effort after the HBOT	100% O_2 concentration at 1.3 ATA for 75 minutes	/	BLA ↔ RPE ↓* HRV ↑* Power (W) ↑*

Legend: F—female; EG—experimental group; CON—control group; RR—rest recovery; OR—oxygen recovery; HBOT—hyperbaric oxygen therapy; PRT—pre-treatment; PST—post-treatment; VO_2max—maximal oxygen uptake; ATA—absolute atmosphere; NN—normoxic normobaric; DOMS—delayed onset muscle soreness; IMPT—isometric muscle peak torque; PIT—peak isometric torque; BLA—blood lactate; IS—isometric strength; CK—creatine kinase; LDH—lactate

dehydrogenase; RPE—rate of perceived extraction; RPR—rate of perceived recovery; BAP—biological antioxidant potential; ALT—alanine aminotransferase; AST—aspartate aminotransferase; MB—myoglobin; GOT—glutamic oxaloacetate transaminase; HRV—heart rate variability

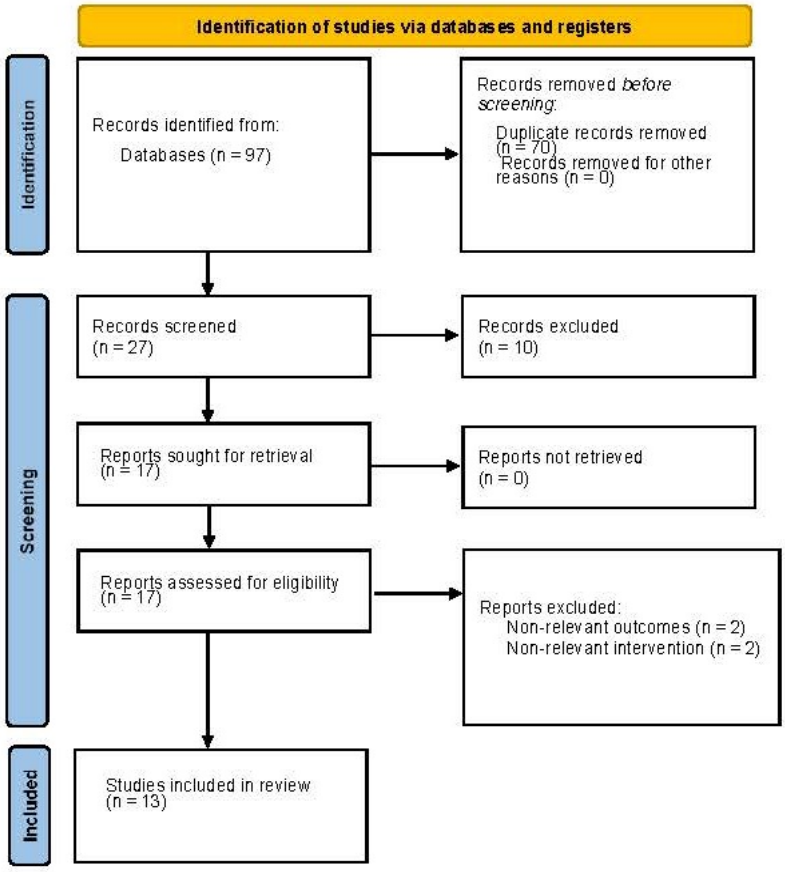

Figure 1. PRISMA flow diagram

Effects of HBOT on Recovery

The studies on post-exercise recovery suggest that hyperbaric oxygen therapy may have positive effects on various recovery parameters, including reduced lactate concentration, improved heart rate recovery, enhanced antioxidant capacity, and accelerated recuperation. Branco (30) investigating the effects of HBOT on post-training recovery in jiu-jitsu athletes suggested that HBOT could enhance post-training recovery processes in athletes, potentially reducing the time required for recuperation. Most recent study (29) highlighted the potential benefits of post-exercise hyperbaric oxygenation on improving recovery for subsequent performance. Earlier study (19) examined the effects of low-pressure hyperbaric oxygen treatment before and after maximal exercise on various recovery indicators, including lactate concentration, heart rate recovery, and antioxidant capacity. It indicated that HBOT could positively influence post-exercise recovery. Furthermore, Sueblinvong (18) investigated the relationship between hyperbaric oxygenation and blood lactate clearance in naval cadets, highlighting the potential of HBOT to aid in post-exercise recovery.

Effects of HBOT on Performance

There are controversial results regarding the performance. While some of the studies showed the potential for HBOT to influence physical performance positively, others did not find significant effects. This is mainly due to differences in participants, exercise type, intensity, and individual participants' characteristics. Hadanny (28) showed that HBOT had positive effects on mitochondrial respiration and physical performance in middle-aged athletes, suggesting a potential role in enhancing physical performance. Shimoda (25) suggested that HBOT could reduce muscle fatigue after intermittent exercise, potentially enhancing physical performance during high-intensity intermittent activities. Additionally, Sueblinvong (18) found improved lactate clearance that could contribute to enhanced physical performance capacity. Woo (27) hinted at

potential impacts on physical performance recovery after intense exercise, specifically in terms of reducing inflammation and muscle damage.

Discussion

The effects of hyperbaric chambers on recovery and performance are a topic of ongoing research with mixed results. The present study aims to identify and summarize relevant literature on hyperbaric oxygen therapy effects on performance and recovery. The main findings of this systematic review are that some studies show positive outcomes in terms of recovery and performance enhancement, while others did not find significant benefits. It is important to consider that the outcomes may vary based on factors such as the type of sport, the condition being treated, and the specific protocols used in the hyperbaric chamber. The mechanisms behind the potential benefits of hyperbaric oxygen therapy may involve improved tissue oxygenation, reduced inflammation, and enhanced recovery processes.

Effects of HBOT on Recovery

Collectively, these studies suggest that hyperbaric oxygen therapy may have a positive impact on recovery from soft tissue injuries, exercise-induced muscle damage, and post-training fatigue. However, the effectiveness of HBOT for recovery may depend on the specific injury or condition and the individual athlete. Studies on the effects of hyperbaric oxygen therapy on muscle damage provide mixed results. While some researchers, such as Webster (24) and Shimoda (25), suggest that HBOT may have positive effects on mitigating muscle damage and reducing fatigue, others, like Mekjavic (23), did not find significant benefits for recovery from delayed onset muscle soreness. Additionally, Woo (27) highlighted the potential for hyperbaric oxygen therapy to improve muscle recovery following intense exercise.

The enhanced recovery observed with hyperbaric oxygen therapy (HBOT) can be attributed to several potential mechanisms. HBOT exposes the body to increased atmospheric pressure, which results in higher oxygen levels being dissolved in the bloodstream. This enriched oxygen supply can promote more efficient oxygen delivery to tissues, aiding in the repair of damaged cells and tissues. HBOT has anti-inflammatory effects. By decreasing inflammation, it can help reduce the extent of swelling and pain associated with injuries or muscle damage, thereby expediting the healing process. Moreover, HBOT may stimulate the production of growth factors and enhance collagen formation. This can lead to more rapid tissue repair, benefiting athletes recovering from soft tissue injuries. Furthermore, the elevated oxygen levels and increased pressure associated with HBOT can improve blood flow. Enhanced blood circulation can transport vital nutrients and oxygen to damaged tissues and facilitate the removal of waste products. Improved oxygen delivery and circulation can help the body eliminate metabolic waste products more efficiently, potentially reducing post-exercise soreness and fatigue.

The results from these studies suggest that the effectiveness of hyperbaric oxygen therapy in mitigating muscle damage may depend on factors such as the type of muscle damage, exercise intensity, and specific HBOT protocols. Further research is needed to determine the optimal conditions and protocols for athletes seeking to utilize HBOT as a recovery strategy for muscle damage.

Overall Discussion on Performance

The studies on HBOT effects on physical performance provide some intriguing insights. Research studies by Hadanny (28) and Shimoda (25) suggest that HBOT might have positive effects on physical performance, potentially through mechanisms like reduced fatigue and enhanced mitochondrial respiration. Sueblinvong (18) indirectly implies that improved lactate clearance may contribute to enhanced physical performance. Additionally, Woo (27) pointed to potential benefits for physical performance recovery after intense exercise.

The potential mechanisms underlying the improvement in physical performance with HBOT are multifaceted and not yet fully understood. However, several mechanisms have been proposed based on existing research. One of the primary effects of HBOT is the delivery of higher concentrations of oxygen to tissues and cells. This enhanced oxygen availability can lead to improved aerobic and anaerobic energy production during exercise. The increased oxygen supply to muscles may delay the onset of fatigue and improve endurance. Additionally, HBOT has been suggested to reduce muscle fatigue and improve muscle function. The increased oxygen levels can help remove metabolic waste products such as lactic acid more efficiently, potentially delaying the onset of muscle fatigue and allowing athletes to maintain higher levels of exertion for longer periods. Furthermore, improved recovery after intense exercise is another mechanism. HBOT may reduce post-exercise muscle soreness and inflammation, allowing athletes to recover more quickly between training sessions or competitions. This can contribute to better overall physical performance. One more possible mechanism is through improved cellular function. HBOT may enhance mitochondrial function and increase ATP (adenosine triphosphate) production, which is critical for cellular energy. This can result in better overall energy levels, potentially improving physical performance.

While most studies indicate the potential for hyperbaric oxygen therapy to influence physical performance positively, it is crucial to consider that the effectiveness of HBOT can vary based on factors like exercise type, intensity, and individual athlete characteristics. Athletes and sports

professionals should carefully assess the relevance of HBOT to their specific physical performance needs and consult with experts to make informed decisions. Further research may be required to refine and validate its use for enhancing physical performance.

Study limitations

The main limitations of the study lie in a small sample of participants through the studies. Moreover, we did not separate the effects of a single session and the effects of several sessions. Furthermore, the participants were athletes but also non-athletes. Therefore, more research is needed to determine the optimal conditions and protocols for athletes to maximize the benefits of hyperbaric chambers in their training and recovery strategies.

Conclusion

Most of identified relevant studies showed improvements in performance and recovery. However, there are studies that showed no effects of hyperbaric chamber. In conclusion, the use of HBOT for recovery and performance is a promising field, but further research is required to establish standardized protocols and to better understand the specific conditions under which hyperbaric oxygen therapy can be most beneficial. Athletes and sports professionals should consider consulting with experts in the field to determine whether hyperbaric oxygen therapy is a suitable and effective option for their individual needs.

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Pregledni rad **UDC: 615.835:796.056.1**
doi: 10.5633/amm.2024.0309

UTICAJ HIPERBARIČNE KOMORE NA OPORAVAK I PERFORMANSE: PREGLEDNI RAD

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Potencijalni mehanizmi koji leže u osnovi poboljšanja fizičkih performansi hiperbaričnom terapijom višestruki su i još nisu u potpunosti shvaćeni. Stoga, cilj ovog sistematskog pregleda bio je da se identifikuje i sumira relevantna literatura o uticaju terapije hiperbaričnom komorom na oporavak i performanse. Da bi se identifikovale potencijalne studije, izvršena je sveobuhvatna pretraga u dvema elektronskim bazama podataka: *PubMed* i *MEDLINE*. Identifikovali smo 13 relevantnih studija sa ukupno 271 učesnikom – ukupno 249 muškaraca i 22 žene. Studije o oporavku nakon vežbanja sugerišu da hiperbarična terapija kiseonikom može imati pozitivne efekte na različite parametre oporavka, uključujući smanjenu koncentraciju laktata, poboljšani oporavak otkucaja srca, povećan antioksidativni kapacitet i ubrzanu rekuperaciju. Studije o efektima hiperbarične komore na fizičke performanse pružaju različite uvide. Iako većina studija ukazuje na potencijal hiperbarične terapije kiseonikom da pozitivno utiče na fizičke performanse, ključno je uzeti u obzir da efikasnost hiperbarične komore može varirati u zavisnosti od faktora kao što su tip i intenzitet vežbanja i individualne karakteristike sportiste. Upotreba hiperbarične komore za oporavak i performanse predstavlja polje koje obećava, ali su potrebna dalja istraživanja da bi se uspostavili standardizovani protokoli i da bi se bolje razumeli specifični uslovi pod kojima hiperbarična terapija kiseonikom može biti najkorisnija.

Acta Medica Medianae 2024; 63(3):71–79.

***Ključne reči**: oporavak, performanse, kiseonik, sportisti*

Review article

UDC: 615.461:616.31]:615.28
doi: 10.5633/amm.2024.0310

METHODS OF ASSESSMENT OF DIMENSIONAL STABILITY OF ELASTOMERIC IMPRESSION MATERIALS AFTER DISINFECTION: A LITERATURE REVIEW

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Precise and dimensionally stable impression materials are crucial for a good impression. Still, the precision of the impression and the accuracy of the reproduction depend on several factors, of which the most important one is the method of disinfection and the duration of the same. The purpose of this review article was to look at different recommended procedures for the disinfection of elastomeric dental impressions and the most commonly used methods and equipment for evaluating their dimensional stability after disinfection. To prepare this paper, we performed an electronic search of databases MEDLINE (Pub Med) and Google Scholar for articles published in the period from 2011 to 2022. Thirty-nine papers were selected for being the most current, relevant and focused on the disinfection of elastomeric materials for impressions as well as on methods and equipment applied to the evaluation of dimensional stability of elastomeric impressions after their disinfection. Our analysis showed that the most commonly used disinfectant materials were glutaraldehyde as well as sodium hypochlorite. Disinfection usually lasted for 10−15 minutes. Regarding the methods used to assess dimensional changes, the microscope was used in 26 papers out of a total of 39 papers. The review of the literature confirmed the non-standardization of the methodologies applied in the research and their great variety.

Acta Medica Medianae 2024;63(3):80−89.

Key words: *elastomeric impressions, disinfection, dimensional stability, tests*

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Introduction

Impression is a routine procedure in all branches of dentistry, especially in fixed and mobile prosthetics (1). It is essential to transfer the condition of the mouth to the model as reliably and accurately as possible (2). Precise and dimensionally stable impression materials are essential for a good impression. Still, the precision of the impression and the accuracy of the reproduction also depend on the way the material is handled, the storage conditions of the impression until the casting of the model, the time spent until the casting of the model, disinfection and its duration (3).

After taking the impression, the dentist must assess whether the impression is sufficiently precise and whether it reliably shows all the structures of the oral cavity. The impression must be taken from a high-precision material because it must faithfully depict all structures, precisely and in detail. Silicone impression materials are widely used due to their excellent physical properties, favourable handling properties and good patient acceptance. Dimensional stability is a characteristic of the impression that indicates the change of the impression after bonding the material to casting with plaster (4). The stability of dental materials refers to the possibility of registration without the influence of time, which gives the operator a chance to obtain an adequate impression (5). This characteristic is an essential requirement in dental and laboratory practice for obtaining accurate replicas and making prosthetic restorations (6).

Literature Review

Dental impressions, contaminated with the patient's blood and saliva, are a potential infection transmission route. Unfortunately, disinfection of

dental impressions was not a routine procedure for many years, but it began to be used on a large scale already at the end of the 20th century. Subsequently, to control the spread of diseases, the American Dental Association (ADA) and the World Dental Federation (FDI) recommended disinfection of dental impressions immediately after removal from the mouth, either by immersion or spraying, using disinfectant solutions (7, 8). The best-known solutions for impression disinfection are sodium hypochlorite, glutaraldehyde, iodine preparations, phenols and chlorhexidine digluconate. In addition, new disinfection methods have been introduced, such as microwave chambers, autoclaving chambers, ultraviolet (UV) light, and ozone. Dimensional stability and precision of two silicone impression materials after chemical disinfection by immersion in disinfectant were investigated. Pronounced changes in the dimensions of all samples were determined as a function of time as well as in the function of the application of the disinfectant. Measurements were taken sequentially using a Canon G9 camera with the Remote Capture software options so that time series photographs of the same print were obtained (9).

The effect of the disinfection method and storage of the tested samples for 6 months after pre-disinfection of one group and sterilization in an autoclave of the other group were investigated and the authors concluded that there were no clinically significant changes in the dimensions of the samples during the storage period using stereomicroscope measurements (10).

The effects of different disinfection methods (UVC, gaseous ozone, commercial solution and spray) on dimensional change in elastomeric materials with different viscosities were compared by Vezgoviec et al. (11). Their results revealed that additional silicones had greater dimensional stability. The study also revealed that similar to standard liquid disinfectants, both UVC and ozone did not affect the physical properties of most silicones. Examination of the changes was performed with Magnuson digital caliper.

Nassar et al. (12) investigated the dimensional stability of 5 consistencies (VPES) stored for up to 2 weeks, with and without the use of a standard disinfection procedure using a microscope at 30x magnification.

According to Guiraldo et al. (13), polysulfide (non-disinfected), polysulfide and polydimethylsiloxane (after disinfection with 0.2% chloramine-T) showed lower mean values of dimensional stability. They came to this conclusion by investigating the stability of four elastomeric materials after disinfection with 0.2% chloramine-T. The entire procedure was performed following the ISO 4823 standard and using an optical microscope.

Soganci et al. (14) compared the dimensional changes of polyether and vinyl polyether siloxane impression materials immersed in two different disinfectants sodium hypochlorite (5.25%) and glutaraldehyde (2%), over three time periods with a 10-micron 3D scanner and 3D software used to assess dimensional changes by superimposition.

Samra et al. (5) analyzed the effect of different disinfection systems on the dimensional stability of commonly used irreversible hydrocolloid and additive silicone impression materials from developing countries compared to materials from developed countries. The disinfectants used were glutaraldehyde, sodium hypochlorite and an ultraviolet chamber, and were examined using a travelling microscope.

Aim

The purpose of this review and study was to review the various recommended disinfection procedures for elastomeric dental impressions and the most commonly used methods and equipment to assess their dimensional stability after disinfection.

Material and Methods

To prepare this paper, we performed an electronic search of MEDLINE (PubMed) and Google Scholar databases and retrieved articles published in the period from 2011 to 2022 using the following keywords: disinfection impression, disinfection method, dimensional stability of impression, type of measurement. From the consulted 45 articles, 39 were selected, the most current, relevant and focused on the disinfection of elastomeric impression materials and methods, and equipment used to evaluate the dimensional stability of elastomeric impressions after their disinfection. *In vitro* studies were of particular interest.

The criteria used to confirm whether a study met the conditions for analysis included in vitro studies focusing on elastomeric materials frequently used in dentistry, the study authors must have followed the protocols of the International Organization for Standards (ISO) 4823 or American Dental Association (ANSI/ADA) Specification No. 19, the materials must have undergone disinfection with the control group consisting of non-disinfected materials, the effect of disinfection on dimensional changes should be explored, and the applied methods evaluated.

Information Analysis

This review included 39 studies related to applied methods for the evaluation of dimensional changes of elastomeric impression materials after their disinfection. Among several methods used for impression disinfection, the chemical immersion method was most commonly used over other methods including microwave irradiation for 3 minutes, steam autoclave for 15 and 30 minutes, UV light for 3, 20 and 40 minutes, disinfection with

ozone and EOV (electrolyzed oxidizing water) for 5, 10 and 20 minutes.

Our analysis showed that the most commonly used disinfectants were glutaraldehyde 0.5%, 2%, 2.25%, 2.5% as well as sodium hypochlorite 0.5%, 1%, 2%, 3%, 4%, 5% and 5.25%. Disinfection lasted from 30 seconds to 5, 10, 15, 20, 30 minutes and 1, 2, 3, 12, 16 and 24 hours. Disinfection usually took 10 to 15 minutes. Regarding the methods used to assess dimensional changes, the microscope was used in 26 papers out of a total of 39 papers. Other authors used other methods.

Table 1. Summary of selected studies indicating the methods for the examination of the effect of disinfection on dimensional stability of different elastomeric impression materials

Autor-Year	Impression material	Disinfection material	Time	Type of measurements	Property Investigated
Carvalhal et al. 2011	PDS, PVS, PS, PE	0.5% NaOCL, 2% glutaraldehyde	10, 20, 30, 60 min	Microscope	Dimensional stability
Surendra et al. 2011	Polyvynilsiloxane	Autoclave 121 °C	15 min	Microscope	Dimensional stability
Ahila et al. 2012	PVS	2.25% glutaraldehyde 5% povidone iodine, 4% NaOCL	10, 30 min 1 h	Microscope	Dimensional stability
Hiraguchi et al. 2013	A-Silicone	2% glutaraldehyde 0.55% ortho-phthalaldehyde	30 min 24 h	Laser scan micrometer	Dimensional stability
Nandini et al. 2013	A-Silicone	2% glutaraldehyde	30 min	Video vision measuring microscope	
Ahila et al. 2014	A-Silicone	2.45%glutaraldehyde, 4% NaOCL			
	C-Silicone	5% povidone iodine	10 min	Travelling microscope	Dimensional stability
Duseja et al. 2014	PE, A-Silicone	Dual phenol	10 min 1 h		Dimensional stability
		2% glutaraldehyde	10 min 1 h	Microscope	
		0.5% sodium hypochlorite	10 min 1 h		
Thota et al. 2014	A-Silicone, C-Silicone, PE	Autoclave	24 h	Stereomicroscope	Dimensional stability
Millar et al. 2014	Silicone(Affinis, Aquasil,	Autoclave 134 °C	30 min	Non-contact scanner	Dimensional stability
	Speedex)	2% Perform-ID solution	10 min		
Sinobad et al. 2014	A-Silicone	0.5% glutaraldehyde benzalkonium-chloride-Sterigum	10 min	Canon G9 camera	Dimensional stability
	C-Silicone	5.25% NaOCl			

Pal et al. 2014	PE, A-Silicone	1% NaOCL, 4% NaOCL 2% glutaraldehyde	10 min	Microscope	Dimensional stability
Goodbole et al. 2014	PVS	UV	10 min	Travelling microscope	Dimensional stability
Kamble et al. 2015	PVS, CS, PE	Autoclave, Microwave irradiation 1% NaOCl	10−15 min	Microscope	Dimensional stability
Khinnavar et al. 2015	PVS, PE, A-Silicone	2% glutaraldehyde, 0.525% sodium hypochlorite	16 h	Leica WILD stereomicroscope	Dimensional stability
Hiragushi et al. 2015	A-Silicone	2% glutaraldehyde 0.55% ortho-phthalaldehyde	30 min 24 h	Three-dimensional coordinate Measuring system	Dimensional stability
Demajo et al. 2016	A-Silicone(PVS)	Glutaraldehyde MD520 alcohol	3 h	Scanning microscope	Dimensional stability
Sinobad T. 2016	A-Silicone, C-silicone	5.25% NaOCL, glutaraldehyde	5, 30, 60 min	Microscope	
	PE	Benzalkonium chloride Ethanol, isopropyl alcohol	24 h	Photometry	Dimensional stability
Nassar et al. 2017	VPES	2.5% buffered glutaraldehyde	30 min	Microscope	Dimensional stability
Guiraldo et al. 2018	PS, PE, PVS Polydimethylsiloxane	2% NaOCl 2% chlorhexidinedigluconate 0.2% peracetic acid	15 min	Microscope	Dimensional stability
Soganci et al. 2018	PE, VPS	5.25% NaOCl, 2% glutaraldehyde	10 min	3D scanner + 3D software	Dimensional stability
Samra et al. 2018	A-Silicone	2% glutaraldehyde, 5.25% NaOCl UV 254 nm frequency	10 min 3 min	Microscope	Dimensional stability
Guiraldo et al. 2018	PDS, PVS, PS, PE	0.2% chloramine-T	15 min	Scanning Tunneling Microscope	Dimensional stability
Azevado et al. 2019	A-Silicone	3% hydrogen peroxide MD 520	10 min 5 min	Visualised with a magnifier glass (Wild/Leica M420), photographed and	Dimensional stability

		1% NaOCl, 5.25% NaOCl	10 min	analysed	
Mahalakshmi et al. 2019	PVS	2% glutaraldehyde,		Microscope	Dimensional stability
		1% NaOCl electrolysedoxidising water EOW	10 min		
Ghasemi et al. 2019	A-Silicone	0.5% NaOCl, Epimax, Deconex	10 min	Digital caliper with 0.01 mm accuracy	Dimensional stability
Nimonkar et al. 2019	PVS	2% glutaraldehyde, 1% NaOCl	20 min	Microscope	Dimensional stability
		UV light	20 min		
Ozdemir et al. 2019	A-Silicone, C-Silicone	1%sodium hypochlorite	10 min	Digital radiography	Dimensional accuracy
	PE	Aldehyde-free disinfectant sol.	3 min		
		Zeta 7 spray			
Khatri et al. 2020	PVS, PE, VPES	2.45% glutaraldehyde	15 min 12 h	Stereomicroscope	Dimensional stability
		3% sodium hypochlorite		Digital Vernier caliper	Surface Detail Reproduction
Asopa et al. 2020	PVS	2% glutaraldehyde	30 min	Travelling stage microscope (NIKON profile projector)	Dimensional stability
		heat sterilization	15 min		
Yousief et al. 2020	A-Silicone	2% glutaraldehyde	30 min	Travelling microscope	Dimensional stability
Vrbova et al. 2020	Variotime Medium Flow Xantopren L Blue	Aseptoprint Liquid	2 min	Light microscope Scanning electron microscope Micro-computed tomography	Dimensional stability
		Zeta 7 solution	10 min		
	Impregum Soft	Silosept	10 min		
		Dentaclean Form	15 min		
Mohd et al. 2021	PVS, VSE	Silosept	10 min	Image analyzer at 20x magnification	Dimensional change
		Microwave Irradiation	3 min		
Sana et al. 2021	A-Silicone	0.2% chlorhexidine	5–10 min	Stereomicroscope	Dimensional accuracy
	C-Silicone	5.25% NaOCL		Digital Vernier caliper	
	Polyether	2% povidone iodine			
		Ozone water			
		Running tap water			

Kuei-Ling Hsu. 2021	PVS	Birex SE Opti-Cide 3 COEfect Minute Spray CaviCide spray	5 min	Computed tomography (CBCT)	Dimensional stability
Abdelhameed et al. 2021	PVS	0.5% sodium hypochlorite	10 min	Three-dimensional analysis computed tomography	Dimensional stability
Alam et al. 2021	Panasil	1% Surfosept 2% Deconex	30 sec	Profile projector	Dimensional stability
Wezgowiec et al. 2022	A-Silicone	UVC	40 min, 254 nm	Magnuson digital caliper	Dimensional change
	C-Silicone	Ozone	10 min		
		Zeta 7 solution	10 min		
		Zeta 7 spay	10 min		
Almuraikhi et al. 2022	VPS, PE	2% glutaraldehyde	30 min		
		5.25% NaOCL	20 min	Stereomicroscope 20x	Dimensional stability
Ud Din et al. 2022	3 PVS (experimental) 5 PVS (commercial)	1% NaOCl	30 min 24 h	Travelling microscope	Dimensional change

*PE- Polyether, PVS- Polyvinyl siloxane, PVES- Polyvinyl ether silicone, VSE- Vinyl siloxanether, VPS- Vinyl polysiloxane, PDS- Polydimethylsiloxane

Discussion

Numerous methods are described in the literature for testing the dimensional stability of elastomeric impression materials depending on various factors. The most famous measurement technique is the one set by the International Organization for Standards (ISO 4823), i.e. American Dental Association (ANSI/ADA Specification No. 19). These standards set the most recognized performance specifications for elastomeric impression materials. The first measurements to assess the dimensional stability of the impression material were performed using a microscope and a micrometer screw. During testing, elastomeric materials were used and an impression was taken from a model that is the same or similar to that described in the reference standards, as well as by taking an impression from different master models that simulate prepared teeth. Measurements were made on elastomeric impressions or plaster models obtained from the impressions. A micrometer screw is a measuring instrument with an accuracy of up to 0.01 mm, while digital indicator instruments have an accuracy of 0.005, 0.002 and 0.001 mm. The micrometer screw is an integral part of many measuring instruments, for example, microscopes and telescopes.

A microscope is a more precise device for 2D measurements. There are many types of measuring microscopes, from the simplest to digital microscopes connected with measuring

software. A profile projector is a measuring instrument that projects an enlarged image and compares it with an enlarged drawing on transparent paper. A coordinate measuring device is a measuring instrument used for spatial measurement in all three coordinate axes (k, i, z).

Radiographic methods (CT and CBCT) are rarely used in research to analyze prints and patterns.

Desktop digital scanners, or laboratory scanners with associated software packages allow us to perform quality analysis and comparison of impressions and cast models.

The effect of chemical disinfection on impression materials depends on the method and duration of disinfection, the type and concentration of the disinfectant and the type of impression material. In general, disinfection affects not only dimensional stability but also affects the humidity of impression materials, as well as the surface quality of plaster models.

Regarding the methods used to investigate the effect of disinfection on the dimensional stability of elastomeric materials, we concluded that the most frequently used is microscope (5, 6, 13, 15−23); then stereomicroscope (1,24−27); travelling microscope (8, 28−31); scanning microscope (32,33). Other methods include: microscope photometry (34); video vision measuring microscope (35); laser scanning micrometer (36); a noncontact scanner (37); Canon G9 camera (9); three-dimensional coordinate measuring system (38); 3D scanner+3D software (14); visualized with a magnifier glass (Wild/Leica M420), photographed and saved for later analyses with the Image (7); digital caliper with 0,01 mm accuracy (11, 39); digital radiography (40); image analyzer (41); computed tomography (42); three-dimensional analysis computed tomography (43); and profile projector (44).

There is no exact provision in the literature regarding which measuring device should be used in assessing the dimensional stability of elastomeric impression materials. Because of the lack of standardization, it is difficult to compare such studies. Microscopes and calipers are most often used as measuring instruments. Conventional print evaluation methods are mainly two-dimensional methods in which linear accuracy is evaluated by measuring the distance between arbitrarily selected points with various measuring devices in order to prove the material's expansion or contraction.

More modern measurement techniques are available today, such as various measurement software that use digital photographs of specimens or laser scanners to measure digitized impressions and the model. Even so, the most common measurements are still those using a microscope. The method and the use of print evaluation system are limited. They depend on the capabilities of the therapist or researcher, the equipment of the office or laboratory, or the availability of different assessment methods. Limiting factor is the price. In research, the choice of systems and methods for the evaluation of prints depends on the needs of the research, on their goals.

The effect of chemical disinfection on impression materials depends on the method and duration of disinfection, the type and concentration of the disinfectant and the type of impression material. In general, disinfection affects not only dimensional stability, but also affects the humidity of impression materials, as well as the surface quality of plaster models.

According to ADA specifications, elastomeric impressions should not produce more than 1.5% dimensional change (6).

Dimensional stability is the ability of a material to maintain its three-dimensional size and shape over time, under appropriate humidity and temperature conditions. Disinfectants can produce a chemical or physical interaction with impression materials, which can effect on their dimensional stability (7).

Dimensional changes can occur in plaster models as a result of the inherent characteristics of the impression material, such as wetting and viscosity. Other possible causes may be the thickness of the material between the oral tissue and the impression tray, hydrophilicity of the material, loss of by-products, polymerization shrinkage and thermal shrinkage due to temperature (13).

Examining dimensional changes on elastomeric materials, under the influence of the immersion period in two different disinfectant solutions (sodium hypochlorite 0,5% and 2% glutaraldehyde) resulted with conclusion that this combination can be used in the dental office as a disinfection method for period of 20 minutes, without interfering the dimensional stability of the impression materials (15).

In a survey conducted to investigate the dimensional stability of elastomeric materials by method of cold sterilization and immersion, the obtained results showed that PVS with thick consistency showed the highest dimensional stability, while polyether showed the lowest dimensional stability (24).

Dimensional changes of plaster casts after immersing the impression taken with a hydrophilic additive silicone with medium viscosity in disinfectant solutions for 30 min and 24 h were investigated. It was determined that the dimensional changes in the models caused by the immersion of the impressions were less than 15 mm. A three-dimensional coordinate system was used for the evaluation (36).

Conclusion

The review of the literature confirmed the non-standardization of the methodologies applied in the research and their great variety.

The most commonly used methods for disinfection are immersing or spraying the impression with disinfectant. The most commonly used disinfectants are solutions of 2% glutaraldehyde and 0.5% and 1% sodium

hypochlorite. The immersion duration for elastomeric impressions in disinfectant is from 10 to 30 minutes.

Regarding the methods used to evaluate dimensional changes, the most commonly used microscopes are light microscope, traveling microscope, scanning electron microscopy, scanning microscope, tool microscope, scanning tunneling microscope and stereomicroscope.

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Pregledni rad **UDC: 615.461:616.31]:615.28**
doi: 10.5633/amm.2024.0310

METODE PROCENE DIMENZIONALNE STABILNOSTI ELASTOMERNIH MATERIJALA ZA UZIMANJE OTISAKA NAKON DEZINFEKCIJE: PREGLED LITERATURE

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Precizni i dimenzionalno stabilni materijali za uzimanje otisaka ključni su za uzimanje dobrog otiska, ali preciznost otiska i tačnost reprodukcije zavise i od još nekoliko faktora; jedan od najvažnijih je način dezinfekcije i njeno trajanje. Svrha ovog preglednog rada bila je da se sagledaju različite preporučene procedure za dezinfekciju elastomernih dentalnih otisaka i najčešće korišćene metode, kao i oprema za ocenu njihove dimenzionalne stabilnosti nakon dezinfekcije. Za potrebe ovog istraživanja izvršili smo elektronsku pretragu *MEDLINE (*PubMed) i baze podataka (*Google Scholar*) i pregledali smo članke objavljene u periodu od 2011. do 2022. godine. Odabrano je 39 najaktuelnijih i relevantnih studija fokusiranih na dezinfekciju elastomernih materijala za uzimanje otisaka, te na metode i opremu primenjene pri proceni stabilnosti dimenzija elastomernih otisaka nakon njihove dezinfekcije. Naša analiza je pokazala da su najčešće korišćeni dezinfekcioni materijali gluteraldehid i natrijum-hipohlorit. Dezinfekcija je obično trajala od deset do petnaest minuta. Kada je reč o metodama korišćenim za procenu dimenzionalnih promena, zapaženo je da je 26 autora od njih 39 najčešće upotrebljavalo mikroskop. Pregledom literature potvrđena je nestandardizacija metodologija primenjenih u istraživanju i njihova velika raznovrsnost.

Acta Medica Medianae 2024; 63(3):80–89.

***Ključne reči**: elastomerni otisci, dezinfekcija, stabilnost dimenzija, ispitivanja*

Case report **UDC: 616.16-002:[616.98:578.834**
doi: 10.5633/amm.2024.0311

AN ADULT WITH HENOCH–SCHÖNLEIN PURPURA SECONDARY TO CORONAVIRUS DISEASE INFECTION

Vesna Karanikolić [1,2], Maša Golubović [1] , Hristina Kocić [1,2]

Previous upper respiratory tract infection has been identified as the most common factor causing Henoch–Schönlein purpura (HSP). The most common causes of infection are streptococci, followed by viral infections. Upper respiratory tract infection with coronavirus disease (COVID-19) could be an HSP-triggering virus.

We present a case of a 39-year-old male who developed HSP in the setting of COVID-19 infection. HSP occurred 14 days after COVID-19 diagnosis and it exhibited itself in the form of the lower extremities and buttocks palpable purpura, lower abdominal pain, nausea and hematuria. The patient was treated with methylprednisone, and meprednisone, which led to rapid clinical improvement. Endothelial damage in patients with COVID-19 viral infection occurs as a consequence of a severe inflammatory reaction. Extremely important place in the inflammatory reaction of the endothelium is occupied by IgA, which can be deposited within the endothelium. This activates other cytokines that can lead to HSP occurrence.

Acta Medica Medianae 2024;63(3):90–93.

Key words: *coronavirus disease, Henoch–Schönlein purpura, adults*

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Introduction

Henoch–Schönlein purpura (HSP) belongs to the group of small blood vessel vasculitis caused by the deposition of the IgA-immune complex. Non-thrombocytopenic palpable purpura, abdominal pain, arthritis and renal involvement are clinical tetrads characterized by Henoch–Schönlein purpura (1). Children account for over 90% of all cases, while in adults it occurs much less frequently with a prevalence of 3.4 to 14.3 cases per million. Such a low incidence in adults may be due to misdiagnosis or insufficient diagnosis (2).

The COVID-19 pandemic was initially described as the common cold. Still, later the clinical picture changed with the appearance of many other clinical symptoms, of which pneumonia occupies the most significant place. Numerous biological abnormalities occur in patients with COVID-19 infection, including hypoxemia, hyperinflammation, hemophagocytosis, high creatinine levels, electrolyte imbalance, disorders of the renin-angiotensin-aldosterone system and cytopenia (3). Consequently, COVID-19 infection can lead to various complications affecting the cardiovascular, respiratory and other systems as well as the skin (4).

We present a case of a 39-year-old male who developed Henoch–Schönlein purpura in the setting of COVID-19 infection.

Case Presentation

A 39-year-old patient with a fever that lasted for two weeks, dry cough, shortness of breath, sore throat, diarrhoea and headache was referred to the Clinical Centre Niš, Serbia for treatment. COVID-19 infection was confirmed by PCR after nasopharyngeal and oropharyngeal swabs.

At the patient's admission, the body temperature was 37.4 °C, respiratory rate was 40 breath/min, pulse rate was 106 beats/minute and blood pressure was 140/90 mmHg. Radiological examination diagnosed bilateral severe COVID-19 pneumonia.

Upon admission to the hospital, the patient received the following therapy: intravenous Ceftriaxone 1 gram every 12 hours, azithromycin 500 mg/24h, lopinavir/ritonavir 400 mg every 12 hours, vitamin C 200 mg every 8 hours, zinc

sulphate 220 mg/24 h and subcutaneous low molecular weight heparin of 40 mg daily. The patient was admitted to intranasal oxygen therapy at the rate of 5 litres/min.

On the tenth day from the beginning of hospitalisation, a skin rash appeared, and it was characterised by non-pruritic palpable purpura over the buttocks, lower extremities, upper extremities and the trunk of the body, showing signs of several weeks duration (appearance of the eruption is shown in Figures 1A and 1B). This was accompanied by intermittent, crampy lower abdominal pain, nausea and hematuria. The patient had a fever and arthralgia. There were no clinical signs of superficial thrombophlebitis or deep vein thrombosis.

The absence of thrombocytopenia was confirmed on several occasions by haematological examination. Microscopic haematuria and proteinuria were confirmed by urinalysis.

The value of serum IgA was 787 mg/dL (normal range of 70–400 mg/dL), while tests for vasculitis, and lupus were negative. Other diagnostic methods were not used because the conditions in the COVID hospital did not allow it.

For that reason, the patient started treatment with IV methylprednisolone, 3 g (1 g/day for 3 days), and then meprednisone, 1 mg/kg/day for 10 days. After the applied therapy, the clinical picture improved as well as kidney function and urinary sediment analysis.

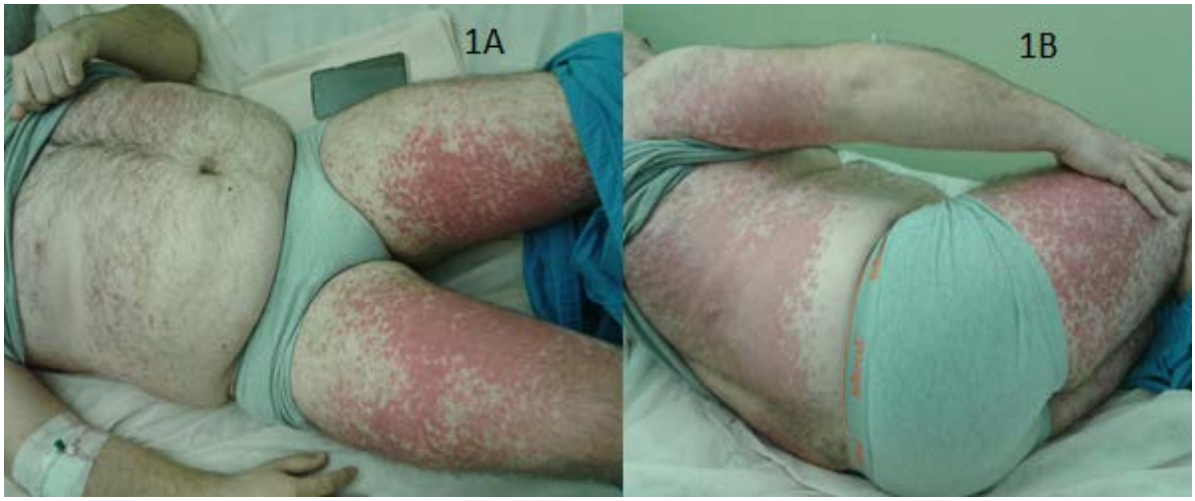

Figure 1. Purpuric rash on the lower limbs of the patient (1A); Purpuric rash on the trunk of the body (1B)

Discussion

Despite a great deal of research, the aetiology of Henoch–Schönlein purpura has not been adequately elucidated to date, however, there are suggestions that several causative agents may be responsible for its occurrence (5). Previous upper respiratory tract infection has been identified as the most common factor causing Henoch–Schönlein purpura. The most common cause of infection is streptococcus, followed by viral infections (6). Upper respiratory tract infection COVID-19 could be an HSP-triggering virus.

IgA vasculitis associated with COVID-19 infection in adults has been reported in several studies. Suso et al. (6) showed the presence of cutaneous vasculitis, nephritic syndrome, and arthritis three weeks after respiratory infection due to PCR-confirmed COVID-19 infection in a 78-year-old man.

Allez et al. (7) describe a 24-year-old man with cutaneous, musculoskeletal and gastrointestinal manifestations of HSP. The patient had an asymptomatic form of COVID-19 infection that was confirmed after PCR testing. Both patients responded successfully to systemic corticosteroid therapy.

IgA vasculitis and COVID-19 infection have also been reported in children. AlGhoozi and AlGhoozi. (8) reported on the clinical presentation of HSP in a four-year-old child who had recently recovered from COVID-19 upper respiratory tract infection.

When the SARS-CoV-2 virus enters the human cell, a systemic inflammatory response is subsequently induced. SARS-CoV-2 IgA is the first immunoglobulin to rise following infection, which may indicate the possible connection between IgA vasculitis and COVID-19 infection (8).

According to the available data, the presence of COVID-19 infection and HSP in adults is exceptionally rare. The published work referred mostly to children and the younger population.

Our patient had the clinical signs and symptoms of HSP, meeting the criteria set forth by the European League Against Rheumatism, the Paediatric Rheumatology International Trials Organization and the Paediatric Rheumatology European Society (9) in 2010.

The patient had non-pruritic palpable purpura predominantly on the lower torso and legs, abdominal pain, arthralgia and impaired renal function.

The IgA serum had a high value, as well. Based on the presented data, it appears that there is a causal relationship between COVID-19 and postinfectious vasculitis.

Conclusion

Endothelial damage in patients with COVID-19 viral infection occurs as a consequence of severe inflammatory reaction. The extremely important place in the inflammatory reaction of the endothelium is occupied by IgA which can be deposited within the endothelium. This activates other cytokines that can lead to HSP occurrence.

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Prikaz bolesnika **UDC: 616.16-002:[616.98:578.834**
doi: 10.5633/amm.2024.0311

HENOH–ŠENLAJNOVA PURPURA KAO POSLEDICA INFEKCIJE COVID-19

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Prethodne infekcije gornjeg respiratornog trakta najčešći su faktor nastanka Henoh–Šenlajnove purpure (*Henoch–Schönlein purpura* – HSP). Najčešći uzročnici infekcije su streptokoke, a za njima slede virusne infekcije. Infekcija gornjih disajnih puteva koju je izazvao COVID-19 mogla bi biti predisponirajući faktor koji izaziva HSP.

Predstavljamo slučaj tridesetdevetogodišnjeg muškarca koji je razvio HSP tokom infekcije COVID-19. HSP se javio 14 dana nakon dijagnoze COVID-19 i ispoljio u vidu palpabilne purpure donjih ekstremiteta i zadnjice, bolova u donjem delu abdomena, mučnine i hematurije. Bolesnik je lečen metilprednizonom i meprednizonom i to je dovelo do brzog kliničkog poboljšanja. Oštećenje endotela kod bolesnika sa virusnom infekcijom COVID-19 nastaje kao posledica teške upalne reakcije. Izuzetno važno mesto u inflamatornoj reakciji endotela zauzima imunoglobulin A (IgA), koji se može deponovati unutar endotela. Ovo aktivira druge citokine koji mogu dovesti do pojave HSP-a.

Acta Medica Medianae 2024; 63(3): 90–93.

***Ključne reči**: koronavirusna bolest, Henoh–Šenlajnova purpura, odrasla osoba*

Case report

UDC: 616-006.44:616.85
doi: 10.5633/amm.2024.0312

PARANEOPLASTIC NEUROLOGICAL SYNDROME IN A PATIENT WITH HODGKIN LYMPHOMA

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Cancer patients can develop paraneoplastic neuropathy, which cannot be explained by tumors, metastases, infections, or side effects of cancer treatment. We present a case of a 38-year-old patient of male gender with weight loss, night sweats, and weakness. He exhibited sensory loss, paresthesias, and allodynia in both lower extremities. White blood cells were 20 x 10^9/L, and C-reactive protein was 40 mg/L. Viral markers indicated no signs of an active infection. Ultrasonography showed several peripheral lymph nodes with reduced echogenicity. Lung computed tomography detected aggregated lymph nodes in jugular regions and mediastinum. The physical examination revealed swollen lymph nodes in the right supraclavicular region. Brain and spinal magnetic resonance were normal. Cerebrospinal fluid cytology ruled out infectious and malignant involvement. Nerve conduction studies revealed decreased amplitude in the lower extremities, with the inability to elicit sensory and motor responses. Nerve conduction studies revealed decreased amplitude in the lower extremities, with the inability to elicit sensory and motor responses and diminished F response. The patient had symmetrical, ascending neuropathy with absent deep tendon reflexes, indicating sensory-motor polyneuropathy. The biopsy of the lymph node confirmed mixed cellularity Hodgkin lymphoma. The patient started on a chemotherapy regimen including doxorubicin, bleomycin, vinblastine, and dacarbazine. Intravenous immunoglobulins were administered. Partial improvement was noted, with prolonged physical therapy. When neurological symptoms are associated with a tumor or positive onconeural antibodies, paraneoplastic neuropathy can be diagnosed. Timely recognition is crucial since any delay in treatment can be detrimental.

Acta Medica Medianae 2024;63(3): 94–99.

Key words: *lymphoma, cancer, neuropathy, onconeural antibodies*

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Introduction

Hodgkin lymphoma is a monoclonal lymphoid neoplasm characterized by an excellent prognosis. Neurological symptoms linked to different lymphoma subtypes can appear at any stage of the disease, impacting various parts of the nervous system. In Hodgkin lymphoma, the involvement of the peripheral nervous system is a key aspect.

Neurologic symptoms in Hodgkin lymphoma may arise from nervous system invasion due to chemotherapy and radiotherapy, mass compression, infection, or as paraneoplastic neuropathy (1). Paraneoplastic neuropathies, occurring in cancer patients, lack direct and localized consequences of the underlying tumor and are not attributed to metastasis, opportunistic infections, or adverse events of the treatment (1, 2). The prevalence of paraneoplastic neuropathy in Hodgkin and other lymphomas is less than 1% (3).

Hodgkin lymphoma is linked to distinct paraneoplastic conditions, such as primary central nervous system angiitis, limbic encephalitis, and degeneration of the cerebellum (3). Early detection of the underlying tumor is crucial for improving or stabilizing paraneoplastic neuropathy.

This work aims to present a case study of a patient who developed sensorimotor neuropathy during the early stages of Hodgkin's disease, emphasizing the importance of timely tumor detection.

Case report

We present a case of a 38-year-old patient of male gender, with weight loss, night sweats, and weakness, with complaints that started gradually and asymmetrically, and pains and tingling in the distal parts of the hands. Soon after, severe pain, paresthesias, allodynia, and exhibited sensory loss in the lower extremities begin, in both lower extremities. He complained of asymmetric numbness in the lower limbs, as well as gastrointestinal dysmotility. Detailed examinations were undertaken. The physical examination revealed swollen lymph nodes in the right supraclavicular region. Paraneoplastic etiology was suspected in this patient. In laboratory findings, white blood cells were elevated (20 x 10^9/L), and C-reactive protein 40 mg/L. Viral markers indicated no signs of an active infection. Ultrasonography showed several peripheral lymph nodes with reduced echogenicity. Ultrasonographic observations indicated multiple hypoechoic peripheral lymphadenopathies. Abdomen and lung computed tomography revealed detected aggregated lymph nodes in jugular regions, anterior chest wall, mediastinum, and abdominal para-aortic lymphadenopathy (Figures 1 and 2).

The biopsy of the lymph node confirmed mixed cellularity Hodgkin lymphoma. A neurological examination indicated significant limb weakness, a small reduction in vibratory and joint position sensations, areflexia, and the involvement of pinprick, temperature, and light touch sensations with severe joint position and vibratory

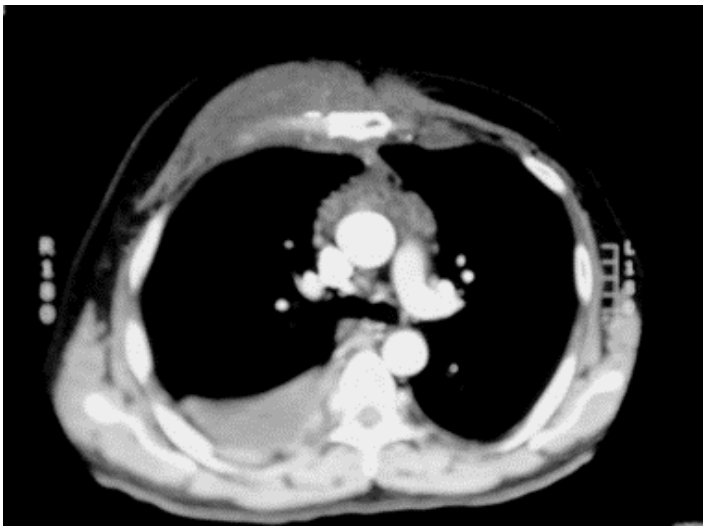

Figure 1. Thoracic computed tomography shows mediastinal lymph nodes, tumor masses in the anterior chest wall, and conglomerated lymph nodes in jugular chains

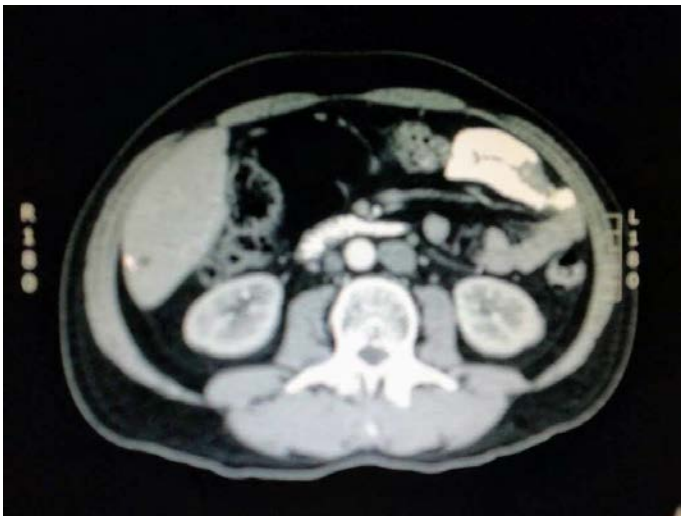

Figure 2. Abdominal computed tomography (para-aortic lymph nodes)

impairment. He exhibited symmetrical ascending neuropathy and deep tendon reflexes were absent. There were no respiratory complaints, and the cranial nerves were normal. Electrical tests revealed reduced distal motor delay and sensory-evoked potentials with standard sensory speed and marginally reduced motor conduction velocities. Nerve conduction investigations in the lower extremities revealed reduced amplitude, with the inability to elicit sensory and motor responses. The F reaction was decreased. The patient had rapidly progressive sensory neuropathy. Cerebrospinal fluid cytology was used to rule out infectious and malignant involvement. Analysis of the cerebrospinal fluid revealed a protein level of 327 mg/dL and no leukocytes. Brain and spinal magnetic were normal. Laboratory tests were positive on serum anti-Hu antibodies, and the other onconeural antibodies were negative. The findings supported sensorimotor neuropathy. Immunomodulatory therapy for neuropathy was administered alongside antineoplastic treatments. Intravenous immunoglobulin was given at a dosage of 400 mg/kg/day for 5 days. A chemotherapy regimen consisting of vinblastine, bleomycin, doxorubicin, and dacarbazine was started. Partial improvement was noted following the initial treatment, and the patient continued with prolonged physical therapy to enhance recovery and support overall function. After being in remission for eight months, the patient suffered paresthesias and gradual weakening over two weeks three weeks after an upper respiratory tract infection occurred. The clinical examination indicated mild to moderate fatigue with just minor sensory impairments. During the monitoring period, identical episodes occurred once more (24 months).

Discussion

Paraneoplastic neuropathies typically manifest before cancer diagnosis or in the early stages, allowing for potential treatment interventions, although they can also develop post-cancer diagnosis (2). These neuropathies may selectively target specific neuron types, leading to pure motor, sensory, or autonomic neuronopathies (4). The majority of cases involve autoimmune mechanisms (1, 2), where an autoimmune reaction develops due to shared antigenic characteristics between the underlying tumor and the nervous system. While antibodies targeting neural antigens have been identified in paraneoplastic neuropathy cases, it is noteworthy that the disorder can occur without the presence of antibodies (5, 6). The complexity of the processes underlying paraneoplastic neuropathies extends beyond the known onconeural and neuronal surface antibodies.

Lymphomas, stemming from abnormal lymphoid cell growth, can give rise to tumors (1). Neurologic manifestations associated with Hodgkin lymphoma are infrequent and are typically observed in advanced stages of the disease (1). Neurologic abnormalities in Hodgkin lymphoma may arise from nervous system invasion due to chemotherapy and radiation, mass compression, infection, or as paraneoplastic neuropathy (7). Considering the underlying immunological disturbance, autoimmune origins are more likely for peripheral neuropathy in Hodgkin lymphoma. In this context, subacute sensory neuronopathy is the type of paraneoplastic neuropathy that occurs most frequently, often presenting with a range of sensory deficits and neuropathic pain (8).

Notably, neurologic neuropathies in the presence of a tumor should not automatically be classified as paraneoplastic syndromes (1). A diagnosis of paraneoplastic neuropathy is established when the disease is associated with a tumor or when onconeural antibodies are detected (1). In our patient, the clinical definition of neuropathy relied on the presence of sensory and motor signs, coupled with reduced or absent deep tendon reflexes without pathological reflexes. The confirmation of neuropathy was obtained through nerve conduction studies. This comprehensive approach aids in understanding and characterizing the intricate relationship between Hodgkin lymphoma and associated neurological complications.

In lymphomas, neuropathies predominantly coincide with monoclonal gammopathy, encompassing conditions like amyloidosis, polyneuropathy organomegaly endocrinopathy monoclonal gammopathy with skin abnormalities, type I cryoglobulinemia, anti-myelin-associated glycoprotein neuropathies, and Waldenström's disease (4, 6). Diagnostic indicators, such as onconeural antibodies (Hu-antibodies, Yo, Ri, Ma, anti-CV2/CRMP5), elevated cerebrospinal fluid protein levels, and the presence of oligoclonal bands in cerebrospinal fluid, aid in discerning the nature of the disease. A thorough investigation into any underlying cancer is imperative (9). A precise definition of paraneoplastic neuropathies is crucial to prevent confusion.

In seronegative sensory neuronopathies, Hu-antibodies, anti-CV2/CRMP5, and anti-amphiphysin antibodies are frequently detected (8). The updated diagnostic criteria for paraneoplastic neurologic syndromes have replaced the term "onconeural antibody" with categorizations based on risk levels: high-risk antibodies (> 70% association with cancer) and intermediate-risk antibodies (30–70% association with cancer) (6). Utilizing a scoring system known as the Paraneoplastic Neurologic Syndromes—Care Score, which incorporates clinical phenotype, the presence of neuronal antibodies, and the presence of cancer, employs a scoring system to classify diagnostic certainty into three levels: possible, probable, and definite paraneoplastic neuropathy (6). Importantly, the presence of cancer is a prerequisite for establishing definite paraneoplastic neuropathy (6). This refined approach enhances diagnostic accuracy and facilitates a comprehensive understanding of the intricate relationship between lymphomas and associated neuropathies.

A diagnosis is established when these neuropathies are associated with malignancies or when oncologic neuronal antibodies are identified. To diagnose definitive paraneoplastic neuropathy in non-classic neuropathies, including sensory and motor neuropathy, the presence of onconeural antibodies should be confirmed, or neuropathy symptoms should show improvement with the treatment of the underlying tumor (2). Detecting onconeural antibodies is challenging in most patients, rendering a diagnosis of unequivocal paraneoplastic neuropathies in lymphomas often impossible (2). Other potential causes of sensorimotor neuropathy comprise infections, autoimmune non-paraneoplastic diseases, malignancies, neurodegenerative disorders, toxins, metabolic issues, alcohol, diabetes, and chronic idiopathic axonal polyneuropathy (6, 8). Chemotherapy-induced peripheral neuropathy stands as a crucial differential diagnosis for paraneoplastic neuropathies after cancer treatment. Emerging challenges in the peripheral nervous system are noted due to various anti-cancer medications, including targeted and immune checkpoint inhibitor therapy (8).

The general therapeutic approach for paraneoplastic neuropathies operates under the assumption that detecting and removing cancer can ameliorate neurological symptoms (8). In our case, the patient underwent intravenous immunoglobulin therapy (2 g/kg in divided doses for 4 to 5 days, repeated monthly) to impede further progression of neuropathy, aligning with current recommendations (10). Determining the precise cause of the regression in neurological findings remains challenging since immunotherapy and treatment of the underlying malignancy were administered concurrently in our patient. Neuropathies that exhibit improvement with tumor therapy are uncommon and occur across various cancers (5). While paraneoplastic neuropathy often manifests before or in the early stages of cancer and may be treatable, studies indicate that it can also develop after cancer diagnosis or in advanced stages (2). Long-term follow-up is imperative (2). In our patient, the identification of paraneoplastic neuropathy coincided with the diagnosis of Hodgkin lymphoma, emphasizing the complexity and importance of managing neurological complications in the context of lymphomas.

Conclusion

The association between paraneoplastic neuropathy and lymphoma is infrequent. The diagnosis of paraneoplastic neuropathy is typically established when the disease is linked to a tumor or when oncologic neuronal antibodies are detected. This case is noteworthy due to the identified correlation between Hodgkin lymphoma and sensorimotor paraneoplastic neuropathy. Timely detection of paraneoplastic neuropathy is paramount, as delays in therapy may occur. A comprehensive clinical examination plays a crucial role in differentiating paraneoplastic sensorimotor neuropathy from other potential causes. This underscores the importance of vigilance in recognizing and addressing neurological complications in the context of lymphomas, contributing to a more nuanced understanding of these complex relationships.

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Prikaz bolesnika

UDC: 616-006.44:616.85
doi: 10.5633/amm.2024.0312

PARANEOPLASTIČNI SINDROM KOD BOLESNIKA SA HODŽKINOVIM LIMFOMOM

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Paraneoplastična neuropatija javlja se kod osoba sa malignim bolestima i ne može se objasniti prisutnim tumorom, metastazama, infekcijama i neželjenim dejstvom terapije osnovne bolesti. Prikazujemo slučaj bolesnika starog 38 godina koji se javio na pregled zbog gubitka telesne težine, noćnog znojenja, gubitka senzibiliteta u donjim ekstremitetima, bolova, parestezije, alodinije i gubitka čula dodira u donjim ekstremitetima. Laboratorijska analiza krvi pokazala je da je vrednost leukocita bila $20x10^9$/L, a C-reaktivnog proteina 40 mg/L. Markeri virusnih infekcija nisu ukazivali na aktivnu infekciju. Ultrasonografijom je otkrivena višestruka hipoehogena periferna limfadenopatija. Kompjuterizovanom tomografijom pluća otkriveni su konglomerati limfnih nodusa u jugularnim jamama, medijastinumu i prednjem torakalnom zidu. Fizikalnim pregledom utvrđena je bezbolna i umerena limfadenopatija desne supraklavikularne regije. Magnetna rezonanca glave i vrata dala je nalaz koji je bio u granicama normalnih fizioloških vrednosti. Analizom cerebrospinalnog likvora isključeni su infekcija i malignitet. Smanjena amplituda u studijama nervne provodljivosti pronađena je u donjim ekstremitetima, a senzorni i motorni odgovori nisu mogli biti dobijeni. F-odgovor bio je smanjen. Bolesnik je imao simetričnu, uzlaznu neuropatiju i negativne duboke tetivne reflekse. Nalazi su ukazali na senzomotornu distalnu polineuropatiju. Biopsija limfnih žlezda rezultirala je dijagnozom Hodžkinovog limfoma mešovite celularnosti. Započet je polihemoterapijski protokol (doksorubicin, vinblastin i dakarbazin). Neuropatija je lečena intravenskim imunoglobulinima, dozom od 2 g/kg. Zapažen je delimičan oporavak i sprovedena je produžena fizikalna terapija. Kada su neurološke tegobe poput ovih kod prikazanog bolesnika povezane sa malignitetom ili sa pozitivnim onkoneuronskim antitelima, može se postaviti dijagnoza paraneoplastične neuropatije. Važno je blagovremeno prepoznati bolest da bi lečenje bilo uspešno. Rana dijagnoza tumora najbolja je garancija za poboljšanje ili stabilizaciju paraneoplastične neuropatije.

Acta Medica Medianae 2024; 63(3):94–99.

***Ključne reči**: limfom, malignitet, neuropatija, onkoneuronska antitela*

Original article

UDC: 316.47-057.875
doi: 10.5633/amm.2024.0313

DIMENSION OF KINDNESS IN THE STUDENT POPULATION

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The acts of helping others are a manifestation of a personal dimension called kindness, which is of particular importance in medicine. The capacity of people who work in medicine to show kindness is one of the factors that determines the future course of treatment.

The primary goal of the research is to determine the presence of the category of kindness in the group of students of the Faculty of Medicine and the group of students from other faculties of the University of Niš and to determine whether there is a difference in the category of kindness between the two groups of students.

A total of 230 subjects filled out an online questionnaire. The multidimensional instrument Kindness scale was used for the assessment of kindness. Data are presented as mean score values for each aspect of kindness, as well as maximal and minimal values. A comparison between the two groups was performed using Student's t-test for two independent large samples.

The results did not show any difference in any of the studied dimensions of kindness in the groups of respondents. The results provided insight into the nature of kindness, its obstacles, and its importance, and they indicated the necessity of thinking about the dimension of kindness while working with students and in everyday clinical practice.

Acta Medica Medianae 2024;63(3): 100–106.

Key words: *kindness, students, communication*

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Introduction

Contemporary research defines the acts of helping others as a manifestation of a personal dimension called kindness (1). The goal of kindness is to provide support to another human being without expecting a reward and at a certain personal price (2). High levels of kindness reduce anxiety and somatic symptoms, such as colds and even blood pressure. The more altruistic members of the group are deemed popular and respected (3). Kindness encourages healthy social interactions and increases subjective feelings of happiness and life satisfaction (4–9).

These literature data are easy to understand, keeping in mind that good interpersonal contact has its biological consequences, acting upon a ventral vagal system, which serves to foster calm behavioral states by inhibiting sympathetic influences to the heart and dampening the hypothalamic-pituitary-adrenal (HPA) axis (10). Our social engagement system, involving vagus, allows us to feel connected to ourselves, others, and the world around us. When activated, it stimulates physical and psychological responses, reduces heart rate, relaxes laryngeal muscles, calms breathing, and stimulates digestion. This manifestation of the soothing system finds its origin in the attachment process (11,12). The attachment process activates a safety signal-related neural region and reduces pain experience. (13, 14), paving the way to the capacity to regulate arousal in the presence of another human being (15).

Kindness is not just about being nice (16), and it is not in any way a superficial concept. Being kind requires understanding, in the very moment, the needs of other human beings and being online with another human being. On a more profound level, kindness stems from the capacity of the individual to act upon impulses manifesting good internal objects and thus of life instinct (17).

That is where the motivation for this work came from. The basis of motivation is the need to recognize that both social connection and social disconnection broadly shape biological responses and behaviors that are consequential for health.

The category of kindness is of particular importance in medicine. People who visit a health facility are vulnerable, and they experience fear and weakness. When people get challenged in an attempt to survive, they start out trying to use a "social engagement system" to look at each other and resolve things warmly, activating the myelinated vagus parasympathetic circuit (13). The capacity of medical workers to possess and show kindness is one of the factors that determine the future course of treatment of help-seeking people (18). Another important fact is that the concept of kindness cannot be described as a unique construct localized within the individual but as a construct that takes place between the individual and the environment. The concept of kindness connects the person and the social world as a bridge (19, 20).

The primary goal of the research is to determine the presence of the category of kindness in the group of students of the Faculty of Medicine and the group of students from other faculties of the University of Niš. The secondary goal of the research is to determine whether there is a difference in the category of kindness in the group of students of the Faculty of Medicine compared to the group of students from other faculties of the University of Niš.

Material and Methods

Procedure

The research was conducted in March 2024. An online questionnaire was created, and a link to the questionnaire was distributed through social media. Respondents' answers were always anonymous since the questionnaire did not involve personal data apart from the age and faculty in which they were enrolled. It took a student around 10 minutes to fill out the questionnaire. The subjects were well-informed about the nature of the research and agreed to participate in the study. The study was conducted by Ethical norms and was approved by the Ethics committee of the Faculty of Medicine, University of Niš.

Subjects

Students of the Faculty of Medicine University of Niš and students from other faculties of the University of Niš were involved in the research. A total of 230 subjects filled out the questionnaire and the results were included in the analysis. Out of the total number of students, 164 students were from the Faculty of Medicine, and 66 students were from other faculties. Demographic characteristics of the sample included questions related to gender, age, and place of residence.

Research instrument

The assessment of the kindness category was carried out using a multidimensional instrument for measuring kindness consisting of 40 questions (1). The instrument measures four aspects of kindness: Benign Tolerance (BT;13 questions), Empathic Responsiveness (ER; 9 questions), Proactive Principle (PP; 9 questions), and Unkindness (U; 9 questions). Participants were given information to answer each item in relation to the question: How often have you shown a specific behavior and the answers were scored on a Likert scale from 1 - almost never, to 7 - almost always. A higher score on the test reflects a higher category of kindness or unkindness (1).

Statistical analysis

Data are presented as mean score values for each aspect of kindness, as well as maximal and minimal values. For each aspect, data normality distribution was performed (Kolmogorov–Smirnov test), and a Cronbah's alpha was calculated. Comparison between two groups was performed using Student's t-test for two independent large samples and the p values less than 0.05 were considered statistically significant. Statistical data processing was performed using the SPSS software package version 18.0 (SPSS Inc., Chicago, Illinois).

Results

Sample survey

The sample consists of 230 students from the University of Niš, out of which 164 are medical students and 66 are students from other faculties. The structure of the sample in relation to the variables gender, age, and place of residence on the subsample of medical students and students of other faculties is shown in Table 1-3.

Table 1. Sample structure by gender on the subsample of medical student's and other faculties

		Gender		Total
		Female	Male	
Group	Other faculties	56	10	66
	Medical faculty	128	36	164
	Total	184	46	230

Table 2. Sample structure by age on the subsample of medical students and other faculties

		Age				Total
		18-21	22-25	26-30	over 30	
Group	Other faculties	41	18	0	7	66
	Medical faculty	55	93	11	5	164
	Total	96	111	11	12	230

Table 3. Sample structure by place of residence on the subsample of medical students and other faculties

		Place of residence		Total
		Urban area	Countryside	
Group	Other faculties	60	6	66
	Medical faculty	148	16	164
	Total	208	22	230

Table 4. Descriptive statistics, Kolmogorov–Smirnov test, and Cronbach's α reliability coefficient of the data obtained from the instrument

Dimension	Mean	SD	Min	Max	K-S	p	α	N
Benign tolerance	54.63	5.269	31	64	0.80	0.53	0.68	13
Empathic responsiveness	35.66	4.63	14	45	0.58	0.88	0.67	9
Proactive principle	33.49	5.74	16	45	0.76	0.60	0.71	9
Unkindness	*22.21*	*5.32*	*12*	*45*	*1.08*	*0.18*	*0.74*	*9*

K-S - Kolmogorov–Smirnov test; α - Cronbach's α reliability coefficient

Table 5. Comparison of the group of medical students and the group of students from other faculties in relation to the investigated dimensions of kindness (Students` t-test)

Dimension	Group	N	Mean	SD	t	df	*p*
Benign tolerance	Other faculties	66	54.16	5.81	-0.862	228	0.39
	Medicine	164	54.82	5.04			
Empathic responsiveness	Other faculties	66	35.31	4.89	-0.728	228	0.46
	Medicine	164	35.81	4.53			
Proactive principle	Other faculties	66	33.03	6.24	-0.772	228	0.44
	Medicine	164	33.67	5.53			
Unkindness	Other faculties	66	23.04	5.23	1.50	228	0.13
	Medicine	164	21.88	5.33			

In Table 4, the descriptive-statistical measures, the results of the distribution normality test, and the reliability of the measurement scales are presented.

From the data obtained (Table 4), we can see that none of the examined dimensions statistically significantly deviates from the normal distribution. Thus parametric statistical method was used for further data processing. The reliability of the measurement scales was obtained by calculating the internal consistency reliability. The obtained values of Cronbach's α coefficient indicate the marginal but still acceptable reliability of all measurement scales.

In Table 5, the results of the comparison between the group of medical students and the group of students from other faculties regarding the expressiveness of the investigated dimensions of kindness are given. Based on the results of the statistical analysis, we can say that there is no

statistically significant difference between the group of students from other faculties and the group of medical students in terms of the expression of all four examined dimensions of kindness (Table 5).

Discussion

In the present work, we determined the presence of the category of kindness in the group of students of the Faculty of Medicine and in the group of students of other faculties that are part of the University of Niš and determined whether there was a difference in the category of kindness between them.

The applied scale measured 4 components of kindness: (I) Benign tolerance, which includes permissive humanity that is reflected in everyday politeness, acceptance, and love for others and refers to the behavioral component (9,10). (II) Empathic responsiveness, which is more personalized and emotional. It is reactive and takes into account the specific feelings of other individuals. It refers to the affective component (10). (III) The Proactive principle is a category driven more by cognition than by emotion. It is a respectful behavior toward others and is typically proactive behavior, not reactive. It includes altruistic behaviors and refers to the cognitive component (11). (IV) Unkindness as a category not directly opposite of kindness reflects an independent aspect of interpersonal interactions (1).

The obtained results of our research showed that there is no difference in the category of kindness among respondents of students of the Faculty of Medicine and respondents of students of other faculties of the University of Niš (Table 5).

The obtained results of our research are in line with the results of a study performed at The University of Huddersfield (1) however, it does show some differences. The results scores for the BT and PP components were lower in our sample. BT refers to the attitude to live and let live and to permissive humanity revealed in everyday courteousness, acceptance, and love for one's fellows. These results might reflect the fact that tolerance is not a common feature in our social milieu. The component PP is about behaving honorably towards others and is typically proactive rather than reactive, while much of this behavior is considered altruistic. This component scored lower in our sample. Bearing in mind that this component is rather cognitive than emotional, it points to the need for cognitive intervention in education and upbringing as well. The component ER showed similar results and was not expected due to the attitude that people in our milieu are very empathetic. This could be the case, but it is important to mention that the experience of empathy in our sample could be different, and these results deserve further exploration. The Unkindness component was lower in the medical student sample than in the sample of subjects from other faculties of the University of Niš. The results showed lower values of the Unkindness dimension in our sample compared to the results of the University of Huddersfield (1). The initial understanding of these results is that students in our environment live in protective conditions, in a familiar environment and without specific challenges, which is why aggressive impulses that manifested as unkindness are especially activated. Also, we might be satisfied with the obtained results if we bear in mind that kindness is learned through a process, while rudeness, an expression of aggressive content, impulses, and urges, is less subject to learning.

The above results, that the Kindness dimension was equally distributed among the students of the Medical faculty and the other faculties in our sample, could be interpreted in two ways. Firstly, the category of kindness is a construct that is highly susceptible to socially desirable responses, which means that the respondents could have also given socially desirable answers. Another possibility would be that there is no difference in the category of kindness between medical students and students of other faculties because both can be grouped simply as - students. During their studies, students live in similar conditions, have similar needs and demands that are placed before them, and communicate with family and friends, and thus their kindness dimension showed the same characteristics in both groups.

The kindness category denotes gentleness, generalized and genuine empathic response instead of superficial charm, and protective behavior towards others instead of exploiting and manipulating others. People with a higher kindness score choose professions of helping others (1). However, the obtained results of this research are different from the literature suggestions.

After initial mild disappointment with the results, due to the expectation that medical students display more kindness than the students of other faculties, two lines of reflection were placed in front. Initial analysis of the unexpected outcome two major points could be reflected on.

The first line of reflection is about the teaching role of the staff at the Faculty of Medicine. Hidden curriculum entails what students learn in the teaching process. Beyond cognitive content, they learn about emotional processes, both in the patient and in the staff themselves, during healthcare consultations, which can improve or turn off the functioning of either participant in this process in hidden and unconscious ways. Implicit information is far more important and guides decision-making, behavior, and the destiny of both parties in the medical field. Unkindness affects performance. Rudeness hijacks cognitive resources, decreases working memory and attention, and stifles creativity and helpfulness (21, 22). The problem-solving and decision-making are specifically impaired. In the medical field, as unkindness as incidents occur,

the clinician becomes a second victim, which in turn adds to their stress and further worsens cognitive processing and performance (23). At this point, the informal and formal wisdom passed from mentors to mentees is often serendipitous and contagious. Mentors provide the unprompted give of time, energy and guidance to the next generation. Over time, mentees transition to become mentors for others. Mentorship creates virtuous cycles within institutions, spurring contagious kindness.

The importance of courtesy in the practice of medicine cannot be overemphasized. Data from the literature show that kindness is learned. Medical faculties around the world are introducing a mandatory two-year curriculum called Human Kindness. Research shows that students have resistance towards learning attitudes of professionalism (24). The solution is to create an intellectual and interactive space in which students are exposed to the deeper meaning of empathy in a clinical context. Obstacles to kindness in stressful working conditions at the clinic have been analyzed. A model of compassion and empathy is developed, emphasizing the capacity for emotional self-regulation and cognitive coping with automatic emotional responses in complex clinical situations. Students should learn to develop self-awareness, be open to other perspectives, and gain information about the neural basis of empathy, the function of mirror neurons, and the neural basis of emotional regulation (25, 26). In our clinical setting, the content of the course Psychiatry with Medical Psychology and the elective course Communication Skills partially include the above-mentioned topics, trying to provide students with some basic knowledge and help them learn kindness.

The second line of reflection, based on the results obtained that the category of kindness is equally distributed among the students of different fields at the University of Nis, is the opinion that kindness is a global phenomenon. It refers to every human subject, and it has to be such. It was far more important to get that kind of result, showing that all the students belonging to different professional groups share the unique dimension of kindness. The society, due to this distribution, could benefit much more. Beyond the impact of negative affect on decreasing performance, there is evidence that positive affect increases cognitive function and performance (21). Warmth is contagious and spreads in waves; the person who receives it continues to give it to others, and thus, the act of generosity could ripple forward (27). Since people depend on each other not only for survival but also for mutual advancement, possessing kindness in our repertoire is of utmost importance.

Conclusion

This work analyzed the category of kindness measured among students of the Faculty of Medicine and other faculties of the University of Niš. The results did not show the difference in any dimensions of kindness in the groups of our respondents. The results provided insight into the nature of kindness, its obstacles, and its importance and indicated the necessity of thinking about the dimension of kindness while working with students and in everyday clinical practice.

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Originalni rad **UDC: 316.47-057.875**
doi: 10.5633/amm.2024.0313

DIMENZIJE LJUBAZNOSTI U POPULACIJI STUDENATA

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Postupci pomaganja drugima manifestacija su personalne dimenzije zvane ljubaznost, koja je od posebnog značaja u medicini. Sposobnost medicinskih radnika da pokažu ljubaznost jedan je od faktora koji određuju budući tok lečenja bolesnika.

Primarni cilj istraživanja jeste utvrđivanje prisustva kategorije ljubaznosti u grupi studenata Medicinskog fakulteta i u grupi studenata drugih fakulteta Univerziteta u Nišu, kao i utvrđivanje postojanja razlika u kategorijama ljubaznosti između ovih dveju grupa studenata.

Onlajn upitnik je popunilo ukupno 230 ispitanika. Za procenu ljubaznosti korišćen je višedimenzionalni instrument „Skala ljubaznosti". Podaci su predstavljeni kao srednje vrednosti rezultata za svaki aspekt ljubaznosti, te kao maksimalne i minimalne vrednosti. Poređenje dveju grupa izvršeno je Studentovim t-testom za dva velika nezavisna uzorka.

Rezultati su pokazali da među grupama ispitanika ne postoji razlika ni u jednoj dimenziji ljubaznosti. Rezultati su dali uvid u prirodu ljubaznosti, prepreke ispoljavanja ljubaznosti, kao i njen značaj i ukazali su na neophodnost razmišljanja o dimenziji ljubaznosti u radu sa studentima i u svakodnevnoj kliničkoj praksi.

Acta Medica Medianae 2024; 63(3):100–106.

***Ključne reči**: ljubaznost, studenti, komunikacija*

Original article **UDC: 611.779:[57.089.6:547.962.9**
doi: 10.5633/amm.2024.0314

SUBCUTANEOUS TISSUE RESPONSE TO THE TWO IMPLANTED COLLAGEN-BASED MEMBRANES OF DIFFERENT ORIGIN

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Collagen, as the main structural protein in mammals, fulfils the fundamental requirements to be a ssuitable biomaterial component used in tissue engineering. Due to its biocompatibility and biodegradability, collagen can be utilized in various forms for guided soft and bone tissue regeneration. Collagen-based membranes, frequently used for both soft and hard tissue regeneration, can differ in their origin (porcine, bovine, equine), physicochemical characteristics such as architecture, porosity, absorption ability, and manufacturing processes which may influence tissue response and final outcome. In this study, we examined and compared tissue response to the two implanted collagen membranes of different origins: porcine vs. equine. The subcutaneous implantation model in BALB/c mice was used, and tissue response was evaluated 3, 10 and 30 days after implantation. Tissue was analyzed by histological and histomorphometric methods. Our study revealed variations in subcutaneous tissue response, patterns of cell infiltration into collagen membranes, and changes in membrane thickness and resorption that may be attributed to the differences in membrane origin but also to the differences in the manufacturing process. We can conclude that both membranes are suitable for application in guided tissue regeneration.

Acta Medica Medianae 2024;63(3): 107–115.

Key words: *collagen membranes, tissue response, in vivo, subcutaneous implantation*

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Introduction

Collagen is one of the most frequently used components of biomaterials in bone and soft tissue engineering due to its biocompatibility and biodegradability (1, 2). Collagen is a major structural protein in animals and the most abundant protein in the human body (3, 4). Due to that, collagen-based biomaterials are widely used in hard tissue engineering: for bone, cartilage, and osteochondral defects, as well as in soft tissue for regeneration of cornea, skin regeneration and repair of the blood vessels (5). Collagen can be used as a membrane, scaffold, gel or hydrogel, in the form of a liposome, nanosphere, or as a delivery system of cells, drugs, organic molecules or growth factors (1, 2, 5). In guided bone regeneration (GBR) collagen-based biomaterials are often used in combination with bone substitute materials based on hydroxyapatite and calcium-phosphate (6–8).

Collagen-based membranes can serve as a physical barrier to impede the ingrowth of connective and epithelial tissue into the defect site, while also promoting wound healing and providing support for soft tissue augmentation (9, 10). The specific use of each membrane depends on its own characteristics. Various types of resorbable membranes are described in the literature. Collagen-based membranes in tissue engineering are mostly distinguished by species and tissue origin: porcine, bovine, or equine; derived from the dermis, peritoneum, or pericardium (10, 11). Additionally, collagen-based membranes from alternative sources such as some fishes or jellyfish, or human originated were shown as a promising tool for GBR (12, 13).

Besides differences in indication and origin, collagen-based membranes may differ by used additives and manufacturing processes. Many cross-linking methods are used to improve the physicochemical characteristics of collagen and to achieve controllable collagen biodegradability. Collagen-based materials are degraded over time by the enzymes, mostly matrix-metalloproteinases, thus avoidance of secondary intervention can be achieved (9). Chemical cross-linking with agents such as aldehydes, improves the mechanical strength and prolongs the time of degradation. Physical cross-linking treatment with irradiation or using biological agents (transglutaminase and horseradish peroxidase) are non-chemical manufacturing techniques that lead to controllable biodegradability (1, 9, 14). However, it has been shown that modification of collagen by chemical cross-linking techniques can lead to cytotoxicity and may impair biocompatibility (15–18).

Bearing in mind that collagen membranes of different origins are available on the market, and differences in a behavior regarding the origin described in the literature, we aimed to to analyze and compare the tissue response to the two commercially available collagen membranes of different species origin, porcine and equine, in subcutaneous implantation model in mice.

Material and Methods

Collagen membranes

4BONE RCM (MIS Implants Technologies Ltd., Israel) is a resorbable collagen-based membrane composed of collagen type I and III, originating form porcine skin (labeled as PM membrane in the study). The prolonged time of resorption for this collagen-based membrane is achieved by a chemical cross-linking technique using formaldehyde and can be used in guided tissue regeneration as an effective barrier for a 4 −6 months period, based on manufacturers' guidance.

PARASORB RESODONT® (RESORBA Medical GmbH, Germany) is an equine-derived collagen-based membrane, which contains 2.8 mg collagen fibrils per square centimeter (labeled as EM membrane in the study). This membrane is completely absorbable, and produced without the use of chemical cross-linkers.

Animals

The study was performed on animals from the Vivarium of the Scientific Research Center for Biomedicine, Faculty of Medicine, University of Niš, Serbia. All animal procedures in the study were authorized by the local Ethical Commission of the Faculty of Medicine, University of Niš, Serbia based on the approval number 323-07-00278/2017-05/6 of the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (date of approval: July 13, 2017).

In this study, 20 syngeneic male BALB/c mice, aged 8 to 10 weeks, weighing 22−24 g, were used. Animals were kept in standard laboratory conditions with an artificial light-dark cycle of 12 h each and access to water and food *ad libitum*.

Experimental design

Collagen membranes were implanted subcutaneously right below the scapular region of animals. Prior to implantations, the animals were anesthetized by a mixture of ketamine and xylazine according to standard protocols for mice anesthesia and surgical procedure was performed following described protocols (19, 20). Animals were shaved and the area of implantation was disinfected with iodine solution. An incision was made on the back and a biomaterial was inserted in formed subcutaneous pockets below the scapulae. The animals were randomly divided into two experimental groups, with 10 animals per group. Experimental groups were: Group PM – implanted 4BONE RCM porcine-origin membrane and Group EM – implanted PARASORB RESODONT® equine-origin membrane.

The animals were sacrificed and membranes with surrounding tissue were explanted 3, 10 and 30 days after implantation. Tissue explants were fixed in 10% neutral buffered formalin (NBF) and further processed.

Histology

After fixation in 10% neutral buffered formalin (NBF), explant tissue samples were processed in serial ascending ethanol concentrations, cleared in xylene and embedded in paraffin. Paraffin-embedded tissue blocks were cut on a microtome and tissue slides were stained with standard hematoxylin and eosin (H&E) technique, to visualize tissue structures, cells and implanted biomaterial, and Azan trichrome (AT) specific staining technique for collagen. The light microscope Leica DMR was used for histological analysis while micrographs were recorded with a microscope camera Leica DC 300.

Histomorphometric analysis

Histomorphometric measurements were performed on stained tissue slides micrographs made at 10x objective magnification. NIS Elements software version 2.0 (Nikon, Tokyo, Japan) was used to measure the thickness of both examined membranes. Thickness of membranes was measured at 15 different points, calculated and expressed in µm. The results of membrane thickness measurements are shown as mean ± standard deviation (SD).

Statistical analysis

The results of histomorphometric measurements were statistically analyzed by performing a one-way analysis of variance (ANOVA). The results were presented as mean ± SD. The statistical significance was set to $p < 0.05$.

Results

Histological analysis

In Figures 1 to 3, histological images of explanted PM and EM membranes with surrounding tissue are presented. Three days after implantation, in the PM group, a compact membrane structure with randomly distributed pores of unequal size, can be noticed. Mononuclear cells with flattened morphology as well as mononuclear macrophages and different inflammatory cells were found on the material surface (Figs. 1a, 1c). On the membrane periphery multinuclear phagocytes also were noticed. In some places mononuclear cells infiltrated the PM membrane.

In the EM group at 3 days numerous pores can be noticed through the whole membrane structure, evenly distributed, with a thin layer of mononuclear cells, mostly with flattened morphology, on the membrane surface with cells started to infiltrate the membrane pores. Rarely presented inflammatory cells were noticed in some spots.

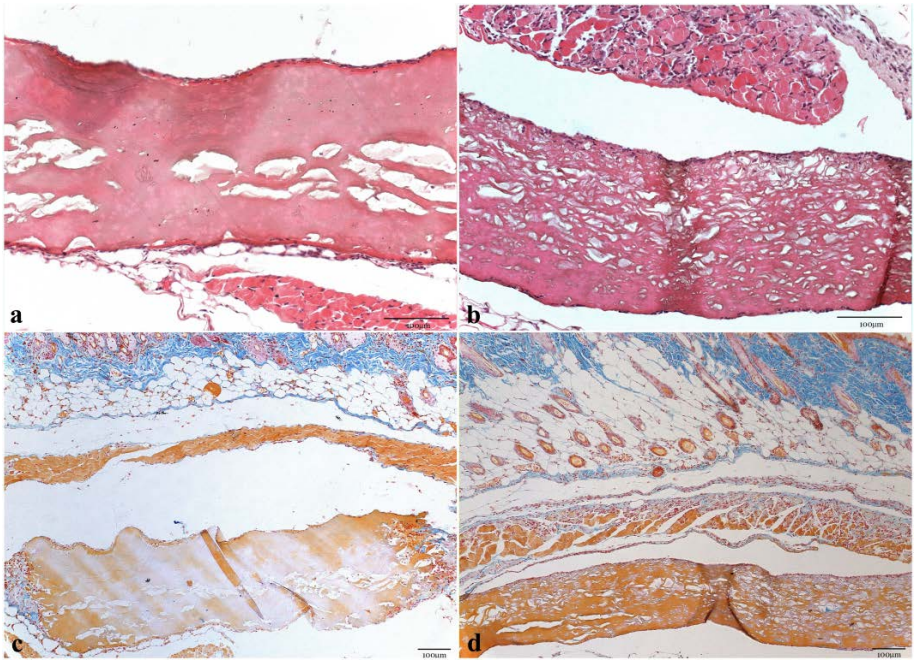

Figure 1. Tissue sections of PM (a, c) and EM (b, d) implants 3 days after implantation, stained with H&E (a, b), objective magnification 20x, 100 µm scale bar, and AT technique (c, d), objective magnification 10x, 100 µm scale bar

Ten days after implantation, PM collagen membrane still looked like a stabile barrier, but was less compact than earlier, with more pores than at 3 days. Mononuclear macrophages have been noticed on and within the membrane. A layer of fibroblast-like cells and inflammatory cells on the membrane surface, as well as cells infiltrated into large pores through the whole membrane are observed (Figs. 2a and 2c).

The infiltration process was noticed in the EM group as well, with cells mostly maintained in peripheral parts of the membrane with visible migration zones towards the inside of the membrane and sporadically cells infiltrated in the center of the membrane (Figs. 2b, 2d). In some spots on the periphery of the membrane, inflammatory cells of leukocyte type were seen. EM membrane was completely stained in blue with the Azan staining method (blue color refers to the stained collagen) and the membrane structure closely resembles the structure of native collagen fibers (Fig. 2d).

Thirty days after implantation, initial membrane structure was disturbed. Only remnants of examined collagen membranes were noticed in both groups, with larger parts of PM membrane presented compared to the EM membrane (Figure 3). This indicates that the degradation process occurred which was more pronounced in the case of EM membrane. Greater

stability, even at this time point, was observed in the case of PM collagen membrane compared to the EM collagen membrane. The remnants of both membranes are populated with cells. Resorption of both membranes is noticed over time, which was more pronounced in the case of EM membrane.

The results of histomorphometrical measurements

In both experimental groups statistically significant increase in membrane thickness was observed from 3 to 10 days (Fig. 4), but there was no statistically significant differences between the PM and the EM membrane at examined time points. The measurement of membrane thickness at day 30 was not performed due to the resorption of membranes and the presence of remnants of membranes only.

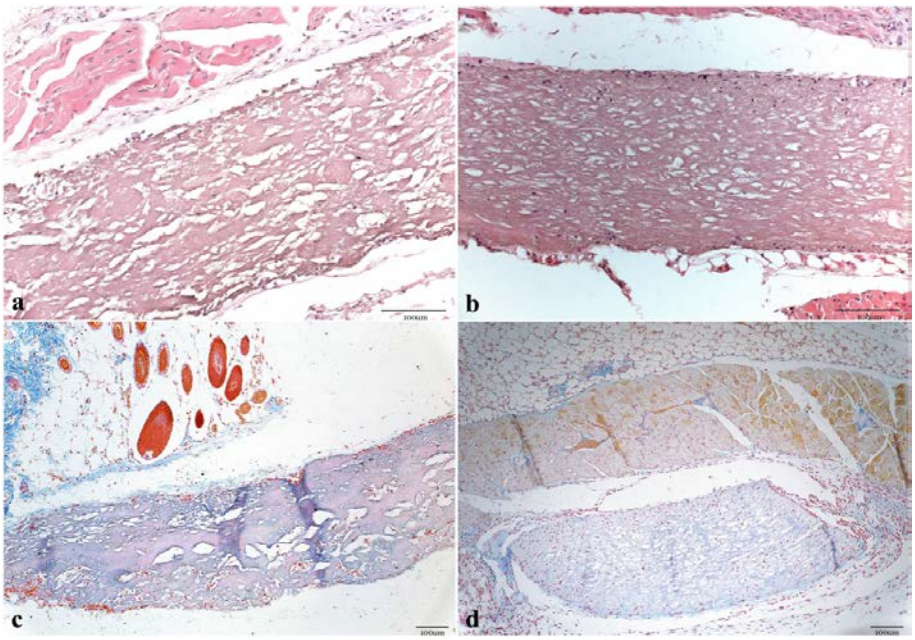

Figure 2. Tissue sections of PM (a, c) and EM (b, d) implants 10 days after implantation, stained with H&E (a, b) objective magnification 20x, 100 µm scale bar, and AT technique (c, d) objective magnification 10x, 100 µm scale bar

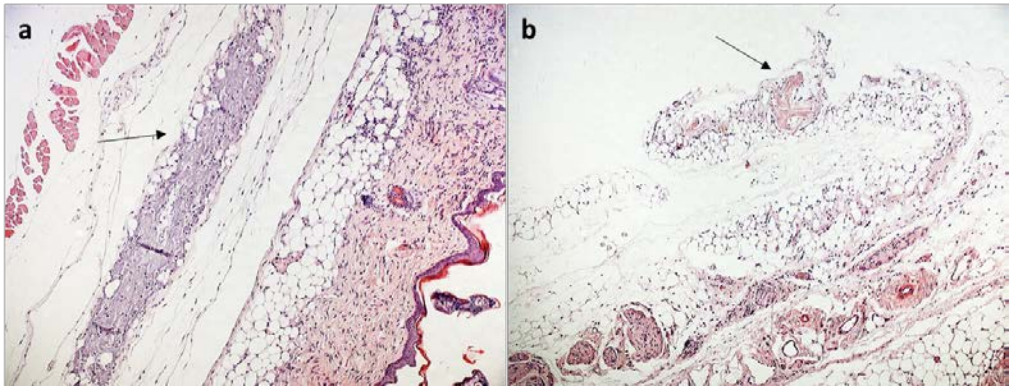

Figure 3. Tissue sections of PM (a) and EM (b) implants 30 days after implantation stained with H&E, objective magnification 10x, 100 µm scale bar, arrows indicate remnants of collagen membranes

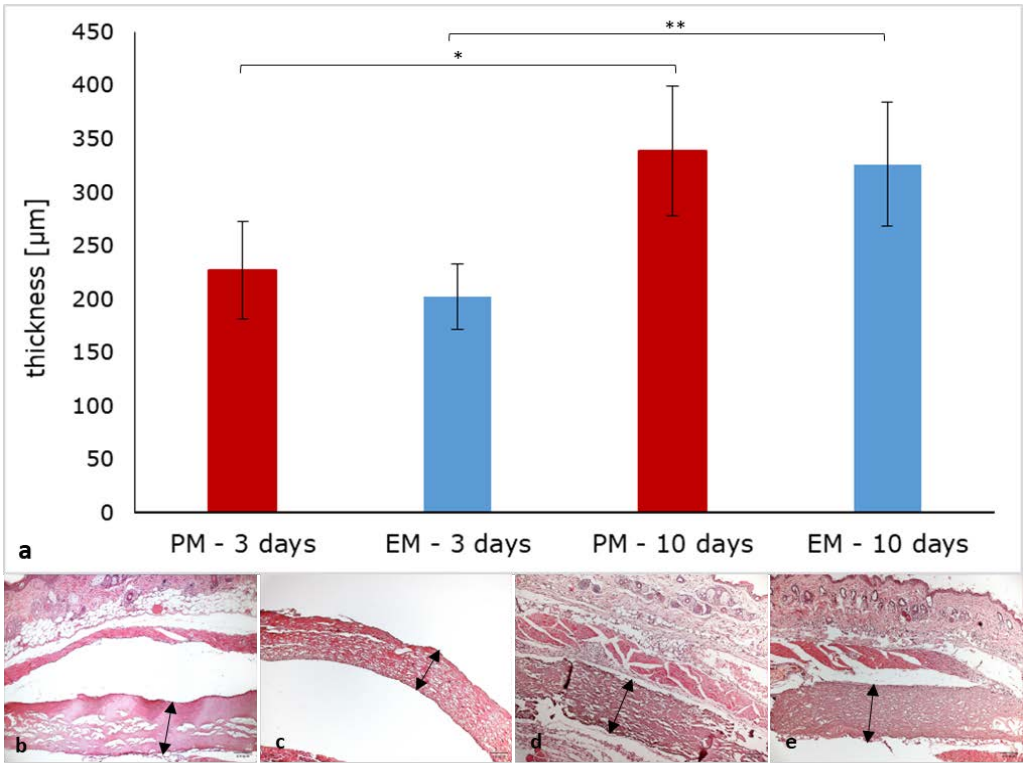

Figure 4. The results of histomorphometrical measurements of membrane thickness (a), results are presented as mean ± standard deviation. Black arrows on histological images (b-e) indicate measured membrane thickness, H&E staining, objective magnification 10x, scale bar 100 µm; (*) $p < 0.05$, (**) $p < 0.01$

Discussion

Tissue regeneration guided by biomaterials requires good integration with surrounding peri-implant tissue and induction of the regeneration process. Collagen-based membranes have a great application in both bone and soft tissue regeneration, in maxillofacial and oral surgery. Biomaterials based on collagen can induce chemotaxis, adhesion, and angiogenic process but also can stop peri-implant tissue ingrowth into the bone defect when serving as a barrier (9, 10, 21). Degradation of collagen biomaterials is caused by enzymes, mostly matrix metalloproteinases (MMPs), which can be released by activated polymorphonuclear leukocytes, fibroblasts, and mononuclear phagocytes (4, 9, 15). There are a lot of commercially available membranes of heterologous origin, with differences in physicochemical characteristics, examined in different studies *in vitro* and on various animal models of implantation (9, 10). Collagen of different species and tissue origin can diverge in amino-acid sequence which can affect the biostability and resorption time of collagen-based biomaterials (22). Bozkurt et al. (23) state that it is difficult to define unique conclusions about the biodegradability and biocompatibility of collagen membranes from numerous studies, citing that different degradation period of the same collagen membrane of porcine origin, is reported in the literature from different studies. They indicated the importance of a direct comparison of different collagen membranes, in the same study, in the same animal model as well as applying the same surgical procedure (23). Thus, in this study, we examined and compared two collagen membranes of different species origin: porcine vs. equine in a subcutaneous implantation model in mice, implanted by the same researchers at the same time.

The histological findings showed different membrane architectures, which may be the basis for diverse cell behavior, and expected tissue response. Despite differences in the porosity of these membranes, they looked like a stable barrier at earlier examined time points. PM membrane seemed to be more compact in structure with a lower number of unequal - sized pores compared to EM membrane, where numerous pores, more

equal in size and evenly distributed are noticed. It is known that the number, architecture, and distribution of pores are important factors that affect cell infiltration and resorption of implanted biomaterial. Different membrane architecture in examined groups was followed by infiltration of the same type cells, but with the difference in infiltration pattern, number, and period of appearance.

A greater number of inflammatory cells was noticed in the PM group at a 3-day term compared to the EM group. The time point of 3 days refers to the inflammation phase after tissue incision and biomaterial implantation, followed by infiltration of mononuclear cells, polymorphonuclears, leukocytes, macrophages and others (24). The noticeable greater infiltration of mononuclear cells was observed in both examined membranes after 10 days compared to the 3-day time point. Cell penetration was noticed in bigger pores mainly in the PM membrane, while cells were evenly distributed in the EM group. On the other hand, mononuclear cells were noticed in a greater number in the EM group than the PM, at 10 days.

At 10 days, a thin layer of fibrous tissue within the boundary of both materials appeared, which showed the beginning of tissue integration.

Histomorphometric analysis showed a statistically significant increase in membrane thickness at 10 days compared to 3 days. An increase in the thickness of both examined membranes, and a change in color when stained with AT method from yellowish to blue which is the color of collagen stained by AT method, went parallelly with the phase of cell infiltration. We assumed that an increase in cell density together with produced collagen by infiltrated fibroblasts and exposure to body fluids, is the main reason for the increased thickness of membranes and observed change in AT staining. It is known that exposure to the body fluids can also affect membrane thickness. In the study of Willershausen et al. (4), it was shown that the thickness of collagen biomaterials of porcine origin can be changed significantly after swelling in NaCl and was different in dry, wet, and *in vivo* conditions (4).

There are literature data showing no statistically significant differences between membrane thickness of porcine origin between 3 and 10 days, with a sign of degradation beginning after 10 to 15 days (25, 26). In the study where subcutaneous rat implantation was performed, it was shown that equine-derived collagen hemostatic sponges, which contain twice as much content of native non-crosslinked equine collagen fibrils compared to the EM membrane, decreased in thickness at 3 and 15 days, followed by cell infiltration, new vascularization formation, and degradation process up to 30 days after implantation (27). Hence, it is expected that PM and EM membranes go through the process of degradation under the influence of the collagenase enzymes in longer observation periods.

Literature data suggest that the desirable time of membrane degradation *in vivo* should be between four weeks and a few months depending on the clinical outcome that needs to be accomplished (9). Different biodegradation period was reported for Bio-Gide® collagen membrane of porcine origin, between four weeks (18, 28) and three months (29). In mice and rat subcutaneous implantation models, this membrane was shown to be a stable barrier in the period of two months (15, 23). In our study, the PM membrane is more stable than the EM membrane, from the beginning, and 30 days after implantation this membrane has retained its structure, was completely populated with connective tissue cells and more membrane remnants were presented compared to EM membrane.

In addition, in the study of subcutaneous implantation in rats, it was shown that modification of an equine collagen-based sponge to a flatted shape by pressing, led to different patterns of cell infiltration, the degradation rate of implanted material, as well as alternation of the inflammatory response (27). These findings indicated that physical modification of material may affect tissue response to biomaterial and the rate of collagen biodegradability (27, 30).

Overall, the results of our study showed that both examined membranes are suitable for guided tissue regeneration.

EM membrane is more suitable for the cellular environment than PM during the examined period. The intensity of blue color after AT staining is higher in EM than in PM group, which is consistent with more fibroblast-like cells observed that may indicate a higher rate of collagen production in EM compared to PM, or better recovery of membrane collagen fibers after implantation, up to 10 days. This can also be related to the manufacturing process of examined collagen membranes since the PM membrane was chemically cross-linked while the EM membrane was produced without the use of chemical cross-linkers, which are known to prolong the degradability time of collagen-based materials. In the context of these observations, we can assume that these membranes can be applied for different indications in guided tissue regeneration. According to the obtained results, the EM membrane is better used as a collagen matrix for soft tissue engineering, supporting the initial phase of wound healing, due to a high level of cell penetration and faster degradability rate. On the other hand, the PM membrane is better to be used as a barrier membrane, due to its lower rate of cell infiltration, and greater stability over time.

Conclusion

The results of our study revealed that both examined collagen membranes are suitable for guided tissue regeneration. Examined membranes are biocompatible, with differences in the pattern of cell infiltration and degradation rate, probably due to their different origin, physicochemical characteristics, and different manufacturing processes. Nevertheless, further preclinical studies with longer observation periods and other models

of implantation, as well as clinical studies, are required to clarify all observed differences in the behavior of these collagen membranes and their impact on tissue regeneration in various clinical conditions.

Acknowlegments

This study was financially supported by the Ministry of Science, Technological Development and Innovations of the Republic of Serbia (Contracts No. 451-03-68/2020-14/200113, 451-03-9/2021-14/200113, 451-03-68/2022-14/200113, 451-03-47/2023-01/200113, 451-03-65/2024-03/200113 and 451-03-66/2024-03/200113).

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Originalni rad **UDC: 611.779:[57.089.6:547.962.9**
doi: 10.5633/amm.2024.0314

ODGOVOR POTKOŽNOG TKIVA NA DVE IMPLANTIRANE MEMBRANE NA BAZI KOLAGENA RAZLIČITOG POREKLA

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Kao glavni strukturni protein kod sisara, kolagen ispunjava osnovne zahteve da bude odgovarajuća komponenta biomaterijala koji se koriste u tkivnom inženjerstvu. Zbog svoje biokompatibilnosti i biorazgradivosti, kolagen se može koristiti u različitim oblicima u vođenoj regeneraciji mekog i koštanog tkiva. Membrane zasnovane na kolagenu, koje se često koriste za regeneraciju mekih i tvrdih tkiva, mogu se razlikovati po svom poreklu (svinjske, goveđe i konjske), fizičko-hemijskim karakteristikama kao što su arhitektura, poroznost, sposobnost apsorpcije, kao i po proizvodnim procesima, koji mogu uticati na odgovor tkiva i konačni ishod. U ovom istraživanju ispitali smo i uporedili odgovor tkiva na dve implantirane kolagenske membrane različitog porekla: svinjskog i konjskog. Koristili smo model potkožne implantacije kod BALB/c miševa, a odgovor tkiva je analiziran tri, deset i trideset dana posle implantacije. Tkivo je analizirano histološkim i histomorfometrijskim metodama. Dobijeni rezultati su pokazali da postoje varijacije u odgovoru potkožnog tkiva, obrascima ćelijske infiltracije, kao i da postoje promene u debljini membrane i brzini resorpcije; to se može pripisati razlikama u poreklu membrane, ali i razlikama u procesu proizvodnje. Možemo zaključiti da su obe membrane pogodne za primenu u vođenoj regeneraciji tkiva.

Acta Medica Medianae 2024; 63(3):107–115.

***Ključne reči**: kolagenske membrane, odgovor tkiva, in vivo, potkožna implantacija*

Review article **UDC: 618.11-006-085**
doi: 10.5633/amm.2024.0315

CONTEMPORARY THERAPEUTIC PRINCIPLES IN THE MANAGEMENT OF PATIENTS WITH POLYCYSTIC OVARY SYNDROME

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Ovarian functional disorder is caused by an imbalance or disorder in the production of sex hormones, leading to irregularity in the menstrual cycles, as well as a reduced ability to conceive or carry to term. There are two types of ovarian dysfunction, i.e. primary, caused by the existence of ovarian pathophysiology, or secondary, which stems from thyroid or pituitary gland dysfunction. Polycystic Ovarian Syndrome, or PCOS, is an endocrine disorder of great complexity, and it was first described in 1935. The syndrome represents the most frequent cause of secondary amenorrhea and ovulatory dysfunction in reproductive-aged women. 'Syndrome' signifies a phenotype, or a set of clinical characteristics. Polycystic ovary syndrome involves classic phenotypes with specific characteristics that include the clinical signs of increased serum androgen concentrations, irregular periods, excessive androgen secretion, and infertility, as a consequence. Furthermore, the syndrome should be observed and treated as a metabolic disorder since it is closely associated with hyperinsulinemia and insulin resistance. A complex and individualized therapeutic approach is necessary to combat the complexity of the various disorders observed in various phenotypes. This review has been based on the literature research found in available databases. It presents a review of all the contemporary therapeutic options for managing patients suffering from polycystic ovary syndrome. Still, more studies are required to fully reveal the complex pathophysiology of the polycystic ovary syndrome. For this reason, this subject needs to be tackled in prospective epidemiological studies.

Acta Medica Medianae 2024;63(3):116–126.

Key words: *polycystic ovary syndrome, inositol, metabolic profile, insulin, LH, FSH*

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Ovarian Dysfunction

Ovarian dysfunction, otherwise known as ovarian functional disorder, is a condition caused by a disorder or imbalance in sex hormone production, leading to a reduced conception possibility, irregular menstrual cycles and an inability to carry to term. Ovarian dysfunction may primarily occur as a result of ovarian pathophysiology. Its secondary manifestation is a result of the dysfunction of different glands, such as the pituitary glands or thyroid glands (1). In essence, ovarian dysfunction is an imbalance in the secretion of estrogen and progesterone, which could lead to altered ovulation, a changed menstrual cycle, or a disrupted uterine function during pregnancy (2).

First described in 1935, Polycystic Ovarian Syndrome PCOS is a highly complex endocrine disorder. It represents the most common cause of ovulatory dysfunction and secondary amenorrhea in women who are at a reproductive age. Considering the fact that the syndrome's etiology is still unknown, the European Society of Human Reproduction and Embryology and the American Society for Reproductive Medicine developed a set of diagnostic criteria in Rotterdam in 2003, which was when the Rotterdam Criteria were established. Two out of the following three criteria need to be present for making the polycystic ovary disease diagnosis: oligo-anovulation, clinically manifested by the menstrual cycle disorder, biochemical or clinical hyperandrogenism, or a polycystic ovarian morphology detected with the use of ultrasound. In addition to the occurrence of at least two of the aforementioned criteria, it is

necessary to exclude other etiologies, such as hypothalamic-pituitary dysfunction, congenital adrenal hyperplasia, androgen-secreting tumours and the Cushing syndrome. The Rotterdam criteria are universally recognized in the diagnosis of polycystic ovarian disease and are also widely accepted by gyneacological, pediatric, endocrinological, and cardiological associations (3, 4).

The disorder is complex and is characterized by hirsutism, infertility, obesity, as well as different types of menstrual dysfunctions, such as amenorrhea, oligomenorrhea and anovulation. PCOS is frequently associated with bilaterally enlarged ovaries, which contain atretic follicles, instead of cysts. This condition is characterized by enlarged ovaries, infertility, menstrual issues, a high male hormone level, acne, excess facial and bodily hair, and obesity. Women who have polycystic ovaries are at an increased risk of high blood pressure, diabetes, cardiac diseases and endometrial cancer (5).

The clinical symptom complex involves the presence of multiple ovarian cysts, amenorrhea or oligomenorrhea, and anovulation, and is frequently followed by abnormal hair growth on the body (hirsutism), infertility obesity and insulin resistance (6, 7). It has been proved that patients suffering from this syndrome are at an increased risk of cardiovascular diseases and carbohydrate metabolism disorders, as well as of developing dyslipidemia, obesity and insulin resistance. Thus, PCOS represents a significant metabolic entity, in addition to being an endocrinological one.

Pathophysiological Aspects of Polycystic Ovarian Disease

The pathophysiological aspects of polycystic syndrome are still not fully understood. There are three theories concerning the pathogenesis of PCOS: 1) disordered ovarian steroidogenesis theory, 2) insulin resistance theory, and 3) hypothalamus-pituitary axis theory (8). Previous findings indicate that the syndrome may have a genetic component causing PCOS development (a mother who suffers from PCOS during pregnancy has a higher androgen level, inducing changes in the ovaries of the female fetus, which leads to the possibility of the girl developing her mother's symptoms later in life), as well as exogenous factors, such as obesity, that may also play a key role in the development of PCOS. It has been noted that the levels of androstenedione in ovarian theca cells are twenty times higher in women suffering from PCOS than in healthy women. Moreover, the levels of 17α-hydroxyprogesterone (17OHP) and progesterone are also increased. This local ovarian hyperandrogenism is caused by the increased transcription of genes encoding steroid enzyme cytochrome P450-11A1, cytochrome P450-17α-hydroxylase/17-20 lyase and 3β-HSD in PCOS theca cells (9). CytochromeP450-17α-hydroxylase is the key enzyme in androgen synthesis both in the adrenal glands and in the ovaries. Anovulatory and ovulatory cycles also have increased androgen synthesis in polycystic ovarian theca cells. Thus, it cannot be the only reason for anovulation (10). Furthermore, increased LH secretion and excess androgen production are noticeable both in normal-weight women and in obese ones, though they are present in women suffering from PCOS, leading to elevated levels of insulin, which has not been implicated in the pathogenesis yet. Considering the fact that ovarian theca cells are sensitive to insulin, a vicious circle is formed, since obesity development is stimulated by ovarian androgen hyper-production. All these processes in which insulin resistance seems to be the critical point result in the polycystic appearance of ovaries (11).

Different polycystic ovarian disease phenotypes can be seen in clinical practice. Phenotypes A and B are classical phenotypes, although type A is characterized by hyper-androgenism, anovulation and polycystic ovaries, whereas type B does not necessarily possess such a specific ovarian morphology. A combination of hyperandrogenism and ovarian polycystic morphology is the characteristic of phenotype C, also known as the hyperandrogenic ovulatory phenotype. Finally, the characteristic of phenotype D, or the normoandrogenic anovulatory type, is the ovarian polycystic structure and anovulation.

The abnormal values of gonadotropin-releasing hormone (GnRH) in women suffering from PCOS lead to the hypersecretion of luteinizing hormone (LH) prior to ovulation. Then, oocyte ovulation failure is caused by the perturbation in the LH. This is one of the reasons why women with PCOS encounter menstrual issues. Some women suffering from PCOS have irregular ovulation, while others do not experience these at all. Such ovulatory issues decrease fertility in patients suffering from PCOS. In addition, anovulation may result in a continuous increase in estrogen levels, subsequently leading to increased endometrial thickness and the suppression of follicle-stimulating hormone production. Progesterone also plays a role in endometrial protection against the effects of estrogen, so it cannot be produced in women experiencing anovulation. All these factors might lead to the development of endometrial hyperplasia, which elevates the risk of possible progression to endometrial cancer in women suffering from PCOS (12).

PCOS also features increased levels of circulating insulin/hyperglycemia/hyperinsulinemia, produced in the β-cells of the pancreas. Hyperglycemia is believed to be caused by lifestyle or environmental factors (diet and obesity), and it eventually progresses to PCOS. Hyperinsulinemia stimulates the anterior pituitary, thus releasing LH and ovarian thecal cells, which leads to the production of ovarian androgen. In addition, it stimulates the hypothalamus to release

adrenocorticotropic hormone (ACTH) for the induction of adrenal androgen. Insulin inhibits the synthesis of insulin-like growth factor binding protein 1 (IGFBP-1) in the granulosa cells of the ovaries, resulting in the increased bioavailability of insulin-like growth factor 1 (IGF-1), and consequently the increased secretion of ovarian androgen. In addition, insulin is also able to increase the activity of cytochrome enzyme P450 17A1, which is an enzyme that synthesizes androgen in the ovaries. Furthermore, insulin inhibits the production of IGFBP-1 in the liver and elevates free IGF-1 which stimulates ovarian androgen synthesis. The production of sex hormone-binding globulin (SHBG) in the liver is also limited by insulin, which increases free testosterone levels, leading to hyperandrogenism (13, 14).

Meanwhile, high levels of testosterone, being one of the androgen hormones subtypes, and androgen receptor changes, such as the ones in the beta-3 adrenergic receptors and the alpha-2 (α2) adrenergic receptors, facilitate catecholamine-induced lipolysis in visceral adipose tissues. As a result, high levels of fatty acids accumulate in the liver, leading to obesity, which could eventually lead to complications, such as cardiovascular diseases (15).

Preventive and Therapeutic Strategies

In order to treat PCOS adequately, medication needs to be applied to increased tissue insulin sensitivity (a decrease in insulin resistance – insulin sensitization agents) (16, 17). Depending on the fertility status and the clinical manifestation of the disease, there are different types of current treatment protocols for managing PCOS. These may include oral hormonal contraceptives, lifestyle modification, ovulation induction protocols, application of leptin, the ovarian hippocampal signal path block, inositol or resveratrol treatment (18, 19). Most therapeutic protocols are efficient in alleviating certain symptoms but have no effect on others.

Lifestyle Changes

According to epidemiological data, insulin resistance is present in approximately 60–80% of the women suffering from PCOS. What is more, approximately 95% of obese women show insulin resistance, diabetes and increased cardiovascular risk (20). The first-line treatments for PCOS management in these patients are lifestyle changes. In this sense, it is recommended that a restrictive diet be administered, i.e. there should be a reduced daily caloric intake. The recommended intake should be in the range of 1,200–1,500 kcal/day, followed by a minimum of 30 minutes of moderate physical activity five times per week. The purpose of the lifestyle changes should be the reduction of the body mass index and lower alcohol and cigarette consumption to reduce insulin resistance and regulate menstrual cycles (21). In the randomized studies to date, certain authors have investigated the effects of diet and physical activity, demonstrating that there was a significant improvement in the ovary functions in obese patients suffering from PCOS after four months of lifestyle changes. The mentioned patients experienced a 6% reduction in the body mass index by implementing dietary changes, 3% achieved this by exercising, and 5% did so through combined treatments. It has also been established that the testosterone level was reduced in 69% of the patients. Such investigations have shown that reduced dietary intake, combined strategies, and moderate physical activity are effective in improving reproductive function in obese and overweight patients suffering from polycystic disease. Furthermore, a reduced body mass index has significantly contributed to the pregnancy rates and live birth rates in patients suffering from polycystic syndrome.

Oral Contraceptive Therapy

The chief therapeutic option in the management of PCOS is oral hormone contraceptive therapy. Oral contraceptive pills represent the key treatment when combating PCOS. There are currently three oral contraceptive pill types: progesterone-only medication, combined estrogen-progesterone medication and contraceptive pills for extended or continuous use. Contraceptive pills are the most commonly prescribed forms of contraception both in America and worldmide. Nevertheless, oral contraceptives may be utilized for the treatment of other conditions, especially menstrual cycle disorders, such as irregular periods, menstrual pain, endometriosis-related pain, fibroids and menstrual migraines. Certain combination pill brands have obtained official FDA approval for acne treatment. Although the majority of women take OCPs to prevent pregnancy, 14% use them for reasons unrelated to birth control (22–25).

Progestogen negative feedback acts on the hypothalamus in order to slow gonadotropin-releasing hormone pulse frequency. This, in turn, lowers the secretion of follicle-stimulating hormone (FSH) and the decrease in the luteinizing hormone (LH) (26, 27). Should a follicle not develop, no estradiol level increase occurs (as the follicle produces estradiol). Progestogen negative feedback and the lack of estrogen positive feedback regarding LH secretion terminate the LH increase mid-cycle. No developed follicle and no LH surge to release the follicles prevent ovulation. Since estrogen slows down FSH secretion, it does have a certain effect on follicle development inhibition owing to its negative feedback on the anterior pituitary gland, though it is not as pronounced as the progesterone effect. All this chiefly affects the reduction of very high luteinizing hormone levels. Moreover, certain

types of oral contraceptives possess extremely potent antiandrogens, such as chlormadinone acetate or cyproterone acetate.

Ovulation Inductors

One of the criteria in patients suffering from polycystic syndrome is ovulatory or anovulatory disorder, and an effective treatment for anovulatory disorder is ovulation induction (28). Ovulation induction drugs are clomiphene citrate, a selective estrogen receptor modulator; letrozole, an aromatase inhibitor; and gonadotropin. The usual initial dose of clomiphene is 50 mg/day, for f 5 days, from cycle day 2 to cycle day 5. Ovulation usually occurs within 5−10 days once the treatment has been completed. The dosage can be gradually increased to 250 mg/day provided ovulation is not achieved. If ovulation still fails to occur, the said patient is considered to be clomiphene-resistant. Approximately 15% of the patients suffering from PCOS fail to react to treatment. For this reason, they are regarded as clomiphene resistant. Clomiphene resistance risk factors include insulin resistance, obesity, elevated serum androgen levels, and old age (29).

Letrozole is an aromatase inhibitor of the third generation, which blocks androgen to estrogen conversion in the ovarian follicle, peripheral tissue and the brain, which creates a positive feedback loop with the estrogen of the hypothalamus-pituitary-ovary axis, leading to an endogenous GnRH release, promotes FSH secretion and stimulates the growth of follicles. The usual Letrozole dose is 2.5–5 mg/ day over five days, starting on days 4−8 of the menstrual cycle (30). Recently conducted studies indicate that selective estrogen receptor modulator clomiphene will be replaced as the first-line ovulation treatment with a letrozole aromatase inhibitor, which possesses a unique advantage for ovulation promotion in obese patients suffering from PCOS. Simultaneously, not only does letrozole exhibit less harmful effects on the cervical mucus, but it can also reduce ovarian hyperstimulation or other adverse effects, such as multiple pregnancies, and these are the unique safety advantages of the drug.

Androgens

Excessive androgen secretion in patients suffering from PCOS is in correlation with the biological synthesis and excessive androgen secretion of theca cells as well as reticular adrenal zone cells secreting testosterone. Women suffering from PCOS, hyperinsulinemia or insulin resistance have increased adrenal and ovarian gland functions. An effective androgen treatment is not free of side effects, especially in patients suffering from PCOS who are not undergoing treatment and who are at risk of developing cardiovascular disease. Regardless of whether a woman is obese or not, high testosterone levels are in direct correlation with hypotension, hyperglycemia and subclinical atherosclerosis. Oral contraceptives are currently the first choice in excessive androgen secretion treatment, although the combination of glucocorticoid (GCR) and norethindrone receptors in the polycystic syndrome will reduce the activity of fibrinolysin, another testosterone derivate and a recently revealed marker, but which has an antiandrogenic activity without any GCR receptor affinity. This derivate is expected to represent a new oral contraception form in the future, but it is being tested in clinical trials at present (31, 32).

Insulin-sensitizing Agents

Clinically, 60−80% of patients suffering from PCOS exhibit differing insulin resistance levels. Evidence suggests that biguanide metformin, an insulin-sensitizing agent, leads to the decrease of elevated testosterone levels through the reduction of glycogen production, thus improving glucose metabolism and inhibiting follicular membrane cells. Moreover, metformin could restore ovulation in approximately 30−50% of the women suffering from PCOS as well as regulate their menstrual cycles, but it was not as effective as clomiphene was when it came to fertility and pregnancy rates (33, 34).

Metformin can be classified as an anti-diabetic drug which improves insulin resistance. The mentioned drug represents the first-line treatment for type 2 diabetes, although it has been used in the pharmacotherapy of PCOS over the recent years (35). The basic action mechanisms encompass the inhibition of gluco-neogenesis and glycogenolysis, which results in the reduction of hepatic glucose production, consequently increasing the peripheral tissue's insulin sensitivity. Slowing down the intestinal absorption of glucose represents another relevant mechanism of metformin's action since the consequently decreased glucose amount is absorbed into the bloodstream. The literature contains a description of the therapeutic effects of metformin in patients suffering from polycystic ovarian disease, as well as the cases of improved ovarian function in such patients. Conversely, certain studies indicate that metformin does not lead to weight loss, as well as that the patients are resistant to the improvement of their lipid status (36, 37).

Vitamin D Deficiency

Literature data cites a correlation between the serum 25 (OH) vitamin D levels and sex hormone- binding globulin levels. This may be employed as a parameter for diagnosing endocrine abnormalities or PCOS metabolism. In patients who suffer from a vitamin D deficiency, vitamin D supplementation increases insulin secretion and insulin sensitivity. At the same time, vitamin D supplement decreases the overall androgen and testosterone levels, which is why it is expected to be an option treatment for PCOS (38, 39).

GLP-1 Polycystic Disease Management Agonists

Available GLP-1 therapy studies regarding e excess body weight management in women suffering from PCOS indicate that liraglutide and exenatide are effective in body weight reduction, either in the form of monotherapy or combined with metformin. Several studies have shown that androgen levels could be modestly decreased and menstrual frequency may be increased. Still, the limited data do not clearly present the obvious effect on blood pressure and lipid metabolism, with the most common sideeffect being nausea, though it does not affect the efficacy of the drug or the treatment (40).

Anti-androgen Drugs

Other drugs used for polycystic ovarian disease management include the application of spironolactone, which reduces triglycerides and increases the 'good' cholesterol levels; flutamide reduces serum total cholesterol, LDL and triglyceride levels; orlistat and acarbose reduce the digestion of polysaccharides in the GI tract.

Hirsutism, as one of the clinical entities, is one of the consequences of elevated circulating androgen levels. Flutamide has proven to be a relevant anti-androgen, although it could also lead to the improvement of the lipid profile. It is a nonsteroid antiandrogen which mediates its effect through the inhibition of androgen hormones in the target tissues. When compared with metformin effects and dietary modifications, flutamil considerably reduces androgens (41).

Spironolactone is another antiandrogen utilized in the pharmacotherapy of PCOS. In addition to the antiandrogenic effect, it has an antimineralocorticoid effect. However, treatment which involves solely spironolactone does not yield significant results, but combined with lifestyle changes, this medication results in reduced insulin resistance.

Assisted Reproduction Therapy

The third-line treatment of patients experiencing fertility issues involves assisted reproduction technology (ART). This therapy chiefly includes artificial fertilization (IUI), oocyte *in vitro* maturation (IVM), *in vitro* fertilization-embryo transfer (IVF-ET), and intracytoplasmic sperm injection (ICSI). IUI is adequate for patients suffering from PCOS who can achieve successful ovulation upon the conducted ovulation induction and may combine the male factor, immunological factor or cervical factor. In accordance with the latest ASPM research, the delivery rate remained stable after IUI, at 8.5% (8.3% in 2011) after the husband/partner's sperm (IUI-H) and 12.0% (12.2% in 2011) after the donor's sperm (IUI-D). Twin and triplet delivery rates related to IUI cycles were 9.0% or 0.4% and 7.2% or 0.5% after the treatment with the husebands and donor's semen. Conventional IVF-ET ovarian stimulation is used to collect a considerable number of follicles by utilizing the GnRH agonist, which is both costly and also poses a risk of ovarian hyperstimulation. It has also been observed in the study that, although IVM was lower than the ones in conventional IVF pregnancy, IVM is the simpler and safer ART method.

Surgical Treatment

As described by Stein and Leventhal in 1935, ovarian wedge resection was the first surgical treatment which was intended for anovulation correction in patients suffering from PCOS (42). Such a treatment could reduce endogenous androgen production and elevate the secretion of FSH, thus achieving natural ovulation. However, the approach has been discontinued owing to the considerable loss in ovarian tissue postoperative adhesion formations.

Inositols

Insulin signal transduction follows three main pathways: phosphatidylinositol (PI) 3-kinase (PI-3-K) pathway, mitogen-activated protein kinase (MAPK) pathway, and protein kinase C (PKC) pathway.

Clinical studies have shown that inositol reduces insulin levels in circulation, improves serum androgens, and regulates certain metabolic disorders (hypertriglyceridemia and elevated blood pressure) in women suffering from PCOS who have a body mass index lower than 26 kg/m^2 (43). A study investigating inositol efficacy (44) on the regulation of metabolic parameters and hormone levels at a dose of 500 mg per day, when administered to overweight patients (BMI > 26), showed a notable reduction in the LH, LH/FSH ratio, testosterone, androstenedione, fasting glucose, basal insulin, as well as the insulin ratio and body weight. During OGTT, insulin concentration is also significantly improved when it comes to the insulin maximal response and the insulin AUC (Area Under Curve) (45−47). These changes were observable in the whole study group, particularly in patients whose family history involved the occurrence of diabetes. Finally, the LH response during the GnRH test was also reduced upon the initiation of the inositol treatment.

The uptake of free inositol by tissues occurs through a membrane-dependent sodium-inositol cotransporter for which Myo-inositol shows a 10 times greater affinity than DCI does. Myo-inositol (MI) and DCI mediate inositolphosphoglicane (IPG) and have the function of second mes-sengers. Then, these mediators are internalized and modify intracellular metabolism and enzymatic activity, mimicking insulin activity (48). Myo-inositol is the most common inositol isomer in the human body. It decreases body weight and leptin secretion, but it increases HDL cholesterol (49); DCI is synthesized by an insulin-dependent

epimerase, which converts myo-inositol into DCI. The deregulation of epimerase activity affects the MI/DCI ratio, as in PCOS, where a defect in the use of Myo-inositol may compromise FSH and insulin signaling. There is a specific MI/DCI ratio related to the function of each organ (50). For this reason, high levels of DCI have been observed in glycogen storage organs. In the ovaries, DCI is responsible for the excess production of insulin-dependent testosterone, whereas Myo-inositol enhances FSH activity through the anti-Mullerian hormone (AMH). Myo-inositol has been found in follicular fluid (51) and it seems to improve oocyte and embryo quality.

The role of Myo-inositol and/or DCI supplementation has been studied in women with PCOS undergoing assisted reproduction technologies (ART) for the purpose of improving embryo quality, oocyte quality and pregnancy chances (52, 53). When applied three months prior to the initiation of ovarian stimulation, myo-inositol reduces the FSH doses required for a follicular response, decreases the concentration of estradiol on the ovulation induction day, by which it lowers the risk of ovarian hyperstimulation (54) and the number of cancelled cycles. Simultaneously, the quality of the ovary cells and the embryo is increased.

Women suffering from PCOS are four times more likely to develop diabetes than women not suffering from this disorder (55). These women are also at a higher risk of gestational diabetes (GD), which occurs in pregnancy. One study made an estimate that the mentioned risk is greater by almost 20% (56). Different research has shown that the MI supplementation intake might decrease GD and blood glucose in women suffering from PCOS and in overweight women (57). One study in gyneacological endocrinology indicated that the number of GD cases in pregnant women suffering from PCOS using MI was 17.4%, when compared to 54% in the women who were not using MI (58).

Clinical practice involves the use of D-chiro-inositol (DCI) for the induction of ovulation in women suffering from PCOS (59). Data obtained recently have confirmed the fact that the molecule acts via two different mechanisms that lead to potentially different outcomes. On the one hand, when viewed from the metabolic perspective, insulin signalling is improved by DCI which restores the physiological level of insulin in resistant individuals. On the other hand, it decreases the expression of steroidogenic enzyme aromatase, at a cellular level, which is responsible for androgen to estrogen conversion. DCI was first described as a molecule and mediator mimicking insulin. Afterwards, its role was discovered as an aromatase modulator in steroidogenesis (60).

Insulin sensitizing achieved through MI and DCI plays a crucial role in patients suffering from PCOS, which is seen as a decrease in the homeostatic model assessment (HOMA) index. Both mentioned isomers are useful in insulin resistance treatment (61).

Manganese pidolate (IUPAC name: manganese(2+);(2S)-5-oxopyrrolidine-2-carboxylate)

Manganese (Mn) is an essential intracellular activity nutrient; it functions as a co-factor for multiple enzymes, including glutamine synthetase (GS), arginase, pyruvate carboxylase and Mn superoxide dismutase (Mn-SOD). Through these metalloproteins, Mn plays a critical role in the development, digestion, antioxidative defence, reproduction, immune response, energy production, and the regulation of neuronal activities. It is found in almost all tissues and is required for blood glucose regulation, digestion, reproduction, and homeostasis (62). Manganese is among the most abundant trace elements included in bone growth, cell energy, the immune system and the healthy metabolism of proteins, lipids and carbohydrates (63). Considering the fact that manganese represents an integral component of manganese catalase and MN-superoxide dismutase (SOD), pyruvate carboxylase and arginase, it helps to minimize oxidative stress by detoxing superoxide free radicals (64, 65). The activities of the mentioned enzymes involve manganese in the metabolism of amino acids, glucose and cholesterol, but its key role is to eliminate reactive oxygen species from the body, as well as bone tissue synthesis and immune response improvement. Preclinical studies have shown that a deficiency of manganese may reduce bone mineral density and impair very bone formation, while supplementation could improve both bone formation and mineral density (65). Studies conducted in patients who are at an average age of 69.3 and suffering from osteoporosis have demonstrated that the levels of manganese were lower (20 mcg/L) in comparison with the healthy controls, at the mean age of 64.5 (40 mcg/L) (66). In another randomized clinical study involving postmenopausal women, manganese levels were correlated with bone mineral density (67).

In combination with vitamin K, manganese has a significant role in blood clotting and hemostasis. It is involved in carbohydrates, glucose and lipid metabolism performing the role of a cofactor of several enzymes. In addition, manganese deficiency may affect carbohydrate metabolism, leading to glucose tolerance abnormalities. Therefore, scientists have examined whether the status of manganese affects the risk of diabetes. A case-control cohort study in China has suggested a U-shaped association between type 2 diabetes and plasma manganese levels. 1,614 adults with type 2 diabetes (mean age of 52.5 years) and 1,614 adults without diabetes (mean age of 54.7 years) participated in this study. In comparison with the middle tortile of plasma manganese concentration (4.21−6.84 mcg/L), those in the lowest tortile (≤ 4.21 mcg/L) were 1.89 times more likely to have type 2 diabetes, while those in the group with the highest

tortile (≥ 6.84 mcg/L) were 1.56 times more likely to have type 2 diabetes (68).

Resveratrol

Resveratrol is a phytoalexin and a stilbene compound synthesized by plants when they respond to stressful stimuli, which are frequently caused by infection. RSV can be found in red wine, sherries and ports, red grapes, peanuts, blueberries, itadori tea, as well as pistachios hops, and in cranberry and grape juices. An increased action of the glucose transporter in the cytoplasmic membrane seems to result in the anti-hyperglycemic effects of resveratrol. What is more, resveratrol enhances the levels of adiponectin, which could be a potential mechanism for insulin sensitivity improvement (69). It is believed that resveratrol has beneficial effects on the cardiovascular system, since it has been found that it improves ischemic preconditioning and vasodilatation, both of which seem to be the result of endothelial NO synthase enzyme activation, and that it inhibits both vascular smooth muscle cell proliferation and platelet aggregation. Supplementation performed using a combination of resveratrol and alpha-lipoic acid exerted positive effects on the BMI, total and trunk adipose mass, as well as lean tissue mass, in obese women suffering from PCOS. Supplementation with the use of these dietary substances may be beneficial for glycemic control, weight loss, or both (69).

Conclusion

The pathophysiology of the PCOS is highly complex, but it has not fully been defined yet. Due to a variety of pathophysiological mechanisms, it is accompanied by a wide spectrum of symptoms. This is why an individual approach is a basic principle in the treatment of patients suffering from polycystic ovaries. Namely, on the basis of the phenotype, characteristics of the phenotype, biochemical indicators, and specific symptoms, the treatment mostly involves different therapeutic combinations. Furthermore, during regular check-ups, it is common to realize that certain therapeutic option combinations have not completely achieved our therapeutic goal. It is for this reason that novel combinations have to be considered. Such situations are not uncommon, but it must be taken into consideration that the treatment of patients with polycystic ovaries is a dynamic long-term process, and we trust that new therapeutic approaches will be available in the near future.

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Pregledni rad **UDC: 618.11-006-085**
doi: 10.5633/amm.2024.0315

SAVREMENI TERAPIJSKI PRINCIPI U LEČENJU BOLESNICA SA SINDROMOM POLICISTIČNIH JAJNIKA

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Poremećaj funkcije jajnika predstavlja stanje u kojem dolazi do neravnoteže, odnosno poremećaja stvaranja polnih hormona; usled toga nastaju poremećaji u menstrualnom ciklusu i smanjena sposobnost začeća ili održavanja trudnoće. Poremećaj funkcije jajnika može biti primaran, usled patofiziologije na nivou jajnika, ili sekundaran; tada poremećaj nastaje kao posledica poremećaja drugih žlezda poput hipofize i štitne žlezde. Sindrom policističnih jajnika složen je endokrinološki poremećaj, a prvi put je opisan 1935. godine. Najčešći je uzrok sekundarne amenoreje, a smatra se i najčešćim uzrokom ovulatorne disfunkcije žena u reproduktivnom periodu. Termin sindrom odnosi se na skup kliničkih karakteristika ili fenotip. Specifične karakteristike klasičnih fenotipova sindroma policističnih ovarijuma podrazumevaju kliničke znakove viška androgena, povišene koncentracije androgena u serumu, neredovne menstruacije i neplodnost kao posledicu. Takođe, zbog česte udruženosti sa insulinskom rezistencijom i hiperinsulinemijom mora se posmatrati i lečiti i kao metabolički poremećaj. Upravo je zbog kompleksnosti različitih poremećaja koji se mogu videti u različitim fenotipovima terapijski pristup vrlo kompleksan i individualizovan. Ovaj pregledni rad zasnovan je na pretraživanju celokupne raspoložive literature u dostupnim bazama podataka i praktično daje prikaz svih terapijskih opcija u lečenju bolesnica sa sindromom policističnih ovarijuma koje su danas dostupne. U svakom slučaju, potrebno je sprovesti još istraživanja da bi se razjasnila složena patofiziologija sindroma policističnih ovarijuma. Stoga, neophodne su dodatne prospektivne epidemiološke studije.

Acta Medica Medianae 2024; 63(3):116–126.

***Ključne reči**: sindrom policističnih jajnika, inozitol, metabolički profil, insulin, LH, FSH*

Original article **UDC: 616-007**
doi: 10.5633/amm.2024.0316

CLINICAL FEATURES OF 22Q11.2 DELETION SYNDROME: A LITERATURE REVIEW AND CASE SERIES REPORTS

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The 22q11.2 deletion syndrome is the most common microdeletion syndrome. The clinical features show a broad spectrum of multisystem manifestations and include congenital heart defects, hypoparathyroidism associated with hypocalcemia, hypoplasia of the thymus and subsequent immunodeficiency, distinctive facial features, velopharyngeal insufficiency, and developmental delay or learning disabilities. Retrospective analysis of medical data was conducted and the spectrum of clinical manifestations of case series of five patients with 22q11.2 deletion syndrome is presented. A small series of patients with a 22q11.2 deletion syndrome is described, but still a sufficient number that undisputably displays a recognizable spectrum of manifestations. All patients expressed elements of facial dysmorphism and signs of immune system dysfunction which ranged from lymphopenia and recurrent respiratory infections to congenital defect in T cell immunity. Almost all of the reported patients had associated conotruncal congenital heart defects, and the majority of cases presented with hypocalcemia and elements of motor and developmental delay. Increased awareness of multisystemic features of 22q11.2 deletion syndrome is pivotal for early recognition and early initiation of comprehensive care and treatment.

Acta Medica Medianae 2024;63(3): 127–132.

Key words: *22q11.2 deletion syndrome, thymus, congenital defect in T cell immunity, hypocalcemia, congenital heart defects*

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Introduction

The 22q11.2 deletion syndrome is the most common microdeletion syndrome occurring with an estimated frequency of 1:3000 to 1:4000 live births. The 22q11.2 deletion syndrome is a multisystemic disorder that includes physical, cognitive, and behavioral problems. Previously, several disorders with a specific deletion in 22q11 region have been reported in the literature, such as velocardiofacial syndrome, DiGeorge syndrome, and a group of disorders described by the acronym CATCH22 (cardiac defect, abnormal facial features, thymic hypoplasia, cleft palate, hypocalcemia) (1, 2).

Chromosome 22 is an acrocentric chromosome and deletion occurs on the proximal part of the long arm of one chromosome from the chromosome pair. The 22q11 region contains several low-copy-number repeat sequences and among them deletion of regions may occur. Heterozygous deletion of the 22q11.2 region in most patients includes the loss of region size of 2.54Mb in which there are approximately 40 genes (3). So far haploinsufficiency of the TBX1 gene (T-box transcription factor 1 gene) in this region has been linked to many of the manifestations in 22q11.2 deletion syndrome. The TBX1 gene provides instructions for the synthesis of proteins which play important roles during embryonic development, including the migration of neural crest cells and formation of the pharyngeal arch and pouches (2, 4).

The spectrum of clinical manifestations in the 22q11.2 deletion syndrome shows multisystem involvement and significant variability in the severity of their presentation (Table 1). The main phenotypic features include the presence of a congenital heart defect, hypoparathyroidism associated with hypocalcemia, hypoplasia of the thymus and an immune deficiency, distinctive facial features as well as cleft palate or velopharyngeal insufficiency. Affected individuals may also have developmental delays, including

Table 1. Characteristic phenotypic features in patients with 22q11.2 deletion syndrome (1−3, 6−8)

Organ system involvement	Phenotypic Features	Frequency in 22q11.2 deletion
Immune disorders	Athymia/Thymic hypoplasia/Ectopic thymus Impaired T cell production and function CD4+ lymphopenia/ and lower CD3 counts Severe combined immunodeficiency Reduced natural T regulatory cells/autoimmunity	25−75%
Endocrine disorders	Thyroid gland aplasia/ hypoplasia Parathyroid gland dysfunction Growth hormone deficiency	30−65%
Genitourinary anomalies	Renal agenesis Multicystic dysplastic kidney Hydronephrosis Absent uterus/Hypospadias/Cryptorchidism	6−35%
Cardiovascular anomalies	Tetralogy of Fallot Isolated aortic arch anomaly Hypoplastic left heart syndrome Valvular pulmonary stenosis Ventricular septal defect	35−85%
Palatal anomalies	Velopharyngeal insufficiency and hypotonia Cleft palate/Submucous cleft palate/Bifid uvula	more than 67%
Gastrointestinal disorders	Gastroesophageal reflux Esophageal atresia Hirschprung disease	about 30%

delayed speech development or learning disabilities (5).

The facial dysmorphism is characterized by a constellation of several phenotypic features, however sometimes mild and not so evident. These features include an elongated face, hypertelorism, wide nasal bridge, hooded eyelids, epicanthus, long nose with a bulbous tip, small mouth and low-set, posteriorly rotated, small ears.

The 22q11.2 deletion syndrome is the most common cause of syndromic palatal anomalies and velopharyngeal dysfunction, but also one of the most frequent causes of developmental delay and congenital heart disease (2). Additionally, it is most commonly associated with conotruncal heart defects such as tetralogy of Fallot, pulmonary atresia with ventricular septal defect, truncus arteriosus and aortic arch anomalies (6).

The aim of this report was to present the spectrum of clinical manifestations of the 22q11.2 deletion syndrome in our patients.

Material and Methods

A retrospective analysis of patients diagnosed with 22q11.2 deletion syndrome examined during regular outpatient visits to the Genetics Clinic, Clinic of Pediatrics, University Clinical Center in Niš during one year was conducted. Medical data from outpatient medical records during the year 2023 were reviewed and collected. This evaluation included only patients with a confirmed diagnosis of 22q11.2 deletion syndrome.

Data extracted from patient medical reports included demographic data, perinatal events and neonatal history, developmental milestones, presence of facial dysmorphism, presence of congenital heart defects or other congenital anomalies, signs of immune system dysfunctions, disorders of calcium metabolism and other disorders of importance. All extracted data were summarized, evaluated and presented.

Results

In this retrospective analysis 5 patients (3 boys and 2 girls) with confirmed diagnosis of 22q11.2 deletion syndrome were evaluated. The age of the patients ranged from 4 months to 11 years (Table 2).

In the majority of reported patients, a diagnosis of 22q11.2 deletion syndrome was confirmed at an early age (in 3 patients within the first 6 months of life, in 1 patient at the age of 22 months). On the other hand, in one boy (patient number 5) there was a clinical suspicion from infancy, but genetic testing was done later and a characteristic microdeletion was identified. There were no identifiable significant risk factors in prenatal and perinatal periods. All patients were born at term, with appropriate birth weight and good parameters and vital signs at delivery.

The spectrum of clinical manifestations in our group of patients is presented in Table 3. All children had distinctive facial features, which in one case were discrete and mild (patient number 3). Three of five patients presented with complex

Table 2. The main characteristics of our patients with 22q11.2 deletion syndrome

Patient	Gender	Age at the time of examination	Age at diagnosis confirmation
No. 1	Female	4 months	1 months
No. 2	Male	9 months	2 months
No. 3	Female	11 months	6 months
No. 4	Male	4.5 years	22 months
No. 5	Male	11 years	10.5 years

Table 3. The main clinical manifestations of our patients with 22q11.2 deletion syndrome

Patient	No. 1	No. 2	No. 3	No. 4	No. 5
Facial dysmorphism	Typical	Typical	Mild	Typical	Typical
Congenital heart disease	Interrupted aortic arch. VSD	ASD. VSD	ASD	T.Fallot	T.Fallot
Serum calcium level	-	Hypocalcemia	Hypocalcemia, neonatal seizure	-	Hypocalcemia
Disorder of immune function	Defect in T cell immunity	Defect in T cell immunity	Lymphopenia	Recurrent infections	Recurrent infections
Motor/intellectual development	-	Slight motor delay	Slight motor delay	Motor delay speech delay	Global develop-mental delay
Other manifestations	-	-	Velopharyngeal insufficiency	-	Hypospadias, renal ectopia

*VSD: ventricular septal defect; ASD: atrial septal defect; T.Fallot: tetralogy of Fallot

congenital heart defects at birth, such as interruption of the aortic arch (patient number 1) and Tetralogy Fallot (patients 4 and 5). These patients underwent cardiac surgery. In the remaining two children, acyanotic congenital heart defects (atrial and/or ventricular septal defect) were detected. Hypocalcaemia was found in three children (patient's number 2, 3 and 5) during the early neonatal period and just in one case manifested with seizures. However, the hypocalcemia in this patient was transient and resolved following short therapy followed by a gradual increase in oral dietary calcium intake.

A broad spectrum of immune system dysfunction was registered in the children presented in this report, ranging from lymphopenia and recurrent respiratory infections to an innate defect in the T cell immune response. All patients were referred to an immunologist for the assessment of immune dysfunction and further follow-up including surveillance of vaccination. All patients except one (patient number 5) were included in the preventive respiratory syncytial virus (RSV) immunoprophylaxis.

Two out of three of our patients at age below one year showed a slight delay in acquiring motor skills. On the other side, the older patients (first at the age of four and half years, and second at the age of eleven years) showed elements of motor and speech delay or even global developmental delay that required the initiation of a development stimulation program.

Discussion

The 22q11.2 deletion syndrome manifests with broad phenotypic variability, making early diagnosis challenging. However, the diagnosis of 22q11.2 microdeletion is often suspected in the presence of congenital heart defects, palatal defects and symptomatic early hypocalcemia associated with varying facial dysmorphism (9).

In this report we present a small case series of patients with a 22q11.2 deletion syndrome, but still a sufficient number that undisputably display a recognizable spectrum of manifestations.

Almost all of our patients had a congenital heart defect, described in the literature as being associated with 22q11.2 deletion syndrome, while an isolated atrial septal defect was detected only

in one patient. According to the literature, cardiovascular conotruncal anomalies are present in almost 80% of neonates with 22q11.2 deletion syndrome (6, 10). The most common anomalies include interrupted aortic arch, tetralogy of Fallot, truncus arteriosus, and ventricular septal defects, which corresponds to the frequency and spectrum of cardiac anomalies detected in our group of patients.

Infants with 22q11.2 deletion syndrome require diagnostic evaluation regarding immunodeficiency, considering that this problem is caused by aplasia or hypoplasia of the thymus, or disorders in the number and function of thymocytes (10). Immunodeficiency, even in cases of hypoplasia or aplasia of the thymus, can be mild to moderate and occurs in 40−77% of patients, while only 0.5−1% of patients have severe immunodeficiency (11). Recurrent infections show the highest frequency in the first years of life. After that period the risk of frequent infections decreases. Medical records of our patients showed T cell deficiency in two patients, while the other patients showed mild immune disorders, taking into account lymphopenia and frequent respiratory infections. Although the majority of children with 22q11.2 deletion syndrome have T cell lymphopenia, immunodeficiency is highly variable and dynamic, and immune status should be determined before immunization with live vaccines (12). At the same time, during the first 2 years of life RSV immunoprophylaxis should be recommended, especially in the presence of congenital heart disease (13). RSV immunoprophylaxis was administered to almost all our patients to prevent serious respiratory disease, including the patient presented with atrial septal defect and coexisting velopharyngeal insufficiency.

Hypoparathyroidism with subsequent hypocalcemia was detected in approximately 60 −70% of children with 22q11.2 deletion syndrome. Hypocalcemia may be manifested at any age but is most severe during the neonatal period presenting as hypocalcemic seizures, or during periods of stress, perioperative period or acute illness (9, 12). In our group of patients, 3 out of 5 (that corresponds to 60%) exhibited hypocalcemia during the neonatal period, including the occurrence of neonatal seizures in one case. Dietary calcium intake and vitamin D supplementation should be considered in those patients and monitoring of calcium serum levels, parathyroid hormone and vitamin D is highly recommended (12).

Palatal abnormalities with velopharyngeal dysfunction can be detected in about two-thirds of children, including cleft palate and may be related to pharyngeal hypotonia, feeding and swallowing disorders, or even obstructive sleep apnea symptoms (9). The group of children presented in this report showed a low frequency of velopharyngeal dysfunction, except in one child with consequent severe feeding problems.

Among 22q11.2 deletion syndrome patients some less frequent symptoms may appear, such as: renal abnormalities, laryngeal-tracheo-esophageal abnormalities, vertebral abnormalities, polydactyly, scoliosis, thrombocytopenia, hearing loss or microcephaly (14). In our group of patients one child presented with hypospadias and renal ectopia.

Children with 22q11.2 deletion syndrome often present with developmental, cognitive, speech-language and communication disorders (12). During adolescence behavioral abnormalities can occur as manifestations of psychiatric disorders such as anxiety and depression (14). Almost all of our patients have manifestations related to motor skills delay, or even global developmental delay with behavioral problems. Neurologic evaluation, early interventions and stimulation of development (physical, occupational and speech therapies) can optimize better neurodevelopmental achievements.

Conclusion

Increased awareness of pediatricians about the spectrum of phenotypic and multisystemic features in 22q11.2 deletion syndrome is essential for an early suspicion, recognition and detection of disease, as well as early initiation of comprehensive multidisciplinary care and treatment.

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Originalni rad **UDC: 616-007**
doi: 10.5633/amm.2024.0316

KLINIČKE KARAKTERISTIKE SINDROMA DELECIJE 22q11.2: PREGLED LITERATURE I PRIKAZ SERIJE SLUČAJEVA

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Sindrom delecije 22q11.2 najčešći je mikrodelecioni sindrom. Kliničke karakteristike pokazuju širok spektar multisistemskih manifestacija, a najčešće uključuju urođene srčane mane, hipoparatireoidizam sa hipokalcemijom, hipoplaziju timusa i posledičnu imunodeficijenciju, karakteristične crte lica, velofaringealnu insuficijenciju i kašnjenje u razvoju ili smetnje u učenju. Urađena je retrospektivna analiza medicinske dokumentacije i prikazan je spektar kliničkih manifestacija kod serije od pet bolesnika sa 22q11.2 delecionim sindromom. Premda je opisana serija bolesnika sa sindromom delecije 22q11.2 mala, broj ispitanika bio je dovoljan da nesporno dočara prepoznatljiv spektar kliničkih manifestacija u pomenutom sindromu. Kod svih bolesnika bili su prisutni elementi facijalnog dismorfizma, kao i parametri disfunkcije imunosistema koji su se manifestovali od limfopenije i rekurentnih respiratornih infekcija do urođenog deficita T-ćelijskog imuniteta. Skoro svi prikazani bolesnici imali su udružene urođene konotrunkalne srčane mane, a većina obolelih ispoljila je hipokalcemiju i elemente motoričkog kašnjenja i razvojnog kašnjenja. Povećana svest o multisistemskim karakteristikama sindroma delecije 22q11.2 ključna je za rano prepoznavanje i blagovremeno otpočinjanje sveobuhvatne nege, praćenja i lečenja obolelih.

Acta Medica Medianae 2024; 63(3):127–132.

***Ključne reči**: sindrom delecije 22q11.2, timus, urođeni deficit T-ćelijskog imuniteta, hipokalcemija, urođene srčane mane*

Original article

UDC: 612.017:616.155.2-085
doi: 10.5633/amm.2024.0317

THROMBOPOIETIN RECEPTOR AGONISTS IN THE TREATMENT OF PRIMARY IMMUNE THROMBOCYTOPENIA: OUR EXPERIENCE

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The primary immuna thrombocytopenia (ITP) is an acquired autoimmune disease characterized by isolated thrombocytopenia PLT < 100 x 10^9/L, and the absence of all conditions and diseases that can result in thrombocytopenia. The first-line therapy in ITP involves the use of corticosteroids, intravenous immunoglobulin or an immunoglobulin anti-D. The second-line treatment includes splenectomy, immunosuppressive drugs and agonists of thrombopoietin receptor (TPO-RA). To describe the treatment results with TPO-RA (eltrombopagin patients with ITP in the Clinic of Hematology UCC NIš. Between March 2018 and December 2023, at the Clinic of Hematology UCC Niš, 6 patients with ITP in which the previous treatment lines did not respond to the therapy or gave side effects were treated with TRO-RA. The indication for the TRO-RA therapy was chronic ITP. The period from the diagnosis to the initiation of the treatment with TRO-RA was on average 71,5 months. The analysis of the average number of platelets after TPO-RA therapy showed an upward trend. The TPO-RA does not show immunosuppression, they lead to an increase in platelet count, stopping bleeding and improving the quality of life. Therefore, TPO-RA are essential medicines for the treatment of ITP after the failure of the first and second - line therapy.

Acta Medica Medianae 2024;63(3):133–139.

Key words: *immune thrombocytopenia, treatment of immune thrombocytopenia, thrombopoietin receptor agonists*

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Introduction

Primary immune thrombocytopenia or idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disease in which platelet destruction occurs under the action of primary antiplatelet antibodies against platelet glycoproteins GPIIb/IIIa, GPIb/IX. These antibodies not only coat platelets but also damage megakaryocytes (1–5).

There is also evidence that ITP is a disease of T lymphocytes (different subpopulations of CD4 T cells) where the loss of immune tolerance to self-antigens is thought to be a consequence of the formation of antibodies and cytotoxic T lymphocytes directed against the patient's own platelets and megakaryocytes. The activation of CD4 T cells can be an initial event in the development of ITP, which is reflected in B cells to produce antiplatelet antibodies (1, 4).

Idiopathic thrombocytopenic purpura is characterized by isolated thrombocytopenia, a platelet count of less than 100 x 10^9/L and the absence of all conditions and diseases that can lead to thrombocytopenia (1, 6).

ITP is more common in women aged 20 to 50 years who were generally healthy until then.

The International Working Group (IWG) defines ITP as: 1. newly diagnosed (the diagnosis established up to 3 months); 2. persistent (3 - 12 months from the diagnosis) or 3. chronic (lasting for more than 12 months) (1, 5). They also clearly defined severe and refractory ITP. Severe ITP is reserved for patients who have clinically relevant bleeding, defined as bleeding at the presentation of sufficient magnitude to mandate treatment or by the occurrence of new bleeding symptoms requiring additional interventions or an increase in drug dose. Refractory ITP is defined as the presence of severe ITP occurring after spleenectomy (7).

The goal of treating patients with ITP is to avoid serious bleeding, excessive treatment and maintain a normal quality of life. The treatment is initiated in patients with a platelet count of less than 30 x 10^9/L, as well as in patients with a higher platelet count who exhibit spontaneous bleeding or have an increased risk of bleeding (6, 8).

The first therapeutic line in the treatment of ITP is corticosteroids at a dose of 0.5−2 mg/kg body weight/day for 2−4 weeks, including a period of gradual reduction of the corticosteroid dose (9). The expected time to respond to the therapy is from a few days to a maximum of 4 weeks, while the use of corticosteroids is accompanied by many side effects such as corticosteroid dependence, diabetes, Cushing's disease, hypertension, gastric disturbances (1, 6, 9, 10, 11) .

The reticuloendothelial blockade is achieved by using intravenous immunoglobulins or anti RhD immunoglobulin in Rh-positive patients (6).

Intravenous IgG immunoglobulins are also an effective method of treatment and are administered at a dose of 1 g/kg/day for 1−2 days, or 0.4 g/kg/day for 5 days. The expected response time after the administration of intravenous immunoglobulins is 2−4 days, but they lead to a temporary increase in the number of platelets for 2−4 weeks. Due to the high cost and short-term effect, their use is limited to emergency cases, preparation for surgical interventions, and in cases where the use of corticosteroids is contraindicated (1, 10, 11).

Administration of anti-RhD immunoglobulin can cause reticuloendothelial blockade only in Rh-positive patients, using a higher dose of about 1.2 mg in adults. It gives a much faster therapeutic response (within a few minutes to a few hours) compared to IV immunoglobulins, but it can be less effective (1, 10, 11).

If no response is achieved with the first line of therapy, the treatment is carried out with the second line (1, 10, 11).

The second therapeutic line is spleenectomy with a frequency of long-term remission of up to 65%, lasting 5−10 years, while about 20% of patients relapse in the first two years. This type of treatment has a double effect, it removes the site of the breakdown of platelets coated with antibodies as well as the main site of the antibody synthesis. In the last 10 years, splenectomy has been performed less often, and the reasons are lifelong immunocompromise and new therapeutic modalities for the treatment of ITP (1, 5, 6, 10, 11).

The second therapeutic line includes immunosuppressive drugs, such as azathioprine, cyclophosphamide, vinblastine or vincristine, cyclosporine A, danazol, anti-CD20 monoclonal antibody and thrombopoietin receptor agonists (TPO-RA) (6, 7).

Thrombopoietin receptor agonists (TPO-RA) represent a new class of drugs in the treatment of ITP. Unlike other drugs for ITP, which achieve their therapeutic effect by reducing the production of antibodies and reducing the breakdown of platelets, TPO-RA stimulate megakryocytopoiesis (5, 12). This group of drugs includes eltrombopag (Revolade) and romiplostin (N-PLATE) (13) which are recommended for patients resistant to conventional treatment, i.e. the first line of therapy, or splenectomy (9). Eltrombopag is a small peptide molecule that exerts its effect by activating the same signalling pathways as endogenous thrombopoietin, stimulates the proliferation and differentiation of megakaryocytes and their precursors and thus leads to an increase in the number of platelets, cessation of bleeding and improvement in the quality of life in 80% to 90% of patients with chronic ITP (4, 12, 14, 15, 16). It is administered orally, in a dose of 25−75 mg daily and the therapeutic response is achieved after 1 to 5 weeks from the beginning of the administration. The most common adverse reactions (headache, weakness, arthralgia, elevated transaminase values, bone marrow fibrosis, thrombosis, etc.) are mild to moderate (2, 14, 16). Sometimes they can cause renal weakness, which requires screening of kidney function (9).

Aim

The aim of the study was to present the treatment results of patients with ITP using TPO-R agonists in the Clinic of Hematology, Allergology and Clinical Immunology.

Material and Methods

Six patients with chronic ITP, with no response to the previous therapeutic lines and without side effects, were treated using eltrombopag, at the Clinic of Hematology, Allergology and Clinical Immunology from March 2018 to December 2023. No patient was spleenectomized. Since 2017. patients have been treated at the expense of the Republic Health Insurance Fund.

The criteria for chronic and refractory ITP are defined by the recommendations of the IWG.

The TPO-R agonist (eltrombopag) was administered in the range of 25 mg to 75 mg orally, daily with a permissible dose escalation up to 75 mg or dose reduction up to 25 mg according to recommendations. The dose of eltrombopag is individual, adapted to each patient, in order to achieve and maintain the number of platelet count above 50 x 10^9/L for at least 4 weeks with the aim of reducing the risk of bleeding (1, 8, 10).

The treatment was initiated at a platelet count of less than 20−30 x 10^9/L, as well as in patients with a higher platelet count who exhibited spontaneous bleeding or had an increased risk of bleeding. Patients were prohibited from using aspirin, non-steroidal anti-inflammatory drugs, intramuscular injections and inappropriate physical activity (8).

The drug was administered 2 hours before or 4 hours after a meal while avoiding foods with a high calcium content. During the therapy, it was necessary to control the blood count once a week until the platelet count reached above 50 x 10^9/L, and then once a month with regular control of transaminases and bilirubin. After 2 weeks of starting therapy with eltrombopag, the number of platelets increased, and after the termination of the treatmentafter 1 to 2 weeks the number of platelets decreased.

The response to the therapy was defined as CR-complete response (a platelet number greater than 100 x 10^9/L and the absence of bleeding); PR-partial response (a platelet count greater than 30 x 10^9/L with the absence of bleeding) or no response (a platelet count less than 30 x 10^9/L, with hemorrhagic syndrome and/or corticosteroid dependence (1). A stable increase in platelets > 50 x 10^9/L was considered a good therapeutic response (13, 17).

Results

Six patients with ITP were treated with eltrombopag, out of whom 2 (33%) were male and 4 (67%) were female (Figure 1).

The average age of all patients at the time of starting eltrombopag therapy was 45.6 years. The youngest patient was 22 years old, and the oldest was 70 years old.

The indication for the introduction of TPO-RA therapy (eltrombopag) was chronic ITP. Three patients previously used 4 therapeutic lines (corticosteroids, azathioprine, cyclophosphamide, vinca alkaloids), two patients used 2 therapeutic lines (corticosteroids and azathioprine), and one patient used 3 therapeutic lines (corticosteroids, azathioprine, cyclophosphamide) (Table 1).

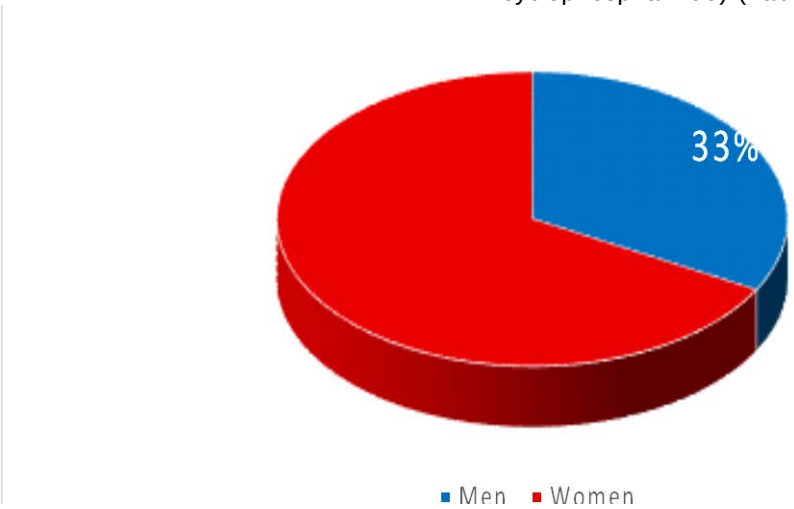

Figure 1. Patients with primary immune thrombocytopenia by gender

Table 1. Previous lines of therapy in patients with primary immune thrombocytopenia

Patients with ITP	Splenectomized patients	Therapeutic lines before the introduction of eltrombopag
1.	No	Corticosteroids Azathioprine
2.	No	Corticosteroids Azathioprine Cyclophosphamide Vinca alkaloids
3.	No	Corticosteroids Azathioprine Cyclophosphamide Vinca alkaloids
4.	No	Corticosteroids Azathioprine
5.	No	Corticosteroids Azathioprine
6.	No	Corticosteroids Azathioprine Cyclophosphamide

The time from diagnosing ITP to the initiation of the TPO-RA therapy was, on average, 71.5 months (range 24−144) (Figure 2) and number of platelets at the time of introduction of TPO-RA was 21 x 10^9/L (range 8−33).

In one patient, the treatment started with eltrombopag at a dose of 25 mg per day, and after six months the dose was increased to 50 mg by the number of platelets; in two patients, the treatment was carried out with a dose of eltrombopag 50 mg, which was maintained during the duration of the therapy; in three patients, the treatment started with 50 mg of eltrombopag and the dose was increased to 75 mg after six months, while in one of them, in addition to the increase of the drug dose, corticosteroid therapy was also included.

The response time after the inclusion of eltrombopag was 4 to 6 weeks.

The average length of eltrombopag administration was 38.16 months (range 3−69 months), and the average platelet count for that period was 79.12 x 10^9/L (range 13 −333 x 10^9/L).

The analysis of the average number of platelets after the introduction of TPO-RA showed the following trend: for the first patient, the average number of platelets was 122 x 10^9/L (range 33−189); for the second patient, the average number of platelets was 91 x 10^9/L (range 28−141); for the third patient, the average number of platelets was 24.1 (range 8 −71); for the fourth, the average number of platelets was 88.4x10^9/L (range 28−223); for the fifth patient, the average number of platelets was 73.9 x 10^9/L (range 13−333); for the sixth patient, the average platelet count was 75.3 x 10^9/L (range 16−139) (Table 2).

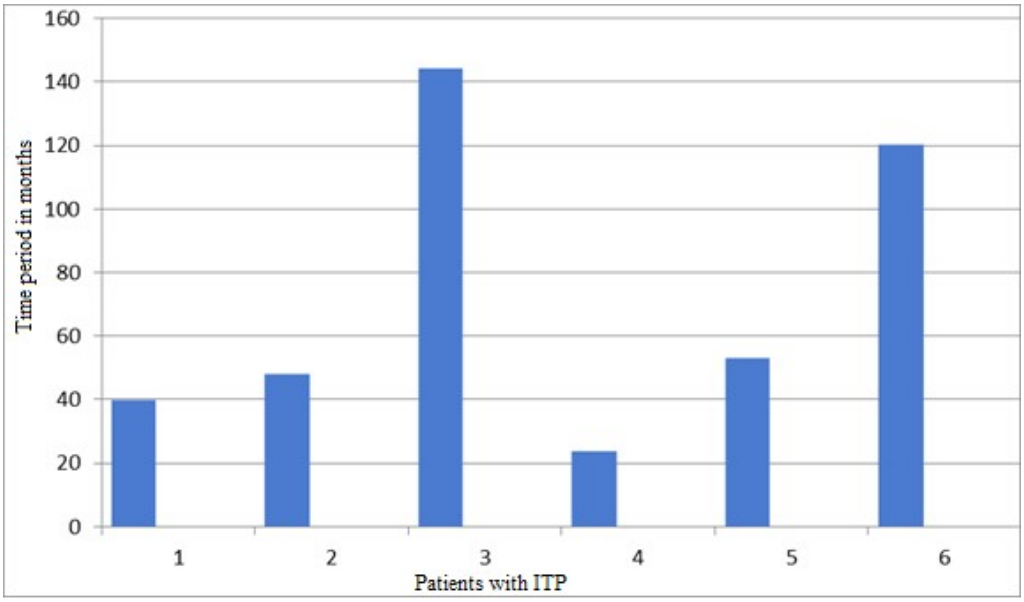

Figure 2. Time period in months from diagnosis of ITP to initiation of therapy with thrombopoietin receptor agonists

Table 2. Effect of eltrombopag therapy in patients with primary immune thrombocytopenia

Patients with ITP	Platelet count before eltrombopag administration	Platelet count after eltrombopag administration	Concomitant therapy	Side effects
1.	33x10^9/L	189X10^9/L(CR)	Without therapy	No
2.	28x10^9/L	149X10^9/L(CR)	Without therapy	No
3.	8x10^9/L	71X10^9/L (CR)	Corticosteroids	No
4.	28x10^9/L	223X10^9/L	Without therapy	No
5.	13x10^9/L	333x10^9/L	Without therapy	No
6.	16x10^9/L	139x10^9/L	Without therapy	No

The patients showed good therapeutic response; 5 patients completely responded to the included therapy, except for one patient who showed a partial response, but without hemorrhagic syndrome (in the absence of bleeding).

There were no unwanted effects during the administration of the drug.

Discussion

Immune thrombocytopenia is a disease that is more common in women (17), with a median age at the time of diagnosis of 56 years, with an incidence that increases with age. In our small group of patients, the median age was 45.6 years.

The aim of ITP treatment is to achieve a stable number of platelets that prevent the development of bleeding. TPO-R agonists are second-line drugs for the treatment of chronic ITP. The effect is manifested from of 2 to 5 weeks from the beginning of the application, in our group of patients from 4 to 6 weeks, which corresponds to the data from the literature (17, 18). Eltrombopag is well tolerated, and the most common adverse reactions are headache, nausea, and nasopharyngitis. Reversible increase in transaminases may occur, although no adverse reactions were recorded in our small group of patients.

All our patients responded to the therapy with a good therapeutic response after 4 to 6 weeks. In one patient, a stable response was maintained by adding corticosteroids. In one patient with a platelet count above 200 x 10^9/L, the dose was reduced to 25 mg, and the possibility of discontinuation of therapy or maintenance therapy was considered. The well-known data in the literature are that in most patients, discontinuation of TPO-R therapy leads to relapse of the disease, but about 20% maintain remission I after discontinuation of therapy (19).

Conclusion

The second-line therapeutic modalities available in Serbia for the treatment of chronic ITP are few (azathioprine, cyclophosphamide, vinca alkaloids), and treatment results are variable and unpredictable (therapeutic response 30−35%), and side effects are numerous. The efficacy and safety of drugs second-line have not been verified in randomized, double-blind, placebo-controlled studies (8, 10). TPO-R agonists do not show immunosuppression, they activate the same signaling pathways as endogenous TPO and lead to an increase in the number of platelets, cessation of bleeding and improvement in quality of life (17, 18). Their efficiency is 80−90% and they are well tolerated. For this reason, TPO-R agonists are necessary drugs for the treatment of ITP after the failure of the first and second line of therapy (16, 18).

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Organilani rad **UDC: 612.017:616.155.2-085**
doi: 10.5633/amm.2024.0317

AGONISTI TROMBOPOETINSKIH RECEPTORA U LEČENJU PRIMARNE IMUNSKE TROMBOCITOPENIJE: NAŠE ISKUSTVO

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Primarna imunska trombocitopenija (ITP) jeste stečena autoimuna bolest koja se odlikuje izolovanom trombocitopenijom Tr < 100 x 10^9/L i odsustvom svih stanja i bolesti koje mogu dovesti do trombocitopenije. Prva terapijska linija podrazumeva primenu kortikosteroida, intravenskih aplikovanih imunoglobulina ili anti-D imunoglobulina. Druga terapijska linija obuhvata splenektomiju, imunosupresivne lekove i agoniste trombopoetinskih receptora (TPO-RA). Cilj ove studije bio je da prikaže rezultate lečenja bolesnika sa ITP-om koji su na Klinici za hematologiju Univerzitetskog kliničkog centra u Nišu lečeni agonistima trombopoetinskih receptora (eltrombopagom). U periodu od marta 2018. do decembra 2023. godine primenom eltrombopaga lečeno je šest bolesnika sa ITP-om kod kojih je došlo do izostanka odgovora na prethodne linije terapije ili do ispoljavanja neželjenih efekata. Indikacija za uvođenje eltrombopaga bio je hronični ITP. Vreme od postavljanja dijagnoze ITP-a do otpočinjanja terapije TPO-RA iznosilo je u proseku 71,5 meseci. Analiza prosečnog broja trombocita po uvođenju TPO-RA pokazala je trend porasta broja trombocita, bez neželjenih efekata. Agonisti TPO-RA ne pokazuju imunosupresiju, dovode do porasta broja trombocita, prestanka krvarenja i poboljšanja kvaliteta života. Upravo zato, agonisti TPO-RA predstavljaju neophodne lekove za lečenje ITP-a posle neuspeha prve i druge terapijske linije.

Acta Medica Medianae 2024; 63(3):133–139.

***Ključne reči**: imunska trombocitopenija, terapija imunske trombocitopenije, agonisti trombopoetinskih receptora*

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