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The 2017 Nobel Prize in Physiology or Medicine laureats:
Left - Prof. Jaffrey C. Hall, Ph.D. (born 1945, New York, NY, USA; Affiliation at the time of the award**:** University of Maine, Maine, ME, USA);
Center - Prof. Michael Rosbash, Ph.D. (born 1944, Kansas City, MO, USA; Affiliation at the time of the award: Brandeis University, Waltham, MA, USA, Howard Hughes Medical Institute);
Right - Prof. Michael W. Young, PhD. (born 1949, Miami, FL, USA; Affiliation at the time of the award: Rockefeller University, New York, NY, USA)
They are jointly awarded for their discoveries of molecular mechanisms controlling the circadian rhythm.

ORIGINAL ARTICLES

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Estimation of loneliness in students with visual impairments

Procena usamljenosti studenata sa oštećenjem vida

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Abstract

Background/Aim. Loneliness is becoming more frequent, especially in young people. Some authors believe that visual impairments increase the risk of loneliness. Empirical data on its manifestation in persons with visual impairments are contradictory. The aim of this research was to determine the degree of loneliness in students with visual impairments and their peers from general population. **Methods.** A comparative research was conducted on a sample consisting of 36 visually impaired students and 101 students without visual impairments (control group). Students with visual impairments were divided into 3 subgroups with regard to the degree of visual impairments (students with low vision, legally blind, and totally blind students). University of California Los Angeles Loneliness Scale (which assesses the general loneliness) and Social and Emotional Loneliness Scale (which assesses social loneliness, family loneliness, and romantic loneliness) were used in our investigation. **Results.** The results showed that the general loneliness was significantly lower in the group of students with visual impairments than in the control group ($t = 2.121$; $p = 0.036$). There were no significant differences in the Social and Emotional Loneliness Scale between the group with visual impairments and the control group. No significant differences were determined in the level of loneliness among students with a different degree of visual impairments. There are significant differences in the manifestation level of social loneliness, family loneliness, and romantic loneliness (Wilk's lambda = 0.604; $p \leq 0.000$) in the group of students with visual impairments. Romantic loneliness was manifested the most, followed by social loneliness, while family loneliness was the least manifested. **Conclusion.** With regard to the results of our research, visual impairment alone is not a crucial factor of loneliness in students with visual impairments. Further studies on protective factors of loneliness can contribute to its prevention in young people with visual impairments.

Key words:
visual disorders; vision, low; students; loneliness; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Usamljenost je sve učestalija, posebno u populaciji mladih. Neki autori smatraju da oštećenje vida povećava rizik od usamljenosti. Empirijski podaci o ispoljenosti kod osoba sa oštećenjem vida su kontradiktorni. Cilj našeg istraživanja bio je utvrđivanje stepena usamljenosti kod studenata sa oštećenjem vida i njihovih vršnjaka iz opšte populacije. **Metode.** Komparativno istraživanje je obavljeno na uzorku koji je činilo 36 studenata sa oštećenjem vida i 101 studenata bez oštećenja vida (kontrolna grupa). Na osnovu stepena oštećenja vida, studenti sa oštećenjem vida bili su podeljeni u tri podgrupe (slabovidi, praktično slepi i potpuno slepi). U našem istraživanju korišćeni su *University of California Los Angeles Loneliness Scale* (za procenu opšte usamljenosti) i Skala socijalne i emocionalne usamljenosti (za procenu socijalne, porodične i usamljenosti u ljubavi). **Rezultati.** Analiza rezultata je pokazala da je opšta usamljenost bila značajno manja u grupi studenata sa oštećenjem vida nego u kontrolnoj grupi ($t = 2.121$; $p = 0.036$). Na Skali socijalne i emocionalne usamljenosti nije utvrđena statistički značajna razlika između grupe studenata sa oštećenjem vida i kontrolne grupe. Takođe, nije utvrđena značajna razlika u nivou usamljenosti između studenata sa različitim stepenom oštećenja vida. U grupi studenata sa oštećenjem vida postojala je značajna razlika u stepenu ispoljenosti socijalne usamljenosti, usamljenosti u porodici i usamljenosti u ljubavi (Wilk's lambda = 0.604; $p \leq 0.000$). Najviše je bilo ispoljena usamljenost u ljubavi, sledi socijalna usamljenost, dok je usamljenost u porodici bila najmanja. **Zaključak.** Prema rezultatima našeg istraživanja, samo oštećenje vida nije presudni činilac usamljenosti kod studenata sa oštećenjem vida. Buduća istraživanja protektivnih faktora usamljenosti mogu doprineti prevenciji ove pojave kod mladih sa oštećenjem vida.

Ključne reči:
vid, poremećaji; vid, oslabljen; studenti; usamljenost; ankete i upitnici.

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Introduction

Great technological advances, fast way of life, high aspirations and expectations are all associated with loneliness which is becoming more and more frequent, especially in young people. This may seem strange if we consider the increased exchange and availability of information, easier communication, and new jobs and vocations. Obviously, the main feature of loneliness is not the number of social contacts, but their quality [1].

Loneliness is defined as a negative experience mainly related to interpersonal relations and basic trust formed in the earliest childhood [2]. It is also seen as a result of an unsuccessful social interaction [3], i.e. subjective dissatisfaction with interpersonal relations due to changes in current social relations or changes in needs for social relations [3, 4], or as an unwanted feeling of lack or loss of friendship, an unpleasant aspect of the lack of certain relations and a certain quality level in different relations [5].

Pinquart and Sörensen [6] make a distinction between two types of definitions of loneliness. Definitions of the first type state the feeling of suffering which results from a lack of contact, while the second ones are socially-cognitive definitions which observe loneliness as a discrepancy between interpersonal relations an individual has and wants to have. A complete model of the cause of loneliness is given by Rokach [7] through the following three-cluster model: lack of interpersonal relations; stressful events; personal and developmental variables (determined by the factor of developmental disability…).

While some believe that people differ only in the degree of loneliness, others think that there are different types of loneliness which differ both in their pre-conditions and in their characteristics. Weiss [8] believes that loneliness primarily depends on an individual's perception that his/her needs in relations with others are not satisfied and that social and emotional loneliness are different experiences resulting from deficits in different types of relations. Social loneliness can be caused by the lack of meaningful friendship and unity and is accompanied by boredom and the feeling of social isolation. Emotional loneliness results from the lack of intimate devotion to another person, non-existence of a romantic relationship, and is accompanied by anxiety, distress and the feeling of emptiness. Social loneliness is caused by the lack of close friends, while emotional loneliness is caused by the lack of closeness with friends [9]. Although the differences are obvious, there are still many things which both types of loneliness have in common [10]. After verifying Weiss's [8] distinction, Di Tommaso and Spinner [11, 12] singled out 3 factors corresponding to the domain or the type of relations: family, romantic relationships, and friends.

Coping with visual impairments is a multidimensional process which requires an individual to adapt emotionally, physically and socially. Loneliness can reduce an individual's adaptability [13]. Adolescents with visual impairments are at a higher risk of social isolation, they have fewer friends, and inadequate social skills [14, 15]. Visual impairments can be associated with depression, anxiety and loneliness [16, 17]. There are also beliefs that visual impairments alone need not always cause problems in psychosocial functioning [18].

Bearing in mind general increase in the incidence of loneliness, the increased risk of loneliness in adolescence, and some authors' belief that visual impairment increases the risk of loneliness, as well as contradictory empirical data on the manifestation of loneliness in people with visual impairments, we decided that the subject of our research will be loneliness in university students with visual impairments.

The aim of this study was to determine whether there are differences between university students with and without visual impairments in the degree of loneliness and to determine the level of loneliness in students with visual impairments with regard to the degree of visual impairment.

Methods

The sample

Students with visual impairments (VI) were our target group. The control group (C) consisted of students who were at the same level/year of studies at the same faculties, and who are from the same cities (Belgrade or Novi Sad) as the students in the group with visual impairments. The research was conducted at Faculties of Social Sciences and Humanities at the University of Belgrade, Serbia and the University of Novi Sad, Serbia from July to December 2014.

The criteria for the selection of participants were: university students who meet all their pre-exam obligations, without a disability, except visual impairments in the VI group, or multiple disabilities and without mental health problems.

The additional selection criteria in the VI group were: visual impairment according to the definition of the World Health Organization, the formal status of students with a disability (they have records at the University Centre for Students with Disabilities, which provide them with the opportunity to be included in additional support programs). There were 60 such students at the time of our research. Seven students studying only for financial benefits were excluded.

The sample consisted of 137 participants in total, out of whom 36 were in the VI group, and 101 students who responded to an invitation on a certain day were in the C group. With regard to the degree of visual impairments, there were 12 (33.3%) students with low vision, 10 (27.8%) legally blind and 14 (38.9%) totally blind students.

There were 21 girls and 15 boys in the VI group, while there were 86 girls and 15 boys in the C group. The groups were equal regarding the participants' age. However, they were not equal with regard to the gender of the participants, since students of social sciences and humanities are mostly girls.

Participation was anonymous and voluntary. All the students, among whom there were 36 out of 60 students with VI, who agreed to participate and gave their written informed consent.

The following instruments were used in collecting data:

University of California Los Angeles (UCLA) Loneliness Scale, short form. This short form of UCLA

Loneliness Scale was developed for the purpose of measuring general loneliness, i.e. loneliness as one-dimensional construct. Factor analysis really did single out only one factor [19]. Numerous studies support the stability of the previous versions of UCLA Loneliness Scale on samples of various ages, education levels, and socio-economic statuses [20, 21]. The advantage of UCLA short form is the act that it is applicable to different groups of people since even with a smaller number of items, it has the same level of reliability as its previous versions [19]. This short form consists of 7 items, with responses given on a five-point Likert type scale (1 – I always feel this way; 2 – I often feel this way; 3 – I sometimes feel this way; 4 – I rarely feel this way; 5 – I never feel this way). Theoretical results may vary from 7 to 35. Cronbach's alpha in our research was 0.81.

Social and Emotional Loneliness Scale by Ćubela-Adorić and Nekić [22]. The original instrument – Social and Emotional Loneliness Scale [11] was developed as a result of verifying Weiss's distinction between social and emotional loneliness. It consists of 3 subscales: the social loneliness subscale (13 items), the family loneliness subscale (11 items) and the romantic loneliness subscale (12 items), for which responses are given on a seven-point Likert type scale (1 – I completely disagree; 2 – I mainly disagree; 3 – I disagree to some extent; 4 – I neither agree nor disagree; 5 – I agree to some extent; 6 – I mainly agree; 7 – I completely agree). Theoretical results may vary from 13 to 91 on the subscale of social loneliness, from 11 to 77 on the subscale of family loneliness, and from 12 to 84 on the subscale of romantic loneliness. Cronbach's alpha reliability coefficient in our research was: 0.938 for social loneliness; 0.851 for family loneliness; 0.925 for romantic loneliness.

Data processing

Data obtained in this study were processed by SPSS, version 19. Arithmetic mean differences of groups in the degree of loneliness were checked by *t*-test for independent samples or univariate analysis of variance (ANOVA), and additionally by Welch's F statistics. We used Levene's Test of Homogeneity of Variances for checking variance homogeneity. From multivariate statistical procedures, we used multivariate analysis of variance (MANOVA) and Bonferroni post hoc test.

Results

Table 1 shows that the degree of general loneliness was significantly higher in students without visual impairments than in students with visual impairments.

On the Scale of Social and Emotional Loneliness, there were no statistically significant differences between groups in social, family and romantic loneliness including total loneliness too (Table 2).

The results of ANOVA showed that there were no significant differences in the degree of general loneliness among the subgroups of the VI group ($F(2.33) = 0.675$; $p = 0.516$).

ANOVA showed that there were no significant differences among the subgroups in romantic loneliness ($F(2.33) = 1.180$; $p = 0.320$) and in total loneliness ($F(2.33) = 1.592$; $p = 0.219$). Welch F statistic indicates that there were no significant differences in social loneliness (the significance of Levene's Test of Homogeneity of Variances was 0.010; $F(2.18.930) = 2.076$; $p = 0.153$) and in family loneliness

Table 1

Differences in the degree of general loneliness between the visual impairment (VI) and the control group (C)

Group	n	Degree of loneliness ($\bar{x} \pm SD$)	*t*	df	*p*
VI	36	11.22 ± 4.120	2.121	135	0.036
C	101	13.18 ± 4.951			

$\bar{x}$ – arithmetic mean; SD – standard deviation; df – degree of freedom.

Table 2

Differences in the degree of loneliness on the Scale of Social and Emotional Loneliness between the visual impairment group (VI) and the control group (C)

Type of loneliness	Group	$\bar{x} \pm SD$	*t*	df	*p*
Social	VI	31.00 ± 17.509	-1.102	135	0.276
	C	27.51 ± 12.275			
Family	VI	21.69 ± 8.162	0.677	135	0.500
	C	22.84 ± 8.924			
Romantic	VI	38.44 ± 21.109	-0.340	135	0.734
	C	37.15 ± 18.872			
Total	VI	91.13 ± 37.382	-0.584	135	0.560
	C	87.51 ± 29.861			

For abbreviations see under Table 1.

among the subgroups of the group VI (the significance of Levene's Test of Homogeneity of Variances was 0.006; F(2.15.292) = 3.039; $p = 0.077$).

Table 3 shows average scores of different types of loneliness. Multivariate ANOVA (MANOVA) showed that there were significant differences among different types of loneliness (social loneliness, family loneliness, romantic loneliness) in the degree of their manifestation in the VI group (Wilk's Lambda = 0.604; F(2.34); $p = 0.000$; Eta squared = 0.396).

Bonferroni post hoc test tested the significance of differences among three types of loneliness. Table 4 indicates that there were significant differences among all three types of loneliness in the degree of their manifestation in the VI group. Romantic loneliness was manifested the most, followed by social loneliness, while family loneliness was the least manifested.

Table 3

Differences among different types of loneliness in the degree of their manifestation in the visual impairment group

Type of loneliness	Average score of loneliness $\bar{x} \pm SD$
Social	2.3846 ± 1.34686
Family	1.9722 ± 0.74200
Romantic	3.2037 ± 1.75915

For abbreviations see under Table 1.

Table 4

Differences among three types of loneliness in the degree of their manifestation in visual impairment group

Type of loneliness	Degree of loneliness	
	DM	*p*
Social and family	0.412	0.039
Social and romantic	-0.819	0.029
Family and romantic	-1.231	0.000

DM – difference of means.

Discussion

Some authors have reported on the higher degree of loneliness in young people with visual impairments than in those without such impairments [23–26]. There is some empirical data which is contradictory [27]. Although it was determined that 15% experience very strong feelings of loneliness, the research showed that adolescents with visual impairments experience strong feelings of happiness and that most of them are not very lonely [28]. Some authors agree that blind adolescents experience loneliness more often than those with low vision [27, 29].

The non-existence of a higher degree of loneliness in adolescents with visual impairments, when compared to their peers from the general population, is usually attributed to imprecise scales. However, there are also explanations that worse results associated with the psychosocial functioning of the blind and those with low vision are perhaps the result of stereotypes and stigmatization [30].

Our results show that there are significant differences between the VI and the C group with regard to general loneliness. Unexpectedly, a higher degree of loneliness was found in the group of students without visual impairments. We can make assumptions about the causes of such a result. Students with visual impairments more often need support and are directed to other people whom they rely upon and with whom they communicate regularly, while typically developing students probably spend more time in performing activities on their own (e.g. using the internet, social networks…).

As for the results on three subscales of loneliness, there were no differences found between the VI and the C group. Both groups of students are equally satisfied with their relations with family members, friends, and partners. They probably have an adequate circle of people in their surroundings, who regardless of their number, make them feel satisfied, since loneliness is a subjective feeling which depends on the experience of the quality of relations.

Uneven distribution of the participants with regard to gender may have influenced the obtained results, indicating a higher degree of general loneliness in the control group and nonexistence of significant differences in the degree of loneliness as a multidimensional construct between groups VI and C. There are many more girls in the control group, and studies show that girls are significantly more lonely than boys. It is also possible that students with visual impairments who agreed to participate in this research are more open, communicative, and less lonely than those who refused to participate. We should also consider the possibility that students with visual impairments wanted to present themselves in as positive manner as possible. Why should these reasons be more important for the participants with visual impairments than for those from the control group? Participants with visual impairments are always aware that studies test the effects of visual impairments on…, and that they are "the centre of attention". Testing things that have a negative connotation, such as loneliness, may increase their censorship (giving suitable answers). Doubt in the anonymity of data may be increased by the manner of filling in questionnaires – a participant does it for them, while those who can see do it themselves.

Researches that compare loneliness of the blind and those with low vision are scarce. Huurre and Aro [29] state that blind adolescents feel lonely more often than those with low vision. According to same authors, people with low vision have more difficulties in making friends [23]. Research conducted by Gold et al. [31] dealt with emotional relationships of blind young people and those with low vision and showed that persons with low vision were involved in emotional relationships more frequently than the blind.

In the matter of all this, we expected significant differences in the degree of loneliness among the participants with different visual impairments in our research. Since there were no statistically significant differences found among those with low vision, legally blind and totally blind students either in general loneliness or in specific types of loneliness, our assumption was not confirmed. The methodological reason for this lack of differences could be a small number of participants in the subgroups. However, the arithmetic means are almost identical, which is in favor of the results accuracy. This finding is significant because it proves that visual impairment alone, i.e. the degree of the impairment, is not a crucial factor of loneliness in students with visual impairments.

Having analyzed the results, a significant difference was determined by the level of manifestation of different types of loneliness in students with visual impairments. The results showed that romantic loneliness is manifested the most, followed by social loneliness, while family loneliness is manifested the least. Some studies have dealt with similar problems. Gold et al. [31] found that only 16% of youth with visual impairments aged between 25 and 29 are married, compared to 27% of general population. Young people with visual impairments face problems in starting and maintaining intimate relationships and probably enter into a relationship with a partner at an older age [26, 27].

Visual impairments can affect making and keeping friends. Several studies have identified different barriers in social functioning of youth with visual impairments, which can cause the feeling of social loneliness in our research: rejection by their peers, negative reactions to visual impairments, problems because of underdeveloped social skills and difficulties with mobility [31–33].

More attention should be paid to enabling students with visual impairments to participate in social activities which offer them opportunities to develop their social networks [27].

Students with visual impairments feel the least lonely within their family, which is not surprising since people with visual impairments largely rely on family members. This is also confirmed by other studies, where most adolescents with visual impairments have close relationships with their parents and have the biggest support from their family members [15, 18].

Limitations of this research are a small number of students with visual impairments and unevenness between the VI and the C groups regarding the number and gender of the participants.

Conclusion

Loneliness must be considered seriously since it is a very common condition which leads to a depression. In people with visual impairments, loneliness impedes their adaptation which in many aspects represents a compromise between the requirements of social surroundings and the requirements of the impairment itself.

As a prevention from feeling left out and lonely, young people should have a good network of friends, access to social activities, and appropriate support, which will make further development of social networks easier. Participation in social activities can be achieved by organizing different workshops and creating opportunities for inclusive activities (e.g. interactive learning with mutual support, taking part in cultural performances). Further studies should include a wide range of contextual factors: social status, family size, type of household, family completeness, social support, etc. More qualitative studies need to be conducted on the relation between loneliness and the quality of relationships in persons with visual impairments. Understanding what "protects" youth and adults with visual impairments from loneliness can help both in solving problems when they appear, and their prevention.

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The effect of illegal lead processing on blood lead levels in children living in the mining area

Uticaj nezakonite prerade olova na nivo olova u krvi dece u rudarskoj zoni

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Abstract

Background/Aim. The northern part of Kosovo was one of the largest lead and zinc production industries in Europe. Special attention has been paid to the landfill sites of these metals remained after past industrial activities. The inhabitants of Roma camps in this area are collecting led waste they process by crushing and melting in their shacks in primitively organized working environments. Because of all the aforementioned it was necessary to examine the concentration of blood lead level (BLL) in the children aged less than 6 years inhabiting this area, especially taking care of blood analysis of children living in Roma camps. **Methods.** The study was conducted in the municipality of Leposavić, Province Kosovo and Metohija, Serbia. Totally 78 subjects participated in the study. All the subjects were divided into two groups: the group I consisting of 42 children who lived in the Romas camp, and the group II with 36 children from a city kindergarten. Based on the mathematical model WRPLOT we found out that both groups of patients were in the low risk zone for industrial contamination exposure. Blood analysis was done according to the protocol provided by ESA Lead Care. **Results.** The average age of participants in the study was 4.60 ± 1.63 years. The mean BBL in the children from the group 1 was 19.11 μg/dL and from the group 2 4.87 μg/dL. There was a statistically significant difference in the mean values of BBL between the groups ($U = 39$, $p < 0.001$). All of the children from the group 1 had BBL greater than 5 μg/dL in comparison to 38.9% of the children from the group 2 ($\chi^2 = 35.75$, $p < 0.001$). **Conclusion.** Although both groups were located outside the zone of direct spread of pollution, the results indicate high concentrations of lead in blood of all the examined children. The concentration was higher in the children who lived in the area in which illegal processing of lead waste took place.

Key words:
lead poisoning; child, preschool; serbia; environmental pollutants; blood chemical analysis.

Apstrakt

Uvod/Cilj. Na području severnog Kosova nalazila se jedna od najvećih industrija za proizvodnju olova i cinka u Evropi. Poseban akcenat se stavlja na deponije zaostale nakon ove industrijske proizvodnje. Na ovom području stanovnici u romskim kampovima bave se prikupljanjem olovnog otpada koji prerađuju – drobe i tope, u svojim barakama u primitivno organizovanim radnim sredinama. Zbog svega navedenog, bilo je neophodno ispitati koncentraciju olova u krvi dece mlađe od šest godina koja žive u ovom području, sa posebnim akcentom na analizu krvi dece koja žive u romskim kampovima. **Metode.** Naše istraživanje sprovedeno je na području opšine Leposavić, Kosovo i Metohija, Srbija, uključujući 78 ispitanika podeljenih u dve grupe: grupa I od 42 dece iz romskog kampa, i grupa II od 36 dece iz gradskog vrtića. Na osnovu matematičkog modela WRPLOT dobili smo podatak da se obe grupe ispitanika nalaze u zoni niskog rizika od izloženosti industrijskom zagađenju. Krv za nalizu uzimali smo iz prsta dece. Analiza krvi vršena je prema protokolu predviđenom od ESA *Biosciences Lead Care*. **Rezultati.** Prosečna starost ispitanika iznosila je 4,60 ± 1,63 godine. Prosečna koncentracija olova u krvi u grupi I bila je 19,11 μg/dL, a u grupi II 4.87 μg/dL. Postoji statistički visokoznačajna razlika u pogledu koncentracije olova u krvi

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između ispitivanih grupa (U = 39; $p < 0,001$). Sva deca (100%) iz grupe I imali su koncentracije olova u krvi veće od 5 μg/dL, a iz grupe II ($\chi^2 = 35,75$; $p < 0,001$) njih 38,9%. **Zaključak.** Iako su obe grupe bile locirane van zone direktnog širenja zagađenja, rezultati ukazuju na visoke koncentracije olova u krvi sve ispitivane dece. Koncentracija je veća kod dece koja žive u sredini u kojoj se odvija ilegalna prerada olovnog otpada.

Ključne reči:
trovanje olovom; deca, predškolska; srbija; životna sredina, zagađivači; krv, hemijske analize.

Introduction

The presence of substantial increases in lead (Pb) levels in the environment leads to increased risk of increased blood lead level (BLL) in people [1, 2]. Children under 6 years of age are at a particular risk of environmental lead [3]. Lead gets into a child's body by ingesting or inhaling lead dust. As a consequence of industrial pollution, lead particles fall out of the air to the ground and stick to soil dust, exposing the children to inhaling dust while playing outdoors. Children may also be exposed to lead by eating food contaminated by secondary transfer of lead from soil to plants and animals.

The absorption of lead from the gastrointestinal tract in children is significantly greater than in adults (according to literature data children absorb lead five times more efficiently than adults), and the food intake per unit body weight is more than that of adults [4, 5]. Also, heavy metals are metabolized faster in children than in adults. Children are particularly vulnerable to the toxic effects of lead because of their ongoing growth and development and not fully matured bodies [6]. So far there has been no medical treatment that permanently reverses the neurodevelopmental effects of lead exposure [7]. Evidence suggests BLL ≤ 5 μg/dL are associated with cognitive deficits [8]. Apart from this, exposure to lead may affect a child's IQ [9, 10]. These effects are long-lasting and persist into adulthood even after lead exposure has been reduced or eliminated [11, 12]. Further lead exposure in children, even in low concentrations, may cause slow growth, anemia, hearing and hyperactivity disorders [13–16]. Some authors describe a proof of concept gene-environment interaction studies of early life Pb^{2+} exposure in mice expressing the human mutant form of the disrupted in schizophrenia 1 (DISC-1) gene, a gene that is strongly associated with schizophrenia and allied mental disorders [17].

In 2012, the Centres for Disease Control and Prevention (CDC) changed the "actionable" reference BLL from 10 μg/dL to 5 μg/dL [18].

The northern part of the province of Kosovo was one of the largest lead and zinc production industries in Europe, which caused a legacy of widespread environmental pollution with heavy metals [19–23]. Special attention has been paid to the landfill sites of these metals remained after past industrial activities. The Roma population of this region from the camps collects waste material, including lead. They process the collected lead waste – crush and melt in their barracks in primitively organized workplaces. After waste processing and blending into lead ingots, they are still illegaly sold.

Earlier studies have showed increased BLL in the population of northern part of Kosovo [24]. The World Health Organization Regional Office for Europe (WHO-EURO) assessed in 2004 that 25% of children aged 2–3 years in the general population in the area had elevated (≥ 10 μg/dL) BLL, according to WHO unpublished data. However, new studies have not been conducted so far.

Due to all of these reasons, it was necessary to analyze the BLL in children of this region, especially in Roma children living in the camps where their families are suspected of informal lead smelting activities.

The aim of the paper was to determine BLL in all the subjects and identify the differences in BLL between the children living in Roma camps where informal and unsafe lead processing has been practiced and the children living outside the camps. All the children were from the municipality of Leposavić in northern Kosovo, Serbia.

Methods

The study was conducted in the municipality of Leposavić in northern Kosovo (Figure 1), known for lead and zinc mines and processing and industrial landfill sites that are the

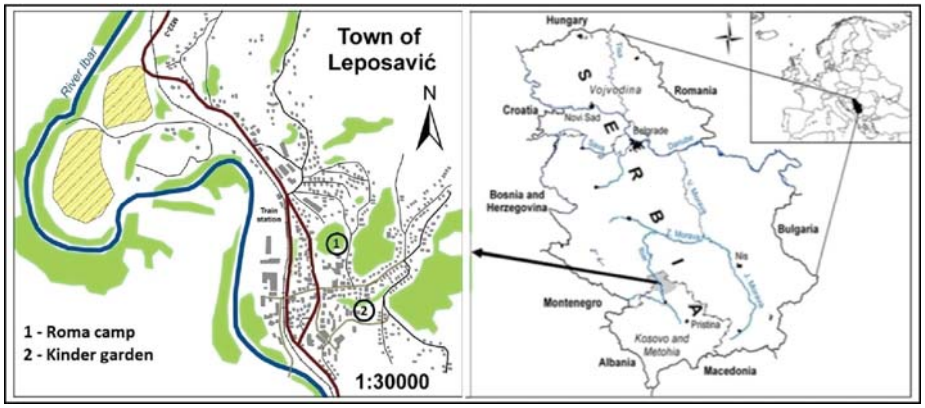

Fig. 1 – Geographical location of the area near Leposavić where blood samples were taken.

major cause of environmental contamination. There is also a Roma camp with illegal and primitive lead waste processing.

The study was conducted in cooperation with Roma people living in the camps aiming at better control of overall health conditions and improvement of their quality of life. The Standard of Good Practice have been applied in the study. The parents of the children had been informed on the procedure of blood sampling and the importance of the study. An informed consent was given to each parent by the doctors involved in the study to be signed on voluntary basis.

Totally 78 subjects participated in the study (47 males and 31 females). All the subjects were divided into 2 groups. One group consisted of children who lived in the Roma camp, the group I of 42 participants, and the group II of children from the kindergarten, the group II of 36 participants. Blood sampling was performed in this institution for faster and more efficient study performance. The average age of the subjects was 4.56 ± 1.52 years.

A difference in pollution diffusion caused by industrial waste depots between the industrial and residential zones was determined by the mathematical model provided in the WRPLOT view TM 7.0.0. software (Figure 2). The children from both groups lived in the zones with lower levels of industrial contamination, but the children from the Roma camps were additionally exposed to lead due to lead waste processing in the camp.

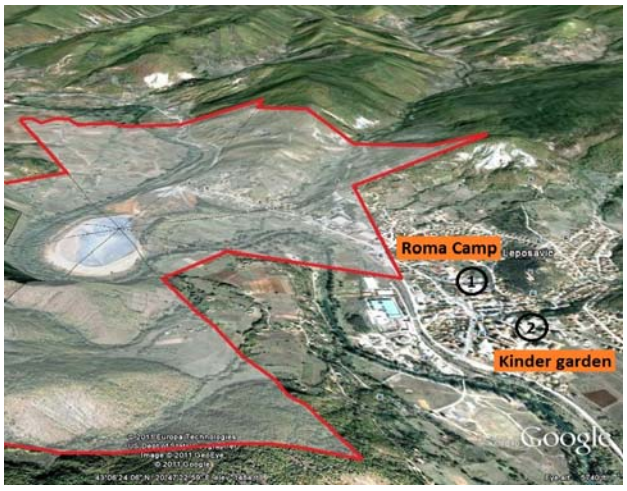

Fig. 2 - Presentation of the zones of propagation of industrial pollution from landfills (shaded – zone of a greater risk from industrial pollution; unshaded – zone of lower risk from industrial pollution; black circle with the number – places of residence of the study children).

In our study, capillary blood samples were collected from fingertips. Children's fingers were prepared by alcohol wipe according to the CDC guidelines and samples were collected into capillary tubes following a finger prick. Capillary whole blood was collected in to metal-free phlebotomy tubes by a registered medical doctor. The sampling was done in one day, in the period from 7 h to 18 h. The process of sampling was made by ensuring there was no lead contamination from the surrounding environment.

Blood analysis in our study was done according to the protocol provided by ESA Biosciences Lead Care [25].This device has been used in some previous studies as well [24, 26, 27]. The maximum BLLs detection limit for the instrument was 65 μg/dL. The ESA lead care instrument was calibrated after the testing of every 48 samples. In order to control the impact of temperature on the BLLs analysis, the samples were analyzed immediately after the sample collection. All blood samples were analyzed at room temperature at the Public Health Institute in Kosovska Mitrovica.

Statistical analysis

The descriptive statistical method, statistical hypothesis testing and dependency testing were used in the study for the analysis of the primary data. The distribution of the sample data was assessed using the Kolmogorov-Smirnov normality test. The descriptive statistical methods included determination of the central tendency (mean, median), measures of variability (standard deviation) and relative numbers (data structure). To test statistical hypotheses, the Mann-Whitney U-test and the χ^2 test were used. The statistical hypotheses were tested for statistical significance at the level $p < 0.05$.

Results

Out of the total number of children involved in the study, 47 (57.7%) were males and 31 (42.5%) females. There was no significant difference according to gender between the groups ($\chi^2 = 0.368$; $p = 0.544$).

The average age in the group II was 5.06 ± 4.26, and in the group I it was 4.14 ± 1.60. There was no statistically significant difference by age between the groups (U = 502.5; $p < 0.001$) (Table 1).

The results obtained using WRPLOT model showed both groups within low-risk areas regarding industrial pollution exposure. We want to emphasize the fact that both groups were within the same risk zone (Figure 2).

The mean BLL value of the children included in the study was 12.54 ± 9.63 μg/dL. The lowest value was 1.1 μg/dL, and the highest one 41.8 μg/dL. The mean BLL in the children from the group II was 4.87 μg/dL (range 1.1–16.6 μg/dL), and the

Table 1

Sociodemographic characteristics of children

Characteristics	Roma camp (Group 1)	Kindergarden (Group 2)	*p*
Age (years), $\bar{x}$ ± SD	4.14 ± 2.60	5.06 ± 1.26	< 0.001
Gender, n (%)			
male	24 (57.1)	23 (63.9)	< 0.001
female	18 (42.9)	13 (36.1)	

$\bar{x}$ – arithmetic mean; SD – standard deviation.

mean BLL in the children from the group I was 19.11 μg/dL (range 6.8–41.8 μg/dL). There was a statistically significant difference in the mean values of lead concentration between the study groups (U = 39; $p < 0.001$). The children from the Roma camp (group I) had significantly higher BLL values than the children from the kindergarten.

Out of a total number of 78 children, 50% had concentrations of lead above 10 μg/dL. In the group II, even 85.7% of children were registered with BLL greater than 10 μg/dL, while in the group I the values of BLL greater than 10 μg/dL were noted in significantly lower percentage (8.3%). The difference was statistically significant ($\chi^2 = 46.43$; $p < 0.001$).

On the other hand, our results show that all the children from the group I had BLL greater than 5 μg/dL in comparison to 38.9% children from the group II. This difference was also statistically significant ($\chi^2 = 35.75$; $p < 0.001$) Table 2.

The distribution of capillary BLL in the children from both groups is shown in Figure 3.

The Regional Office of the WHO-EURO estimated that 25.0% of children, aged 2–3 years, in this area had increased BLL (≥ 10 μg/dL) in 2004 [24].

A study conducted in 1978 and 1980 aimed at determining the concentrations of BLL population near lead smelter in the town of Kosovska Mitrovica [28] showed increased concentration of lead in blood of the subjects (mean BLL value 23.4 ± 15.6 μg / dL, range 1.7–65.0 μg/dL). A recent study conducted in the Kosovska Mitrovica in 2009, 30 km south of Leposavić, was aimed at monitoring the concentrations of lead in blood of internally displaced Roma, Ashkali and Egyptian's children [29]. In this study the average BLL in the subjects was 18.8 μg/dL, in the range 5.9–41.8 μg/dL. Unfortunately, the results have not improved since this research was performed. So, in our sample even 71.8% of children had BLL higher than acceptable 5 μg/dL, which is certainly an alarming data. What is particularly important to note is that even 38.9% of children in the control group had

Table 2

Capillary blood lead levels (BLLs) in children

Characteristics	Roma camp (Group 1)	Kinder garden (Group 2)	*p*
BLL (μg/dL)			
median (range)	18.8 (6.8 –41.8)	4.1 (1.1–16.6)	< 0.001
$\bar{x}$ ± SD	19.11 ± 8.4	4.8 ± 3.1	
BLL > 10 μg/dL, n (%)			
below	6 (14.3)	33 (91.7)	< 0.001
above	36 (85.7)	3 (8.3)	
BLL > 5 μg/dL, n (%)			
below	0 (0)	22 (61.1)	< 0.001
above	42 (100)	14 (38.9)	

n (%) – number (%) of children; $\bar{x}$ – arithmetic mean, SD – standard deviation.

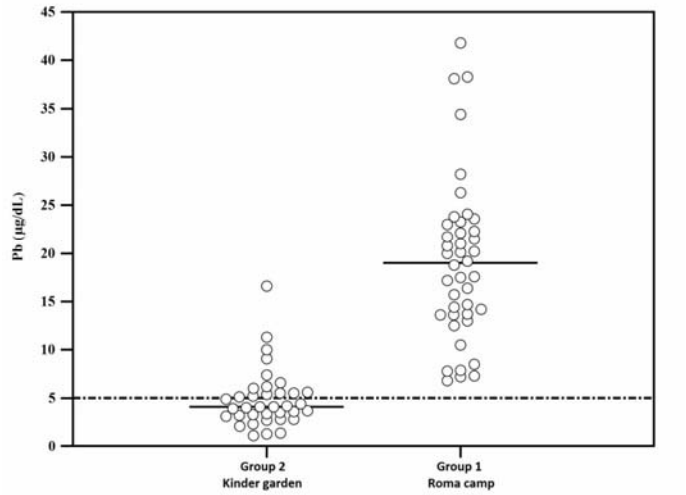

Fig. 3 – Distribution of the concentration of lead in capillary blood of the children from the kindergarden and the Roma camp.

Discussion

The toxicity of environmental lead is one of the most serious health threats to the children worldwide.

Our results show worryingly high BLL in the overall sample. The previous studies show similar results, too. lead levels above the acceptable limits. These data, along with the data from the mentioned studies, clearly indicate the presence of increased concentration of environmental lead which then enters the body. We have already mentioned that children are particularly vulnerable and exposed to this type of poisoning. Despite the fact that mining and ore processing

have greatly been reduced at the region of testing, the main source of pollution are still landfills created after many years of industrial production, that cause pollutants spreading and contamination of the surrounding grounds by erosion of the land surface by wind-blown dust and land degradation caused by rainfalls. Pollutants enter the human body either directly or indirectly by contaminated plants and animals [30]. It is worth mentioning that many studies conducted worldwide have proved that increased BLL are the consequence of increases in lead levels in the environment [31, 32].

There was a statistically significant difference in BLL between the examined groups, indicating that children who live in Roma camps are additionally exposed due to increased concentrations of lead in their immediate environment. Our results show the alarmed concentration of lead in blood of the children (Table 2). Namely, in all the children BLL was found to be higher than the referential value. If we take into account that the average age of children in this portion of the sample was 4.14 ± 1.6 years, we come to the conclusion that health of these children at their earliest age is at serious risk. Additionally, even 85.7% children from this group had BLL greater than 10 μg/dL. Unfortunately, there are no previously conducted studies concerning children from this region to compare our results with.

Limitations of the study

We did not measure the concentration of lead in the environment because we did not have the permission from the people living in the Roma camp.

Children were not clinically examined.

According to "Brief guide to analytical methods for measuring lead in blood", limitations of a portable andic skipping voltammetry (ASV) are: not as accurate as other methods, can determine levels only up to 65 μg/dL.

Conclusion

Although both groups were located outside the zone of direct spread of pollution, the results indicate high concentrations of lead in blood of all the examined children. The concentration is higher in children who live in an environment in which illegal processing of lead waste takes place. As toxic effects of lead on children's health are numerous and extremely dangerous, we can conclude that health of children who live in this area is at extremely high risk. We can assume that health in some of these respondents has already been threatened.

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Health-related quality of life in patients with functional dyspepsia

Kvalitet života i zdravstveno stanje bolesnika sa funkcionalnom dispepsijom

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Abstract

Background/Aim. Recent population based studies have proved that patients with functional dyspepsia (FD) have a significantly impaired health-related quality of life HRQoL as compared to general population. The aim of the study was to evaluate the impact of FD on (HRQoL) in patients treated in primary healthcare settings in Serbia. **Methods**. The study involved 1,448 patients with FD. The diagnosis was made by a general practice physician or gastroenterologist using the Rome III diagnostic criteria. The Serbian version of the questionnaire for the assessment of HRQoL of the U.S. Department of Health and Human Services' Centers for Disease Control and Prevention (HRQoL-4 item CDC) was used for data collection, while descriptive statistical measurements were applied to calculate standard deviation, frequency as well as multiple logistic regression model. **Results**. Out of the total number of patients, 41.8% assessed their health condition as seriously disordered or poor. The mean values of health disorder duration over the last 30 days was 11.8 days, disordered physical health 7.2 days, mental disorder 6.3 days, and activity limitation 5.1 days. Also, 29.7%, 15.2%, 12.8% and 10.7% of the patients reported ≥ 14 unhealthy days, ≥ 14 physically unhealthy days, ≥ 14 mentally unhealthy days and ≥ 14 activity limitation days, respectively. **Conclusion**. From patients' perspective, FD has a significant impact on HRQoL. In this study, 41.8% of the patients described their health status as fair or poor, and FD significantly affects all aspects of life, both mental and physical. The recognition of that impact is probably the most important step towards appropriate treatment and decreasing HRQoL impairment in patients with FD.

Key words:
dyspepsia; quality of life; depression; anxiety; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Rezultati nedavno sprovedenih populacionih studija ukazuju na to da je kod bolesnika sa funkcionalnom dispepsijom prisutno značajno sniženje kvaliteta života u odnosu na opštu populaciju. Cilj ovog istraživanja bio je procena uticaja funkcionalne dispepsije na kvalitet života kod bolesnika lečenih u ustanovama primarne zdravstvene zaštite u Srbiji. **Metode**. U studiju je bilo uključeno ukupno 1 448 bolesnika sa funkcionalnom dispepsijom. Dijagnoza funkcionalne dispepsije postavljena je primenom ROME III kriterijuma od strane lekara opšte prakse ili gastroenterologa. Za prikupljane podataka korišćena je srpska verzija opšteg uputnika za procenu kvaliteta života povezanog sa zdravljem Centra za kontrolu i prevenciju bolesti iz SAD. U statističkoj obradi podataka primenjena je deskriptivna statistika sa izračunavanjem standardne devijacije, učestalosti i procenata kao i multipli logistički regresioni model. **Rezultati**. Od ukupnog broja bolesnika uključenih u studiju 41,8% je ocenilo svoje zdravstveno stanje kao ozbiljno narušeno ili loše. Srednja vrednost trajanja narušenog zdravstvenog stanja u poslednjih 30 dana iznosila je 11,8 dana, narušenog fizičkog zdravlja 7,2 dana, narušenog psihičkog zdravlja 6,3 dana i nemogućnosti obavljanja svakodnevnih aktivnosti 5,1 dan. Takođe, 29.7% bolesnika imalo je narušeno zdravstveno stanje ≥ 14 dana u toku prethodnog meseca. Čak 15.2% imalo je narušeno fizičko zdravlje ≥ 14 dana, 12.8% je imalo narušeno mentalno zdravlje ≥ 14 dana i 10.7% ograničenje u aktivnostima ≥ 14 dana u toku prethodnih 30 dana. **Zaključak**. Funkcionalna dispepsija značajno pogoršava kvalitet života ispitivanih bolesnika. Od ukupnog broja ispitanika uključenih u studiju 41,8% je ocenilo svoje zdravstveno stanje kao ozbiljno narušeno ili loše. Funkcionalna dispepsija negativno utiče na sve aspekte normalnog funkcionisanja. Prepoznavanje funkcionalne dispepsije je verovatno najvažniji korak ka odgovarajućem lečenju i sniženju njenog štetnog uticaja na kvalitet života.

Ključne reči:
dispepsija; kvalitet života; depresija; anksioznost; ankete i upitnici.

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Introduction

Functional dyspepsia (FD) is a functional gastrointestinal disorder (FGID) defined as the presence of symptoms thought to originate in the gastroduodenal region in the absence of any organic, systemic or metabolic disease that is likely to explain the symptoms [1]. According to the ROME III criteria, FD is defined as the presence of one or more of the following symptoms: bothersome postprandial fullness, early satiation, epigastric pain and epigastric burning without evidence of structural disease. Two main differences compared to the ROME II criteria are the duration of symptoms and characterization of FD as postprandial distress syndrome (B1a) and epigastric pain syndrome (B1b) [2]. The etiology of FD remains unclear. According to the biopsychosocial model, FD, like other FGID results from complex interactions between biological, social and psychological factors [3]. FD represents a serious public health problem, with prevalence ranging from 11% to 30% according to large population studies [4–6]. The importance of FD lies in the impairment of health-related quality of life (HRQoL), as confirmed by many studies [7–9]. The aim of this study was to assess the impact of FD on HRQoL in patients treated in primary healthcare settings in Serbia.

Data about FD-related HRQoL in the Eastern European countries have been scarce. The aim of this study was to assess the impact of FD on HRQoL in patients treated in primary healthcare settings in Serbia. The current study, to the best of our knowledge, is the first population-based study regarding HRQoL in the patients with FD ever conducted in Serbian primary health care settings. In addition, according to the iceberg concept, the majority of patients with FD should be treated in the primary healthcare setting. Understanding the nature of HRQoL impairment and risk factors for FD is important for improvement of treatment modalities.

Methods

This study measured HRQoL in patients with chronic non-transmittable diseases, selected from a large cross-sectional survey conducted in the primary healthcare facilities in Serbia from January-December 2011. Sampling was based on random selection of clusters of patients: at the first stage random selection of primary health care centers, and at the second stage, random selection of general practitioners. Patient inclusion criteria were FD as the main reason to visit the doctor. FD was diagnosed using the ROME III criteria by a primary care physician and/or gastroenterologists. The exclusion criteria included the evidence of structural disease, confirmed by upper endoscopy, such as peptic ulcer disease, malignant tumors, etc. These criteria ensured that only patients with FD were eligible for the study. All patients filled-in a questionnaire in the office of their general practitioner (GPs).

In our study, data collection was carried out by using the Serbian version questionnaire of the U.S. Department of Health and Human Services' Centers for Disease Control and Prevention (CDC) which supported population surveillance of health-related quality of life (HRQoL-4 item CDC). We used the back-translation procedure to ensure the cross-cultural validity of the Serbian version and linguistic equivalence of translated items. This questionnaire, often used in surveys, prevention researches and population-based studies, has shown good measurement properties in several populations, languages and settings [10]. Collection of additional data included variables such as age, gender, education, the main reason to visit the doctor and medication adherence.

Data collection and statistical analysis

A total of 1,448 patients with FD were enrolled in this study. The patients self-rated their health status as excellent/very good, good and fair/poor using a questionnaire. In the last 30 days prior to receiving the questionnaire, the patients reported the following symptoms: pain limitation days, depression days, anxiety days, poor sleep days and days feeling well. Also, the patients reported more or equal to 14 days of physical and/or mentally unhealthy days as well as activity limitation days. Most patients used either over-the-counter (OTC) drugs or medications prescribed by a primary care practitioner or specialist.

Descriptive statistics with standard deviation, frequency and percentage were calculated. A multiple logistic regression model was applied with self-rated health status and ≥ 14 unhealthy days in the last 30 days as dependents variables, and sociodemographic characteristics as independent ones. The statistical analysis was performed using SPSS, version 20.

Results

The study group involved 1,448 patients with FD, 667 males and 781 females, mean age 50.7 years. Regarding the education level, 57% had lower education levels that included no education level or primary education level, and 43% had higher education levels that included secondary and tertiary education levels. Of all patients, 605 (41.8%) self-rated their health status as fair or poor.

Data on self-rated health status, a number of unhealthy or healthy days in past 30 days and number of patients who had ≥ 14 unhealthy or healthy days in that period are given in Table 1.

Deterioration of HRQoL was confirmed by the analysis of duration of symptoms over the last 30 days (Table 2). A total of 721 (49,8 %) patients were on medications prescribed by the gastroenterologist, 485 (33.5%) took medication prescribed by primary care practitioners and 162 (11.2%) of the patients were self-administering OTC drugs. Only 80 (5.5%) patients did not take any medications.

Using multiple logistic regression model, and after adjustment for age, sex and education level (Table 3) it was demonstrated that the female FD patients [odd ratio (OR) = 1.31, 95% confidence interval (CI) = 1.04–1.64], aged over 50 years (OR = 3.49, 95% CI = 2.77–4.41) and lower education levels (OR = 0.36; 95%, CI = 0.29–0.46) had a significantly higher prevalence of poor or fair health. The FD patients aged over 50 years (OR = 2.0; 95%, CI = 1.47-2.73) and with lower education levels (OR = 0.47,95%, CI = 0.34-

Table 1

Perceived health status of patients with functional dyspepsia (n = 1,448)

Parameters	Patients, n (%)	Days, $\bar{x} \pm SD$
Self-rated health		
exellent, very good, good	843 (58.2)	
fair, poor	605 (41.8)	
Unhealthy days in past 30 days		
overall		11.8 ± 10.6
physically		7.2 ± 7.3
mentally		6.3 ± 7.5
activity limitation		5.1 ± 7.1
≥ 14 unhealthy days in part 30 days		
overall	430 (29.7)	
physically	220 (15.2)	
mentally	186 (12.8)	
activity limitation	155 (10.7)	
Days with good health in past 30 days		11.8 ± 9.5
≥ 14 healthy days in past 30 days	406 (28.0)	

n – number of patients; $\bar{x}$ – arithmetic mean; SD – standard deviation.

Table 2

Symptoms present in past 30 days reported by patients with functional dyspepsia

Symptoms	Patients n (%)	Days with symptoms ($\bar{x} \pm SD$)
Pain		
≥ 14 days limited by pain	146 (10.1)	5.3 ± 6.4
pain limitation days		
Depression		
≥ 14 days feeling depression	137 (9.5)	5.8 ± 7.2
days with depression		
Anxiety		
≥ 14 days feeling anxiety	203 (14.0)	7.3 ± 7.7
days with anxiety		
Poor sleep		
≥ 14 days with poor sleep	241 (16.6)	8.4 ± 7.7
days with poor sleep		

n – number of patients; $\bar{x}$ – arithmetic mean; SD – standard deviation.

Table 3

Factors influencing quality of life in patients with functional dyspepsia

Dependent variables	Independent variables [OR (95% CI)]		
	Sex (female)	Age	Education
Fair or poor self-rated health	1.31 (1.04, 1.64)	3.49 (2.77, 4.41)	0.36 (0.29, 0.46)
≥ 14 physically unhealthy days	1.2 (0.9, 1.61)	2.0 (1.47, 2.73)	0.47 (0.34, 0.66)
≥ 14 mentally unhealthy days	1.16 (0.85, 1.58)	1.47 (1.07, 2.03)	0.6 (0.43, 0.84)
≥ 14 unhealthy days	1.43 (1.13, 1.81)	1.52 (1.2, 1.92)	0.51 (0.4, 0.65)
≥ 14 activity limitation days	1.31 (0.93, 1.85)	1.99 (1.38, 2.87)	0.34 (0.22, 0.51)
≥ 14 pain limitation days	1.39 (0.98, 1.98)	1.75 (1.21, 2.53)	0.46 (0.31, 0.69)
≥ 14 days feeling depressed	1.67 (1.16, 2.42)	1.7 (1.17, 2.48)	0.49 (0.33, 0.73)
≥ 14 days feeling anxious	1.53 (1.12, 2.08)	1.31 (0.96, 1.78)	0.52 (0.37, 0.72)
≥ 14 days with poor sleeping	1.18 (0.89, 1.57)	1.3 (0.97, 1.72)	0.58 (0.43, 0.78)
≥ 14 days feeling healthy	0.93 (0.73, 1.18)	0.32 (0.25, 0.41)	1.39 (1.09, 1.77)

OR – odds ratio; CI – confidence interval.

0.66) had a higher prevalence of ≥ 14 physically unhealthy days, regardless of gender. Furthermore, similar results were obtained for mentally unhealthy days. The patients aged over 50 years (OR = 1.47; 95%, CI = 1.07–2.03) and with lower education levels (OR = 0.6; 95% CI = 0.43–0.84) had ≥ 14 mentally unhealthy days regardless of gender.

Female gender (OR = 1.43; 95% CI = 1.13–1.81), aged over 50 years (OR = 1.52; 95% CI = 1.2–1.92) and lower education levels (OR = 0.51; 95% CI = 0.4–0.65) were significant predictors for ≥ 14 unhealthy days. For ≥ 14 limitation days, age (OR = 1.99; 95% CI = 1.38–2.87) and lower education levels (OR = 0.34; 95% CI = 0.22–0.51) were significant predictors, regardless of gender. The age of over 50 years and lower education levels were also significant predictors for ≥ 14 pain limitation days.

Female gender (OR = 1.67; 95% CI = 1.16–2.42), older age (OR = 1.7; 95% CI = 1.17–2.48) and lower education levels (OR = 0.49; 95% CI = 0.33–0.73) were predictors for ≥

14 days with depression. Furthermore, female gender (OR = 1.53; 95% CI = 1.12–2.08) and lower education levels (OR = 0.52;95% CI = 0.37–0.72), regardless of age were predictors for ≥ 14 days with anxiousness.

Regarding healthy days, participants aged under 50 years (OR = 0.32; 95% CI = 0.25–0.41) with higher education levels (OR = 1.39; 95% CI = 1.09–1.77) had ≥ 14 healthy days over the past 30 days.

Discussion

FD can be perceived from the perspective of the patient, as well as from the perspective of the physician. FD represents an important health issue due to the facts that it occurs with a high prevalence in general population, causes a significant impairment of HRQoL and demands the excessive use of resources. This study analyzes an influence of FD on HRQoL from the perspective of patients.

HRQoL can be observed using both overall well-being and disease-specific instruments. In this study, we utilized the overall health condition by the Serbian version of the self-administered questionnaire CDC-HRQoL-4. We have already used this questionnaire for the evaluation of patients with gastroesophageal reflux disease (GERD) [11].

HRQoL refers to the consequences of the disease on the everyday activity of patients and their perception of the disease itself. Some studies have shown that the impairment of HRQoL is more severe in patients with FD than in patients with structural abnormalities [12], while other have not found any difference between FD and organic dyspepsia [13].

Etiology of FD is still unknown, although several studies have concluded that multiple factors are involved and that various mental disorders could play a significant role [14]. The diagnosis of FD is based on clinical symptoms and negative findings on upper endoscopy for structural diseases.

It was detected in the study that 5.5% of patients were not under any treatment, while 11.2% of the patients were on self-administered OTC medication. In addition, 33.5% patients used medications prescribed by a primary care practitioner, while 49.8% of patients were treated with medications prescribed by a specialist working in secondary and tertiary health care settings. According to the National Institute for Health and Care Excellence (NICE) guidelines for FD, proton pump inhibitors PPIs are the first-line therapy for FD [15].

In our study, 10.1% of patients were limited by pain for ≥ 14 days over the last 30 days. Tack et al. [16] found that 34% of patients with FD experienced hypersensitivity to gastric distention associated with symptoms of postprandial pain [16]. Mones et al. [8] reported low scores regarding all domains in patients with FD. Even if achieving improvement by decreasing physical pain with therapy (16%), the final score was still lower compared to that in general population.

It was detected that 15.2% of patients had ≥ 14 physically unhealthy days, while 12.8% had ≥ 14 mentally unhealthy days; female gender, older age and lower education levels were predictors in both, which correlates with the findings of other studies. In the study by Talley et al. [7] the mean physical, as well as the mean mental composite scores, were significantly lower when compared with age and sex adjusted to the U.S. national norms.

Our study showed that 14% of the patients had anxiety and 9.5% depression problems; female gender, older age and lower education levels were found to be strong predictors of anxiety in FD patients. These results are in accordance with results from other studies. Huang et al. [17] study results show that anxiety and depression are more severe in FD patients, and that female gender, older age and lower education levels are all risk factors for both anxiety and depression. A Swedish population-based study determined a positive correlation between anxiety and FD but failed to prove a correlation between depression and FD [18].

Based on the multiple logistic regression model, our study demonstrated that physically and mentally unhealthy days, activity limitation days, depression, anxiousness, and poor sleep were associated with female gender, older age and lower education levels, thus leading to the conclusion that all of these factors are predictors of FD. As detected in our study, higher education levels and earlier lifetime period enable a better understanding of one's own disease in both genders, primarily resulting in higher medication compliance. In addition, persons with higher education levels have better financial possibilities as well as a better understanding of the necessity for diet and lifestyle changes that are important in the treatment of FD which in turn leads to a lower impairment of HRQoL. Furthermore, patients aged over 50 years usually have a number of comorbidities which could overlap with or potentiate FD symptoms.

In this study, 41.8% of the patients described their health status as fair or poor, even though majority used medications.

The limitation of our study was the inability to determine patient compliance with medication and the comparison of efficacy among different treatment strategies. Also, overlapping with eventual comorbidities and their impact on FD symptoms were not determined. Further studies should take this into consideration in order to achieve a better assessment of HRQoL in FD patients.

Conclusion

From patients' perspective, FD has a significant impact on HRQoL. In this study, 41.8% of the patients described their health status as fair or poor, and FD significantly affects all aspects of life, both mental and physical. The recognition of that impact is probably the most important step towards appropriate treatment and decrease of HRQoL impairment in patients with FD.

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Comparative clinical evaluation of two different techniques of local anaesthesia in the posterior mandible using 4% articaine with 1 : 100,000 adrenaline

Uporedna klinička procena dve tehnike lokalne anestezije u bočnom segmentu mandibule primenom 4% artikaina sa 1 : 100 000 adrenalinom

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Abstract

Background/Aim. Local infiltration anaesthesia (LIA) is significantly simpler compared to the inferior alveolar nerve block (IAB) and less unpleasant for patients. However, it is not efficient if used in posterior region of the mandible, at least with traditional local anaesthetics. The aim of this study was to compare anaesthetic efficacy of two techniques the LIA in the posterior segment of the mandible, and the IAB, using 4% articaine with 1 : 100,000 adrenaline and to note possible changes in haemodynamic parameters caused by these two techniques. **Methods.** Sixty pre-informed patients were divided into two study groups. Both groups received 1.8 mL of the same anaesthetic solution, 4% articaine with adrenaline 1 : 100,000, with two different techniques of local anaesthesia. The first group received the LIA in projection of root apex of the first lower molar; the second group received the IAB. The examined parameters were: changes in tooth sensitivity after 5 and 30 minutes in relation to the value recorded before administering the anaesthetics, onset of anaesthesia, width of anaesthetic field, and duration of anaesthesia. Also, the impact of the applied techniques on cardiovascular parameters was noticed. **Results.** The LIA group had a statistically significant decrease in sensitivity 5 minutes after application of the local anaesthetic. The decreasing trend continued between 5 and 30 minutes, although without statistical significance. There was no statistically significant difference in sensitivity changes between two groups for the first molar and the first and second premolars. However, there was a statistically significant difference in duration of local anaesthesia in favour of the IAB, while the width of anaesthetic fields was significantly higher after the LIA. Significant changes in hemodynamic parameters were not recorded within the two groups. **Conclusion.** The effect of the LIA on tooth sensitivity of premolars and first molar is quite satisfactory. The IAB was more effective for canine and second molar. None of the tested techniques had any significant effect on the cardiovascular parameters.

Key words:
anesthesia, dental; carticaine; epinephrine; mandible.

Apstrakt

Uvod/Cilj. Lokalna infiltraciona anestezija (LIA) mnogo je jednostavnija u poređenju sa mandibularnom alveolarnom blok anestezijom (MA), a i prijatnija je za pacijente. Međutim, ona nije efikasna ukoliko se daje u posteriorni deo mandibule korišćenjem tradicionalnih lokalnih anestetika. Cilj istraživanja bio je da se uporedi efikasnost dve tehnike anestezije, LIA u bočnom segmentu mandibule, i mandibularne anestezije primenom 4% artikaina sa 1 : 100 000 adrenalina, i uoče eventualne promene hemodinamskih parametara koje ove tehnike mogu da prouzrokuju. **Metode.** Šezdeset prethodno informisanih pacijenata bilo je podeljeno u dve studijske grupe. Obe grupe su dobile 1,8 mL istog anestetika, 4% artikain sa adrenalinom 1 : 100 000, primenom dve tehnike anestezije. Prva grupa ispitanika primila je lokalnu infiltracionu anesteziju u projekciji vrhova korenova prvog donjeg molara. Druga grupa ispitanika primila je mandibularnu anesteziju. Ispitivani parametri bili su: promene u osetljivosti zuba nakon pet i 30 minuta u odnosu na vrednosti zabeležene pre davanja anestetika, početak dejstva anestezije, širina anestetičkog polja i trajanje anestezije. Takođe, zabeležen je i uticaj primenjenih tehnika na kardiovaskularne parametre. **Rezultati.** Grupa ispitanika LIA imala je statistički značajno smanjenje osetljivosti zuba pet minuta nakon primene LIA.

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Trend smanjenja postojao je i dalje, između pet i 30 minuta, iako bez statističke značajnosti. Nije bilo statistički značajne razlike u promenama osetljivosti između dve grupe na prvom molaru i prvom i drugom premolaru. Postojala je statistički značajna razlika u trajanju anestezije u korist MA, dok je širina anestetičkog polja bila značajno veća u LIA grupi. Klinički značajne promene hemodinamskih parametara nisu zabeležene ni u jednoj studijskoj grupi. **Zaključak.** Učinak LIA bio je zadovoljavajući kada su u pitanju premolari i prvi molar. Za anesteziju očnjaka i drugog molara uspešnija je bila MA. Nijedna od ispitivanih tehnika nije imala značajan uticaj na kardiovaskularne parametare.

Ključne reči:
anestezija, stomatološka; artikain; adrenalin; mandibula.

Introduction

Although local anaesthesia is very often used in modern dentistry, it is not always successful, especially when the inferior alveolar nerve block (IAB) is concerned [1–3]. Poor technique is not the only reason for the failure of the IAB; among others, the anatomic variations of the position of the mandibular foramen could be an additional one [4, 5]. Therefore, there is a tendency to minimize the need for the IAB, even more due to possible side-effects and complications related to it, such as injury of medial pterygoid muscle and blood vessels in the pterygomandibular space [6].

The local infiltration anaesthesia (LIA) is significantly simpler compared to the IAB and less unpleasant for patients. However, it is not efficient if used in posterior region of the mandible, at least with traditional local anaesthetics. Articaine hydrochloride is a local anaesthetic of the amide type, introduced in Switzerland and Germany in the mid-seventies, in the US in 2000 and in Australia in 2004 [7]. The specificity is that, instead of benzene, it contains a thiophene ring that allows it high lipid solubility [8]. Additionally, increased lipid solubility provides the enhanced diffusion through hard and soft tissues. This feature enables the passage of the anaesthetic even through compact posterior mandible, enabling a similar analgesic effect when infiltrating locally as it is after the IAB [9, 10]. It was confirmed that articaine achieved effective analgesia compared with lidocaine when applied in the lateral region of the lower jaw [11, 12]. However, it is not known yet whether the level of anaesthesia achieved by infiltration of local anaesthetic in the lower jaw is adequate for use in oral surgery or endodontics.

Electric pulp test (EPT) is a commonly used method for testing the vitality and sensitivity of teeth. Today, electric pulp testers (EPTs) have rheostat which adjusts the intensity of the current applied to the tooth. They vary in scale from 1–9, 1–15, 1–80 [13]. Grossman [14] recommended EPT for clinical purposes to check the quality of anaesthesia prior to commencement of the intervention on the teeth [14, 15].

The aim of this study was to compare anaesthetic efficacy of two techniques, the LIA in the posterior segment of the mandible, and the IAB, using 4% articaine with 1 : 100,000 adrenaline, as well as to note possible differences in haemodynamic parameters caused by these two techniques.

Methods

This prospective randomised clinical study was conducted at the Clinic of Oral Surgery, Faculty of Dental Medicine, University of Belgrade, Serbia. The study was approved by the Ethics Committee of the School of Dental Medicine (no. 36/19 of the 3rd of June 2015), and all participants gave written consent. The study was carried out on systemically healthy persons (ASA I) who had more than one vital teeth on the test side of the lower jaw, without large carious lesions or fillings. In total, 60 patients who participated in the study, aged between 18 to 50 years, were divided into two groups of 30 participants each – the LIA group, with participants who received the LIA in the projection of roots apices of the first lower molar and the IAB group with participants who received the regular IAB block. All participant received 1.8 mL of articaine hydrochloride (40 mg/mL) with 1 : 100000 epinephrine (SEPTANEST, Septodont, France). One researcher carried out administration of anaesthesia while another one did the measurements.

After infiltrating the anaesthetic, following parameters were examined: onset of anaesthesia determined by loss of sensation, changes in teeth sensitivity determined by EPT, duration of anaesthesia, width of anaesthetic field and possible changes of cardiovascular parameters (systolic and diastolic blood pressure and heart rate).

As the most reliable indicator of analgesia, EPT was carried out before applying anaesthetic, and then after 5 , and 30 minutes. A digital tester, which produces a direct electric current, was directed to the probe touching the examined tooth, was used. The examined tooth was firstly dried with sterile gauze and air, then isolated from the adjacent teeth with celluloid strips, while the probe tip was coated with alcohol to increase the conductivity of impulses to the tooth surface. Before applying anaesthesia, sensitivity of canine, and both premolars, the first and the second, were determined. Following administration of the anaesthetic solution, the teeth that did not respond to the EPT were given a value of ten in the research record.

Width of the anaesthetic field in the LIA group of participants was tested in the area of buccal gingiva with the "pinprick" method, using a sterile dental probe 15 minutes after the application of anaesthetic. The width of the field was expressed in millimetres.

Hemodynamic parameters in both groups were monitored at various time intervals: before anaesthesia, during administration of anaesthesia, and 5, 10, 15 and 30 minutes after the administration of anaesthetics.

Statistical analysis was performed using the computer program IBM SPSS Statistics for Window software (version 20.0, IBM Corp., Armonk, NY, USA). The research results are presented in the form of mean values and standard deviations. Differences in characteristics of the achieved anaesthe-

sia between groups were determined by Student's *t*-test and Mann-Whitney U-test. For comparisons within groups, among observed three time measurements, Friedman's test was used for non-parametric data and factor analysis of variance with repeated measures (RM ANOVA) for parametric parameters. Hemodynamic parameters were analysed by ANOVA for repeated measures (Two Way RM ANOVA). To compare the two measurement points, within each of the studied groups, the Wilcoxon's test for related samples was used. *P* values less than 0.05 were considered statistically significant.

Results

Demographic characteristics of all participants are presented in Table 1, showing that both groups did not differ significantly and were comparable.

By analysing parameters of the achieved local anaesthesia, a statistically significant difference in the duration of anaesthesia was noted in favour of the IAB, whereas the width of anaesthetic field was significantly higher when administering the LIA (Table 2).

The results of tooth sensitivity, noticed by use of the EPT, are shown in Table 3 for each of the tooth tested separately. Tooth sensitivity was significantly reduced 5 minutes after application of the IAB, continuing till the end of the observing period (30 min). In the LIA group, a statistically significant reduction in sensitivity was observed within the first 5 minutes ($p = 0.001$). Also, the trend of decreased sensitivity was recorded till thirty minutes ended, indicating effective anaesthesia and the observed differences were not statistically significant.

Tooth sensibility measured 5 and 30 minutes after administration of anaesthesia, regardless of the technique of anaesthesia (the LIA *v*s IAB), did not differ significantly when both premolars and molars were considered. However, tooth sensibility was statistically different before and after anaesthesia in the whole investigated segment of the posterior mandible.

Haemodynamic parameters (systolic pressure, diastolic pressure and heart rate) among participants of both study groups were relatively similar, without statistically significant differences (Figure 1). The most prominent difference was noted in heart rate (Figure 1c), although it was still considered insignificant when both groups were compared.

Discussion

Although the IAB is a local anaesthesia technique of choice for interventions on lower premolars and molars, complete pulp anaesthesia of these teeth is not always achieved [16, 17]. Buccal infiltration of local anaesthetic is ineffective due to the presence of dense cortical bone which prevents adequate diffusion of the solution [18–20].

Available literature indicates that articaine with adrenaline provides similar anaesthetic effect as other amide local anaesthetics with vasoconstrictors [16]. Articaine is the only amide local anaesthetic that contains a thiophene ring that gives it a distinct liposolubility. This property improves penetration of anaesthetic through the lipid membrane of neurons and the surrounding tissues [16, 21]. Kanaa et al. [12] have already demonstrated that articaine is more effective than lidocaine when applied with the LIA in the posterior mandible. Interestingly, the same authors presented later that in the upper jaw, there was no statistically significant difference in the efficiency of these two anaesthetics [22]. Robertson et al. [23] recorded a successful analgesia with articaine administered via LIA from 75% to 92%, which was significantly higher when compared to lidocaine. Even more, supplemental local infiltration with articaine after IAB with lidocaine provided better pulp anaesthesia of lower posterior teeth, enabling longer duration of pulp anaesthesia of the first molar and second premolar [24].

In this study, both techniques (LIA and IAB), articaine proved to be highly successful, in the region of the first molars and both premolars. In other words, there was no statistically significant difference between the two groups, although this difference between the two techniques was noticed on the second molar and canine. The reasons for this may be the individual anatomical nature, such as the increased thickness of the buccal lamella in the region of the second molar, more lingual position of the mandibular canal, as well as the fact that the anaesthetic was applied proximal to the mentioned tooth. Haas at al. [25] reported that there was no difference in achieving pulp anaesthesia in mandibular teeth between 4% articaine and 4% prilocaine, both with adrenaline 1 : 200,000. When it comes to the second lower molar, they noted the success in achieving the pulp anaesthesia after LIA in 63% (12/19) of patients when articaine was used, and 53% (10/19) after 4% prilocaine. Their results support the fact that the buccal lamella is wider in the region of the second molar, and indicate that articaine diffuses better through the compact bone, as the results of the present study show. Results of this research indicate that the anaesthetic effect is similar to both anaesthesia techniques and correspond with results of clinical studies testing the efficacy of articaine applied with the LIA in the lower jaw. Poorni at al. [11] compared the LIA and IAB applying articaine and lidocaine in patients with irreversible pulpitis of lower molars. That study included participants with the present pain caused by the pulp disease, which was a basic measure in assessing the efficacy of two experimental techniques and local anaesthetics. The success of achieved anaesthesia was evaluated on the basis of achieving painlessness in access preparation and pulp extirpation, and the authors concluded that there was no difference.

The amount of the applied solution, can also have effect on the quality of the achieved anaesthesia. In this study, all patients received 1.8 mL of articaine/adrenaline. Applying twice the dosage with the LIA, a higher degree of success could be expected, as shown in the study by Martin at al. [26].

Most of the literal data is related to the achievements by using the pulp anaesthesia for removing pain in irreversible pulpitis. Measuring the success of anaesthesia refers to the percentage of patients in whom the absence of reaction to the maximum pulp test stimulation in two consecutive measurements was achieved. It should be noted that the EPT

Table 1

Demographic characteristics of participants

Characteristics of participants	MBA	ILA	Total
Gender			
male	10	15	25
female	20	15	35
Total	30	30	60
Age (years), $\bar{x} \pm SD$	24.73 ± 4.86	27.20 ± 5.68 *	

MBA – mandibular block anaesthesia; ILA – infiltration local anaesthesia; $\bar{x}$ – arithmetic mean; SD – standard deviation; * – Students's *t*-test, $p = 0.076$.

Table 2

Anaesthesia technique characteristics

Parameters	MBA	ILA
	$\bar{x} \pm SD$, (median)	$\bar{x} \pm SD$, (median)
Time to onset of anaesthesia (min)	1.93 ± 0.79, 2.0*	1.77 ± 0.73, 2.0
Duration of anaesthesia (min)	334.63 ± 98.59, 351.00**	214.7 ± 47.78. 205.00
Width of anaesthetic field (mm)	39.43 ± 17.54, 35.50	57.27 ± 9.16, 53.50***

$\bar{x}$ – arithmetic mean; SD – standard deviation; * – Mann-Whitney *U*-test, $p = 0.379$; ** – Students's *t*-test, $p = 0.01$; * – Students's *t*-test $p = 0.001$.**

Table 3

Influence of mandibular block anaesthesia (MBA) and local infiltration anaesthesia (LIA) on tooth sensitivity estimated by electric pulp testing (EPT)

Tooth	Before anaesthesia	Time of EPT 5 min after	30 min after	*p*
Second molar				
MBA	3.17 ± 1.4	7.97 ± 3	9.27 ± 1.7	0.000 (a, a_1, a_2) 0.011 (a_3)
LIA	2.4 ± 0.9	6.27 ± 3	6.23 ± 2.8	0.000 (b, b_1, b_2) 0.932 (b_3)
*p**	0.023	0.019	0.000	
First molar				
MBA	3.17 ± 1.9	7.58 ± 2.4	9.08 ± 1.4	0.000 (a, a_1, a_2) 0.004 (a_3)
LIA	2.64 ± 1.3	6.84 ± 3	6.88 ± 3.1	0.000 (a, a_4, a_5)
*p**	0.318	0.410	0.017	0.886 (a_6)
Second premolar				
MBA	3.2 ± 1.2	8.13 ± 2.4	9.6 ± 1.2	0.000 (a, a_1, a_2) 0.001 (a_3)
LIA	2.55 ± 1.2	8.24 ± 2.7	8.52 ± 2.5	0.000 (b, b_1, b_2) 0.449 (b_3)
*p**	0.011	0.652	0.135	
First premolar				
MBA	3.14 ± 1.2	7.83 ± 2.8	9.66 ± 0.9	0.000 (a, a_1, a_2) 0.004 (a_3)
LIA	2.48 ± 0.9	7.62 ± 2.9	8.55 ± 2.6	0.000 (a, a_4, a_5) 0.147 (a_6)
*p**	0.001	0.681	0.086	
Canine				
MBA	3.640 ± 1.8	7.93 ± 2.9	9.82 ± 2.5	0.000 (a, a_1, a_2) 0.007 (a_3)
LIA	2.8 ± 1.1	5.13 ± 3	6.07 ± 3.4	0.000 (a, a_4, a_5) 0.096 (a_6)
*p**	0.057	0.001	0.000	

***Mann-Whitney test;**
$^{a1, a4, b1}$ – comparing before and 5 min after anaesthesia
$^{a2, a5, b2}$ – comparing before and 30 min after anaesthesia
$^{a3, a6, b3}$ – comparing before and 5 min and 30 min after anaesthesia.
Second molar: ^{a}Friedman's test; ^{b}RM ANPVA, $^{a1, 2, 3}$ – Wilcoxon Signed Ranks test; $^{b1, b2, b3}$ – Paired simple *t*-test;
First molar: ^{a}RM ANPVA; $^{a1, 2, 3, 4, 5, 6}$ – Paired simple *t*-test;
Second premolar: ^{a}RM ANPVA; ^{b}Friedman's test; $^{a1, 2, 3}$ – Paired simple *t*-test; $^{b1, 2, 3}$Wilcoxon Signed Ranks test;
First premolar: ^{a}Friedman's test; $^{a1, 2, 3, 4, 5, 6}$Wilcoxon Signed Ranks test;
Canine: *Mann-Whitney test; ^{a}Friedman's test; $^{a1, 2, 3, 4, 5, 6}$Wilcoxon Signed Ranks test; $^{a1, 4}$ – comparing before and 5 min after anesthesia applied; $^{a2, 5}$ – comparing before and 30 min after anesthesia applied; $^{a3, 6}$ – comparing 5 and 30 min after anesthesia applied.

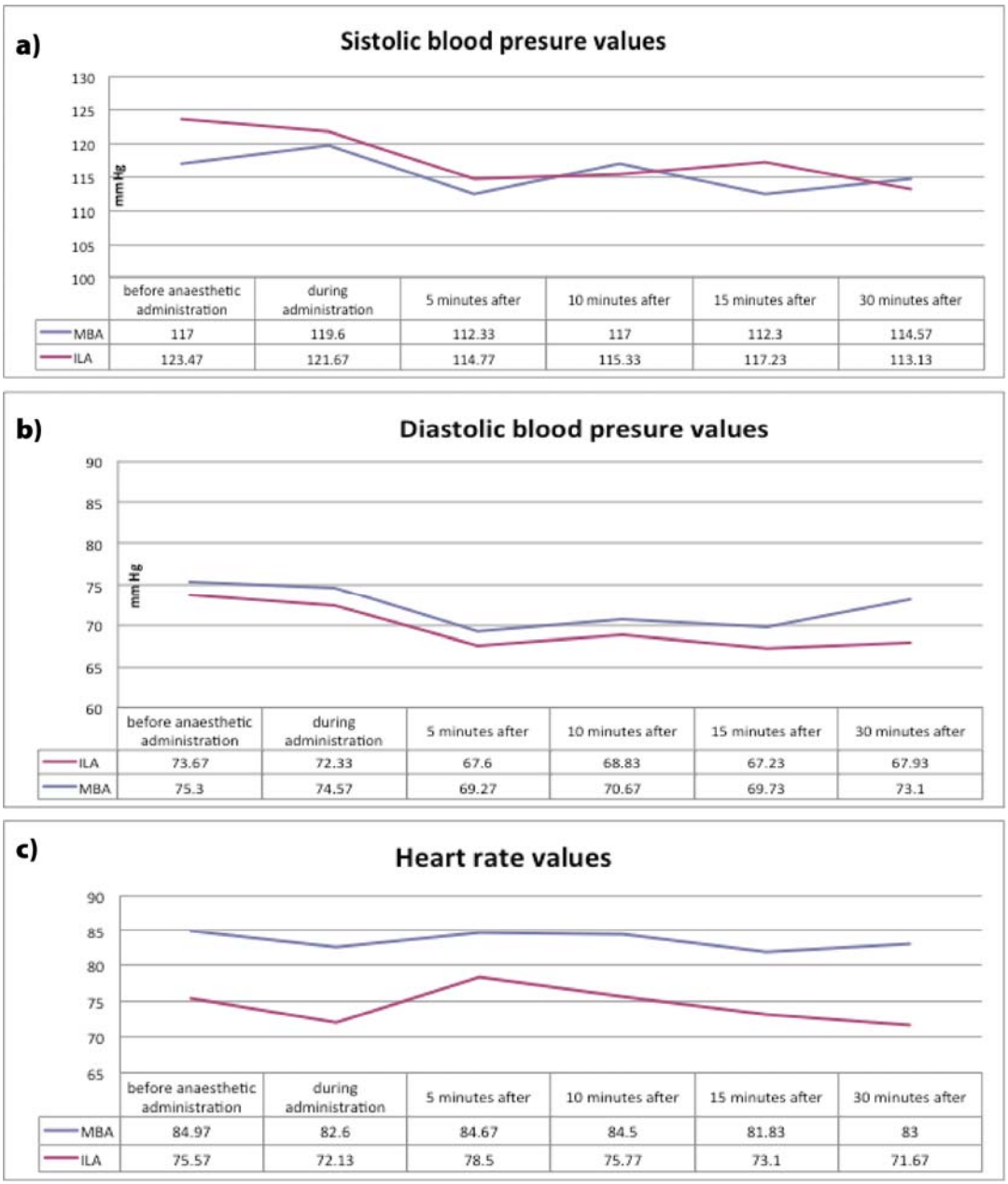

Fig. 1 – Influence of infiltration local anaesthesia (ILA) and mandibular block anaesthesia (MBA) on haemodynamic parameters over observation time period:

a) Systolic blood pressure changes: values were statistically significantly different over time and among different time intervals in each of the study groups ($p = 0.001$; $p = 0.017$), but with no statistically significant difference between the two groups;

b) Diastolic blood pressure changes: there was a statistically significant difference during various time intervals in each of the study groups ($p < 0.001$), whereas the difference in the values of diastolic blood pressure between the two groups was not statistically significant ($p = 0.532$);

c) Heart rate changes: heart rate was also significantly changed over time intervals in both groups ($p = 0.001$), whereas no statistically significant difference between the two different groups was observed ($p = 0.258$).

technique is not a quantitative indicator of the pulp sensitivity [27, 28]. Mental condition of the patient during the day or at different time intervals has an impact on the reliability of testing tooth sensitivity [29].

To the best of our knowledge, there were no previous studies that compared anaesthetic and cardiovascular effects of articaine administered with the LIA and IAB, respectively. Differences in cardiovascular parameters between patients of the LIA and IAB groups were statistically insignificant; it seems that both techniques of anaesthesia are clinically safe for risk patients.

Onset of anaesthesia in our study was slightly shorter in the LIA group, but with no statistical significance.

Duration of anaesthesia after the IAB was significantly longer than that provoked by the LIA, which was expected. This fact is of clinical importance. On one hand, long-term

anaesthesia after administration of the IAB would provide long-lasting analgesia after the intervention in which severe postoperative pain could be expected (for example after oral surgery). On the other hand, in contrast to this, in interventions with smaller or non-existent postoperative pain, the use of the LIA with articaine can avoid unpleasant long-term numbness.

Width of anaesthetic field measured with "pin prick" test was significantly higher after the LIA technique, due to simultaneously achieved buccal nerve anaesthesia; the LIA did not have simultaneous lingual nerve anaesthesia, which the IAB did.

Conclusion

The effect of the LIA on tooth sensitivity of premolars and first molar was quite satisfactory. The IAB was more effective for canine and second molar. None of the tested techniques had any significant effect on the cardiovascular parameters.

Conflict of interest

The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

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Additional impact on muscle function when treating active rheumatoid arthritis patients with high alfacalcidol doses

Dodatni uticaj visokih doza alfakalcidola na mišićnu funkciju prilikom lečenja bolesnika sa aktivnom formom reumatoidnog artritisa

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Abstract

Background/Aim. Hormone D (vitamin D) plays an important role in immunoregulation and musculoskeletal metabolism. The aim of this study was to assess the impact of alfacalcidol (ILD_3) or prednisone use on muscle function and disease activity in active rheumatoid arthritis (RA). **Methods**. The study included 67 RA patients with the active disease, disease activity score (DAS28) > 3.2, on the highest tolerable methotrexate (MTX) dose during last 3 months. Data collected were: DAS 28, muscle function tests [chair rising test (CRT) timed up and go (TUG), 6 minutes walk (6MWT), tandem walk (TW)], efficacy and safety laboratory tests. At enrollment, patients were randomly assigned to three-month supplementation with 1 μg (group A1) or 2 μg (group A2) or 3 μg (group A3) of 1αD3 daily or prednisone (group C) 20 mg daily, for the first month and 10 mg afterward, in addition to MTX. **Results**. After the treatment, we found highly significantly reduced disease activity in all four treatment arms (DAS28 $p < 0.01$). 1αD3 2 μg (A2 group, n = 19) treated patients significantly improved muscle function (TUG, 6MWT), while 1αD3 3 μg treated (A3, n = 16) improved 6MWT ($p < 0.05$), and CRT ($p < 0.01$). Serum 25(OH)D3 significantly decreased in the group C ($p < 0.01$), in contrast to its changes obtained in alfacalcidol treated ones. **Conclusion**. 1αD3 2 μg and 3 μg daily is as effective as prednisone (mean 13.3 mg daily) in RA activity control and also has the additional favorable impact on muscle function.

Key words:
arthritis, rheumatoid; alfacalcidol; muscles; disease progression.

Apstrakt

Uvod/Cilj. Hormon D (vitamin D) igra važnu ulogu u imunoregulaciji i koštano-mišićnom metabolizmu. Cilj rada bio je ispitivanje uticaja alfakalcidola (1αD3) analoga hormona D, i prednizona, na mišićnu funkciju i aktivnost bolesti kod obolelih od aktivnog reumatoidnog artritisa (RA). **Metode**. U istraživanje je bilo uključeno 67 bolesnika sa RA koji su imali aktivnu bolest (indeks aktivnosti bolesti – DAS28 > 3,2), uprkos maksimalno podnošljivoj dozi metotreksata (MTX), tokom prethodna tri meseca. Prikupljeni su podaci o aktivnosti bolesti (DAS28), mišićnoj funkciji [(test ustajanja sa stolice (CRT), test ustani i kreni (TUG), šestominutni test hodanja (6MWT), tandem hod (TW)], kao i zapaljenski i bezbednosni laboratorijski nalazi. Bolesnici su nasumično raspoređeni na tromesečno lečenje: 1 μg (grupa A1) 2 μg (grupa A2) ili 3 μg (grupa A3) 1αD3 dnevno ili prednizon (grupa C) 20 mg dnevno, prvih mesec dana, potom 10 mg, uz MTX. **Rezultati**. Na kraju lečenja u svim terapijskim grupama nađena je visoko statistički značajno snižena aktivnost bolesti ($p < 0,01$). U grupi A2, lečenih 1αD3 u dozi 2 μg/dan značajno su poboljšani TUG i 6MWT, a kod lečenih 1αD3 3 μg (A3 grupa) došlo je do visoko statistički značajnog poboljšanja CRT ($p < 0,01$) i značajnog poboljšanja 6MWT ($p < 0,05$). U grupi C došlo je do visoko statistički značajnog sniženja nivoa vitamina D (25(OH)D3) u serumu ($p < 0,01$), nasuprot njegovom povećanju kod lečenih alfakalcidolom. **Zaključak**. Alfakalcidol u dozi 2 μg i 3 μg dnevno podjednako je efikasan kao prednizon (13,3 mg dnevno) u kontroli aktivnosti RA, a ima i dodatni povoljan efekat na mišićnu funkciju.

Ključne reči:
artritis, reumatoidni; alfakalcidol; mišići; bolest, progresija.

Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease, affecting 0,5-1% of the population [1]. The most prominent feature of the disease is synovitis of diarthrotic joints resulting in severe joint pain, reduced muscle strength and impaired physical function [2, 3]. Autoimmune driven synovium and systemic inflammation lead to cytokine

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dysregulation, elevation (3–100 times) of inflammatory cytokines in both beds [interleukin (IL) 6, interleukin 1 (IL1), tumor necrosis factor alpha (TNFα), etc], even during inactive phases of the disease [4–6]. Active RA also results in downregulation of sex steroids, growth hormone, anabolic factors such as insulin-like growth factor1 (IGF-1), insulin resistance and impairment of lipids metabolism, resulting in deleterious co-morbidities of the disease such as advanced atherosclerosis, muscle wasting-rheumatoid cachexia, osteoporosis [4–7]. Rheumatoid cachexia is reported in two-thirds of all RA patients, as cytokine imbalance, hypermetabolism and inactivity driven accelerated loss of skeletal muscle mass with the elevation of fat mass contribute to functional disability and lowering of the quality of life [6]. The course of RA is typically one of exacerbations and remissions. Aggressive treatment of RA also treats co-morbidities [8]. All RA patients at some point of the disease course require adaptation of disease-modifying antirheumatic drug (DMARD) or corticosteroid (CS) therapy, and combined strength and endurance training. Despite anti-inflammatory, symptomatic and structural efficacy in RA, a CS use is suggested to be as short as possible, due to its adverse effects [8, 9].

The discovery of the immunomodulatory and antitumor properties of D hormone prompted researchers to investigate the possibility of its use as a therapeutic agent for autoimmune and malignant diseases [10–18]. During the last several decades, there were many investigations with the aim to synthesize hormone (vitamin) D analogs [vitamin D receptor (VDR) agonists] with the same biologic activity and even stronger anti-inflammatory properties, but with lower blood calcium-increasing capacity [13, 18]. One of steroidal VDR agonists, alfacalcidol (1αD3) differs from hormone D only by lacking the 25(OH) group. Agonists with the OH group in 1α position having the highest specificity of binding to VDR were developed based on the knowledge about the specificity of biochemical mechanisms of VDR binding with natural D hormone or its analogs[16]. There are numerous preclinical and clinical data on preventive and therapeutic potential of alfacalcidol in the primary and secondary osteoporosis, osteoporotic fractures, neuromuscular functioning, transplantation, displaying its exclusive pleiotropic capacity [11, 14, 19–26]. Recently, greater intake of vitamin D is associated with a lower risk of RA, as well as the lower serum levels of vitamin D with higher disease activity [27, 28]. Hormone D deficiency in tissues, which is present in chronic inflammation, caused by inhibition of α-hydroxylase, can be corrected by alfacalcidol use, due to its activation in the liver or other target organs, bypassing body`s own feedback regulation [29, 30]. It has shown beneficial effects in autoimmune diseases such as RA and Psoriatic arthritis [11, 19]. Positive effect of alfacalcidol on regulating the cytokine homeostasis and increasing suppressor cells has been recognized [11, 19, 31, 32]. Recently, it has been shown that despite previous beliefs, it acts directly on inflammatory cytokine production without being additionally hydroxylated at the 25 position [33]. Due to the multifaceted potential of alfacalcidol in the autoimmune diseases, we wanted to assess its performance on joints and muscular features in clinical setting of active RA, at the point of current therapy adaptation. The aim of this study was to assess the impact of alfacalcidol or prednisone use on the muscle function and disease activity in active RA.

Methods

Study population and protocol

This was the open label prospective study approved by the Institutional Review Board/Ethics Committee (Decision No 29/1-7, February 19th, 2010) and the national Medicine and Medical Devices Agency of Serbia (Decision No 515-04-0544-12-2, September 28th, 2012). Written informed consent was obtained from all patients prior to enrollment.

The study population consisted of 67 active RA patients (46 females) on stable prior methotrexate (MTX) (10–25 mg/weekly) therapy and no steroids use for more than 3 months before enrollment. Patients were randomly assigned to administration of 1 μgr/daily 1αD3 (group A1, n = 17), 2 μg/daily 1αD3 (group A2, n = 19), 3 μg/daily 1αD3 (group A3, n = 16) treatment for 3 months or 20 mg of prednisone/daily for 1 month, followed by 10 mg prednisone/daily for 2 months (group C, n = 15), added to background stable MTX treatment. Alfacalcidol was provided by the investigator as gelatin capsules for oral administration (Alpha D3^{R}, TEVA, Serbia). Alfacalcidol dosing was modified only in case of toxicity, i.e. disturbances in calcium levels in blood or urine [more than 2.65 mmol/L in blood or more than 0.3 g daily in urine (DU) measured in two consecutive samples], drug dose half lowered during 1 week, until calcium and phosphorus levels normalized, then supplementation was re-started with the assigned treatment dose, if not, a patient was excluded from the study.

RA diagnosis was established at least 6 months prior the study started, using American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 criteria [34, 35], and patients were eligible for participation if ESR-DAS28 was > 3.2 [36]. Neither previous history of calcium metabolism disturbances or renal or bladder stones, nor any of following was allowed: psychiatric illness and/or social situation that would limit compliance with study medication and protocol requirements, signs and symptoms of severe, progressive or uncontrolled renal, hepatic, hematologic, endocrine, pulmonary, cardiac, neurologic or cerebral disease, inherited metabolic disease, pregnancy or lactation, presence of malignancy, or history of any malignancy in previous 10 years. Drugs which effect could be influenced or known to influence calcium or phosphorus metabolism, were not allowed. Any of the nonsteroidal anti-inflammatory drugs (NSAIDs), the additional DMARDs or steroid drugs, not present at the enrollment, could not be used during the study. Patients were advised to maintain a daily fluid intake of at least 1.5 L throughout the duration of the study and to intake only dietary calcium.

Study outcomes and safety parameters

Demographic and clinical data were collected, blood samples were taken, muscle function tests and disease activity were assessed at the study entry and after 3 months

after the treatment was assigned per protocol. Disease activity was assessed by the ESR DAS 28, calculated from the erythrocyte sedimentation rate (ESR), number of tender (TJC) and swollen joints (SJC) and the patients` assessment of disease activity (Patient Visual Analogue Scale – PVAS) based on the answer to the question "Given the overall impact of your arthritis to you, how are you feeling today?" Rated on a scale of 100 mm, answer may vary from 0 mm - very good to 100 mm - very poor, i.e. lower score reflected better state [37]. Muscle function was assessed by a battery of 4 tests usually used in the clinical setting:1) chair rising test (CRT), time to rise five times from a standard chair i.e. 46 cm high, with arms folded, shorter time (seconds) represent better muscle power [38], 2) timed up and go (TUG) test, time taken to rise from a chair, walk 3 meters, turn around, walk back and sit down, shorter time (seconds) represent better muscle coordination [39, 40], 3) 6-minutes walk test (6MWT) average distance (meters) of two attempts to walk during six minutes, longer distance represent better functional mobility [41], and 4) tandem walk (TW), time (seconds) of walking heel-to-toe in a 2 meters straight line, shorter time represent better muscle balance [40].

Safety follow-up visits monitoring calcium metabolism were performed 2 weeks apart from the start and then they were followed monthly for the clinical and laboratory findings, as well as adverse events (AE). At each of two follow-up visits, the patients were assessed for any relevant change in the clinical status, weight, arterial pressure, electrocardiogram, ESR (Westergren method), routine hematology (Coulter HmX haematology analyzer), biochemistry such as C-reactive protein (turbidimetry Gilford), glucose, albumin, liver enzymes, creatinine, uric acid, alkaline phosphatase, serum calcium, ionized calcium and 24 h calciuria were tested, while serum 25(OH)D3, (ECL electrochemiluminiscence-Elecsys 2010) and parathormone (PTH) (FPIA- fluorescent polarisation imunoassay–AxSym Abbot) were determined only at the start and at the end of the treatment period.

Statistical analysis

Statistical analysis was performed using the SPSS 20 package [data are presented as mean±SD (min-max)]. Subgroup changes of variables pre and post treatment were analyzed by paired *t*-test or Wilcoxon's test. The subgroup differences were assessed by independent *t*-test, ANOVA or χ^2 test, as appropriate and least significant difference (LSD) method, *post hoc*; $p < 0.05$ was considered statistically significant in all analyses.

Results

Demographic and clinical characteristics of patient population

All patients completed 3 months study period. Out of 67 RA patients included, 46 (68.65%) were females, average age was 56.24 ± 12.423 (23–83), disease duration 7.71 ± 6.68 (1-33) years, MTX dose 15.41 ± 3.28 (10–25) mg/weekly and MTX use of 5.67 ± 5.9 (0.5–15) years, mean disease activity (ESR DAS28) was 5.58 ± 0.905 (3.22–7.55) at the baseline. Baseline DAS28 was > 5.1 in 47 (70.14%) and DAS28 > 3.2 in 20 (29.85%) patients. The patients, randomized in 4 different treatment arms for 3 months, were fully comparable and their demographic and clinical characteristics are presented in Table 1. At enrollment, randomized groups were mutually comparable with respect to the

Table 1

Demographic, disease and muscle function characteristics in study groups

Demographic characteristics	A1 (n = 17)	A2 (n = 19)	A3 (n = 16)	C (n = 15)
M/F ratio, n	5/12	7/12	5/11	4/11
Age (years), $\bar{x}$ ± SD	57.94 ± 12.28	53.79 ± 12.012	53.06 ± 10.91	60.67 ± 13.75
BMI (kg/m^2), $\bar{x}$ ± SD	22.2 ± 1.9	21.9 ± 2.34	23.1 ± 2.76	22.7 ± 2.98
RA duration (y), $\bar{x}$ ± SD	9.82 ± 8.15	7.63 ± 7.12	5.63 ± 4.33	7.53 ± 6.79
Comorbidity, n (%)	6 (35.29)	9 (47.36)	6 (37.5)	7(46.66)
MTX duration (years)	7.82 ± 2.112	6.71 ± 1.567	5.31 ± 2.22	6.89 ± 4.11
MTX average (mg/w)	14.09 ± 1.988	14.45 ± 3.533	16.87 ± 3.48	16.66 ± 2.94
PsDMARD, n (%)	8 (47.05)	12 (63.15)	11 (68.75)	9 (60)
PbDMARD, n (%)	1 (5.88)	1 (5.26)	1 (6.25)	1 (6.66)
Disease activity, $\bar{x}$ ± SD				
DAS28	5.28 ± 0.874	5.81 ± 0.891	5.73 ± 0.87	5.86 ± 0.789
TJC	6.55 ± 2.999	9.21 ± 3.735	8.02 ± 4.33	9 ± 4.309
PVAS (mm)	52.51 ± 16.070	51.89 ± 17.129	51.73 ± 14.99	50.40 ± 15.624
ESR (mm/h)	28.18 ± 20	39.84 ± 23.735	42.88 ± 28.98	43.73 ± 15.895
RF, n (%)	15 (88.23)	13 (68.42)/19	14 (87.5)/16	12 (80)/15
ACPA, n (%)	3 (17.64)	7 (36.84)/19	4 (25)/16	4 (26.66)/15
25(OH)D3 (ng/mL), $\bar{x}$ ± SD	31.88 ± 13.57	28.97 ± 9.914	28.02 ± 14.12	34.02 ± 15.741
PTH (pg/mL), $\bar{x}$ ± SD	41.49 ± 19.64	31.2 ± 9.382	42.74 ± 11.66	40.49 ± 15.49
Muscle function, $\bar{x}$ ± SD				
CRT (s)	11.02 ± 2.91	12.02 ± 5.20	12.28 ± 3.46	12.72 ± 3.75
TUG (s)	6.92 ± 1.17	7.68 ± 2.91	7.22 ± 3.31	8.8 ± 3.138
6MWT (m)	437.3 ± 63.93	411.58 ± 127.35	423 ± 97.18	347.8 ± 15.741
TW (s)	7.93 ± 3.65	8.24 ± 4.05	9.7 ± 2.25	8.12 ± 5.343

n – number of patients; M – men; F – women; BMI – body mass index; RA – rheumatoid arthritis; MTX – methotrexate; W – week; VDMARD – disease modifying antirheumatic drugs; psDMARD – previous syntetic DMARD treatment; PbDMARD – previous biologic DMARD treatment; DAS28 – disease activity score; 25(OH)D3 – serum level of vitamin D; PTH – serum level of parathormone; ESR – erytrocite sedimentation rate; RF – rheumatoid factor positive; ACPA – anti-citrullinated protein atibodies positive; TJC – tender joint count; SJC – swollen joint count; pVAS – patient visual analogue scale; CRT – chair rising test; TUG – timed up and down test; 6MWT – six minute walk test; TW – tandem walk.

total number of the participants per group ($p = 0.881$, $\chi 2$), gender distribution ($p = 0.926$, $\chi 2$), age ($p = 0.257$, ANOVA), body mass index (BMI) ($p = 0.542$, ANOVA), duration of RA ($p = 0.374$, ANOVA), dosage of MTX 12 weeks before the study ($p = 0.061$, ANOVA), activity of the disease ($p = 0.058$, ANOVA), duration of MTX treatment ($p = 0.543$, ANOVA), rheumatoid factor (RF) positivity ($p = 0.567$, $\chi 2$), anticitrullinated protein antibodies (ACPA) positivity ($p = 0.588$, $\chi 2$), the serum levels of 25(OH)D3 ($p = 0.237$, ANOVA), PTH ($p = 0.075$), ESR ($p = 0.188$, ANOVA), comorbidity ($p = 0.690$, $\chi 2$), previous treatment with synthetic DMARD, (sDMARD) ($p = 0.978$, $\chi 2$) and biologic DMARD, (bDMARD) ($p = 0.998$, $\chi 2$), CRT ($p = 0.659$, ANOVA), TUG ($p = 0.173$, ANOVA), 6MWT ($p = 0.074$, ANOVA), TW and ($p = 0.062$, ANOVA).

Influence on muscle function

Assessment of muscle function revealed improvement in the time needed to perform CRT in all therapeutic regimens, while in one group treated with 3 μg of 1αD3, the improvement was highly statistically significant (12.28 ± 3,464 *vs* 8.9 ± 2.33, $p < 0.01$, paired *t*-test) (Figure 1a). Walking distance during 6 minutes (MWT) improved significantly in all treatment groups, in the A1 (437.29 ± 63.933 *vs* 486.35 ± 118.958, $p < 0.05$, paired *t*-test), in the A2, (411.58 ± 12.347 *vs* 483.95 ± 58.228, $p < 0,05$, paired *t*-test), in the A3 (439.3 ± 74.47 *vs* 483.13 ± 51.64, $p < 0.05$, *t*-test), as well as in the C group (347.73 ± 97.17 *vs* 398.73 ± 61.002, $p < 0.05$, paired *t*-test), as shown in Figure 1b. Timed up and go test (TUG), improved in 2 μg, 3 μg 1αD3 and prednisone treated patients, yet only in the A2 group the difference pre-post treatment was statistically significant (7.67 ± 2.91 *vs* 6.02 ± 1.15, $p < 0.05$, paired *t*-test) (Figure 1c). Tandem walk test (TW) slightly improved in all treatment groups, except in the A1 one, yet neither change was statistically significant. Subgroup analysis showed no difference in muscle tests changes pre-post treatment, except for ΔTUG (A2 *vs* C, $p < 0.05$, ANOVA).

Influence on disease activity

Clinical efficacy indices showed marked improvement in term of ESR DAS28 in all therapeutic regimens, with highly statistically significant reduction achieved in the group A1 (5.28 ± 0.874 *vs* 4.16 ± 1.03, $p < 0.01$, paired *t*-test), A2 (5.81 ± 0.89 *vs* 4.32 ± 1.03, $p < 0.01$, paired *t*-test), A3 (5.74 ± 0.87 vs 4.44 ± 0.86, $p < 0.01$, paired *t*-test), as well as in the group C (5.86 ± 0.79 *vs* 4.13 ± 0.913, $p < 0.01$, paired *t*-test). In order to further explore the efficacy of different

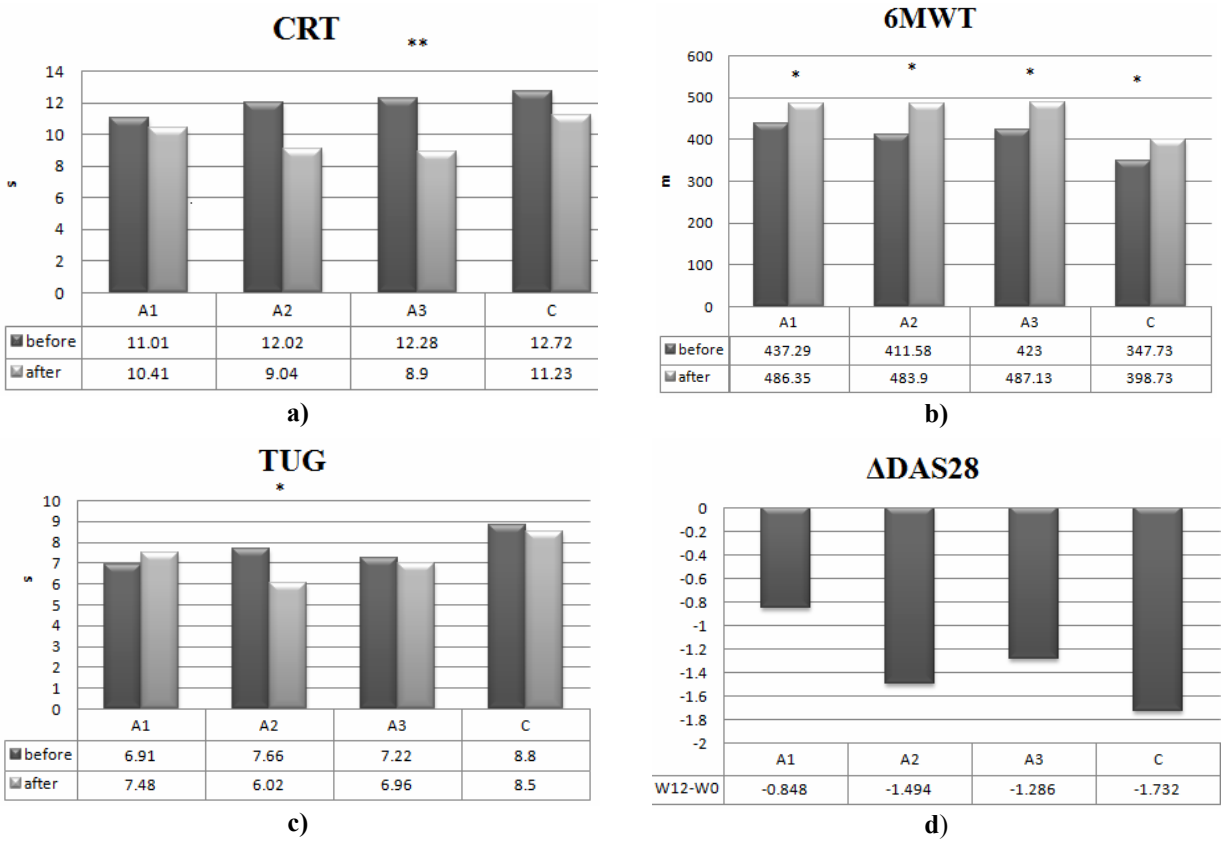

Fig. 1 – Changes of muscle function tests and the disease activity over time; a) Chair rising test (CRT) pre-post treatment; b) Six minutes walk test (6MWT) pre-post treatment; (c) Timed up and go test (TUG) pre-post treatment; d) Difference of disease activity score (ΔDAS28) post-pre treatment; A1 – group supplemented with 1μg 1αD3; A2 – group supplemented with 2 μg 1αD3; A3 – group supplemented with 3 μg 1αD3; C – group treated with prednisone; * – statistically significant difference ($p < 0.05$); ** – highly statistically significant difference ($p < 0.01$).

treatment regimens in term of the disease activity, we used EULAR DAS28 response model [37]. Changes of the disease activity (ΔDAS28) during 12 week study in four different treatment regimens were A1 *vs* A2 *vs* A3 *vs* C, $p < 0.05$, ANOVA, while there was no difference in A2, the A3 groups *vs* C group ($p = 0.437$, post-hoc Fisher's Least Significant Difference – LSD), (Figure 1d). Also, there was no significant difference in the number of patients with the good DAS28 EULAR response, which means the reduction of DAS28 > 1.2, in the groups A2, A3 and C ($p = 0.532$, $\chi 2$).

Influence on calcium metabolism parameters and safety issues

The patients reported overall good tolerability of study treatments, no serious adverse events or any laboratory or clinical adverse events were observed.

Compared to baseline levels, the serum 25(OH)D3 levels raised in all alfacalcidol treated patients, at the end of the study, while in patients treated with prednisone significantly decreased (34.02 ± 15.74 *vs* 21.93 ± 10.90, $p < 0.01$, paired *t*-test). PTH levels significantly decreased in the A2 group (31.2 ± 9.39 *vs* 26.42 ± 10.29, $p < 0.05$, paired *t*-test) and the A3 group (42.88 ± 11.66 *vs* 26.45 ± 13.401, $p < 0,01$, paired *t*-test) at the end of the treatment period.

The serum calcium and ionized calcium follow-up were particularly of interest, both remained unchanged in all treatment groups, while calciuria was significantly raised in the groups treated with alfacalcidol 2 μg and 3 μg, only in the latter group, the upper limit of normal exceeded slightly (ULN) of 0.3 g/DU (in the A2 0.18 ± 0.077 *vs* 0.27 ± 0.09, $p < 0.01$, in the A3 group 0.13 ± 0.047 *vs* 0.32 ± 0.11, $p < 0.01$, paired *t*-test). Alfacalcidol daily dose was corrected from 3 μg to 1 μg daily for 1 week in 4 patients, due to an increase in calciuria registered in 2 patients in the A3 group, 2 weeks, and 8 weeks apart from the start of the study, respectively. After daily dose reduction, calciuria normalized, and study treatment was continued as before.

The serum glucose levels raised pre-post treatment in the C group by 1.7 mmol/L (about 40% compared to baseline), yet not above ULN, in contrast to 1αD3 treated patients, who had about 2% lower glucose levels.

Discussion

As RA is associated with the significant physical disability, which has a negative impact on employment and health related quality of life. A primary goal for many patients is to maintain and improve physical function [42]. In the mutifactorial model of disability, skeletal muscle wasting and weakness in RA patients have a large influence [2, 3, 6]. Data on the impact of RA therapy on the skeletal muscle function are lacking. Contemporary aggressive sDMARD and bDMARD RA treatment, accompanied with exercises is effective, as for other metabolic co-morbidities, yet the challenge is to identify and treat any modifiable factors that contribute to physical disability [8, 36, 43]. Vitamin D3 (hormone D) deficiency may be one of it [20, 25].

Evidence from recent meta-analysis of 16 randomized controlled trials (RCTs) of vitamin D treatment influence on muscle function, do support the beneficial effect of vitamin D supplementation on muscle strength and function in the elderly, vitamin D insufficient subjects, with major lack of data on the possible effect in younger people [43]. Identified studies were heterogeneous with regard to most aspects including the indices measured. Yet, some analogy can be made with the study of Lips et al. [44] who used a battery of physical performance tests including TUG, CRT, 6MWT, in 226 ambulatory patients, in which 8400 IU vitamin D3/weekly or placebo were applied for 4 months. They observed a rise in baseline vitamin D3 serum levels, yet no significant positive effect on muscle function was observed, compared to placebo. The average age of their study population was 78 years, with no data about co-morbidities. We had much younger (56 years), ill (RA) and vitamin D replete population (31.56 ng/mL) in our study. We observed better muscle functioning in all of our patients, except for TUG test in the 1 μg αD_3 subgroup (0.57 s), with the significant improvement in CRT, 6MWT (in 3 μg αD3 treated patients) and 6MWT and TUG (in 2 μg αD3 treated ones), accompanied with statistically significant serum 25(OH)D3 elevation and decrease of PTH in the same subgroups and highly statistically significant reduction of RA activity, also. Alfacalcidol 1 μg treated ones had significant reduction of disease activity (ΔDAS28 -0.84) accompanied with improvement in walking distance as measured by 6MWT, similar to findings of some improvement in muscle function noticed in prednisone treated ones, yet, only walking distance in 6MWT significantly increased, even they decreased the disease activity the most (ΔDAS28 -1.73). Our data additionally support the evidence of 1αD3 efficacy in RA, as we got highly significant improvement of the disease activity assessed by DAS28, in all alfacalcidol treated patients, as one of the most cited open labeled trial of alfacalcidol 2 μg use for twelve weeks in the active RA patients (n = 19), that showed significantly fewer swollen joints and improvement in two symptom scale scores, the Ritchie articular and Lee indices, with *in vitro* immunomodulatory effects on their lymphocytes [11].

Scharla et al. [26] showed that 1 μg alfacalcidol use for 3 months in RA vitamin D replete patients resulted in increase of lower extremity muscle power by isometric knee extension measurement (60%) compared to native vitamin D 1000 IU use (18%). We also observed an increase in CRT as a measure of muscle power in all alfacalcidol treated patients. It is of importance that no endurance nor strength training in any of our subjects was applied.

Study of muscle biopsies in osteoporotic patients showed that 1 μg alfacalcidol treatment for 3–6 months, induced an increase in the relative number and cross-sectional area of fast-twitch type IIA muscle fibers, accompanied by the reduction of fast-switch type IIB fibers, altogether with the improvement in activities of daily living [45]. We did not perform any imaging or morphological analysis of muscle tissue, while the clinical impression of the reduced disease activity index and increased mobility in our patients was im-

pressive. This represents the starting point for future investigations of $1\alpha D_3$ in RA patients in a placebo-controlled manner, including whole body densitometry scan with an estimation of lean/fat mass pre-post treatment.

We can say that the most gain of three-month study treatment in active RA patients was observed when 2 μg and 3 μg 1αD3 were used, as a dual action on inflammation and muscle performance was documented. Dual effect of alfacalcidol treatment is observed in osteoporotic subjects, both in primary and corticosteroid induced osteoporosis [23, 24, 46–48]. Meta-analysis of those studies showed a reduction of fracture rates as the result of improved bone mass, quality and a reduction of falls by improvement of muscle function, which was the exclusive action of alfacalcidol [24, 48]. Hypercalcemia as the adverse effect is rare in alfacalcidol use, as shown in post-marketing surveillance of 13550 osteoporotic patients [49]. We used high doses of alfacalcidol, but also found no hypercalcemia, yet, reversible calciuria occurred in 2 μg and 3 μg treated subjects, not of clinical importance.

Prednisone treatment (average 13.3 mg daily, for 3 months) in our study resulted in a highly significant reduction of serum level of 25(OH)D3, which was definitely not a desirable state, as it was closely negatively related to at least RA disease activity [28, 50]. On the contrary, anti-inflammatory effects of all three alfacalcidol treatment regimens in our study resulted in increased levels of serum 25(OH)D3, lower serum PTH, the state which is known to be related to better muscle mass and strength, reduced falls rate, better physical performance as was shown in systematic review of Schacht and Richy [51] and in 3 year`s prospective study of Visser et al. [52]. All the benefits of the disease activity, PTH metabolism and muscle function of 1αD3 supplementation in active RA, did not result from correcting vitamin D deficiency since the patients were already vitamin D replete. We assume that direct effect of high doses of alfacalcidol on the nuclear VDRs in different target tissues produced such effects. Corticosteroids are widely used due to their symptomatic, anti-inflammatory and structural effects in active RA [8, 9].

Conclusion

Based on our findings, alfacalcidol might find its place as bridging therapy instead of corticosteroids, in at least selected group of RA patients. Three-month alfacalcidol treatment is as effective as prednisone in the disease control and has the dose dependent positive effect on muscle function in vitamin D replete active RA patients with a good safety profile.

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Specific bronchial hyperreactivity and hypersensitivity in patients with allergic asthma

Specifična bronhijalna hiperreaktivnost i kožna preosetljivost kod bolesnika sa alergijskom astmom

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Abstract

Background/Aim. Bronchial asthma is a disease that is characterized by the variability of the clinical picture, physical and functional status and the existence of bronchial hypersensitivity and hyperreactivity with varying degrees. Bronchial responsiveness and sensitivity are tested in patients with clinically suspected existence of asthma and normal spirometry test. The aim of the study was to analyze the patients with atopic asthma and study test results of skin sensitization to inhaled allergens, nonspecific bronchial hyperreactivity and specific hyperreactivity estimated by bronchial provocation tests with inhalant allergens. **Methods**. The prospective study at the Pulmonology Clinic of the Military Medical Academy in Belgrade Serbia, during 2014, included 70 male subjects aged 18–30 years, who had perennial asthma symptoms. All subjects were nonsmokers, with normal spirometry findings, with normal radiological chest findings and with no symptoms of respiratory infection over the past two months. All respondents were tested with skin prick tests with inhalant allergens and nonspecific bronchial provocation test with histamine. On the basis of histamine test, subjects were divided into two groups: the group I, in which there was a slight degree of hypersensitivity [provocation concentration of histamine causing a 20% fall in forced expiratory volume – PC_{20} = 6.09 ± 1.1 mg/mL], and the group II with negative histamine test (PC_{20} = 14.58 ± 6.34 mg/mL). Specific bronchial provocation test was performed in all patients, and the selection of the allergens was carried out based on the results of testing of skin hypersensitivity. **Results.** Results of skin sensitization show the highest incidence of mites *Dermatophagoides pterronissinus* (83.3% group I and 85.0% group II) followed by grass pollen (53.3% group I and 52.0% group II), and house dust (33.3% group I and 50.0% group II). There were no statistically significant differences in allergens between groups ($p > 0.05$). In both groups, spirometry findings were within normal values [forced vital capacity – FVC and forced expiratory volume 1 – FEV1 > 80% predictive value], but statistically significant difference was found in FEV1 between groups ($p < 0.05$). Specific bronchial provocation tests with solutions of inhaled allergens in both groups caused a significant decline in FEV1 (≥ 20%) in all patients individually. No statistically significant differences were found neither between groups, nor between individual allergens (average decline in FEV1: Group I 32.9 ± 2.4% and group II 31.5 ± 2.2%). **Conclusion**. There is no relationship between the degree of specific and non-specific bronchial hyperreactivity in patients with allergic asthma.

Key words:
asthma; bronchial spasm; hypersensitivity; allergens; skin tests; histamine; spirometry.

Apstrakt

Uvod/Cilj. Bronhijalna astma je bolest koja se odlikuje varijabilnošću kliničke slike, fizičkog i funkcijskog statusa i postojanjem bronhijalne hipersenzitivnosti i hiperreaktivnosti različitog stepena. Bronhijalna reaktivnost i senzitivnost ispituje se kod bolesnika sa kliničkom sumnjom na postojanje astme i normalnim spirometrijskim testom. Cilj rada je bio da se kod bolesnika sa atopijskom astmom analiziraju rezultati testova kožne preosetljivosti na inhalacione alergene, nespecifične bronhijalne hiperreaktivnosti i specifične hiperreaktivnosti procenjene bronhoprovokacijskim testovima sa inhalacionim alergenima. **Metode**. Prospektivnom studijom u Klinici za pulmologiju Vojnomedicinske akademije tokom 2014. godine, obuhvaćeno je 70 ispitanika muškog pola starosti od 18 do 30 godina, koji su imali višegodišnje simptome astme. Svi su bili nepušači, urednog spirometrijskog i radiološkog nalaza na snimku grudnog koša, bez simptoma i znakova respiratornih infekcija tokom prethodna dva meseca. Svim ispitanicima rađeni su testovi kožne preosetljivosti sa inhalacionim alergenima i nespecifični bronhoprovokacijski test sa histaminom. Na osnovu

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histaminskog testa ispitanici su podeljeni u dve grupe: I grupu, kod koje postoji lakši stepen hipersenzitivnosti [provokaciona koncentracija histamina koja uzrokuje 20% smanjenja forsiranog ekspiratornog volumena u jednoj sekundi (PC_{20}) = 6,09 ± 1,1 mg/mL] i II grupu sa negativnim histaminskim testom (PC_{20} = 14,58 ± 6,34 mg/mL). Kod svih ispitanika izvršeno je specifično bronhoprovokacijsko testiranje, a izbor alergena vršen je na osnovu rezultata testiranja kožne preosetljivosti. **Rezultati.** Rezultati kožne preosetljivosti pokazuju najveću učestalost grinje *Dermatophagoides pterronissinus* (83,3% u grupi I i 85% u grupi II), zatim polena trave (53,3% u grupi I i 52% u grupi II) i kućne prašine (33,3% u grupi I i 50% u grupi II), bez statistički značajne razlike u zastupljenosti alergena između ovih grupa ($p > 0,05$). U obe grupe spirometrijski nalaz bio je u granicama referentnih vrednosti [forsirani vitalni kapacitet (FVC) i forsirani ekspiratorni volumen u jednoj sekundi (FEV21) > 80% prediktivne vrednosti], ali je postojala statistički značajna razlika u vrednosti FEV1 između grupa ($p < 0,05$). Specifičnim bronhoprovokcijskim testovima sa rastvorima inhalacionih alergena kod obe grupe ispitanika izazvano je značajano smanjenje FEV1 (≥ 20%) kod svih ispitanika. Nije bilo statistički značajne razlike u plućnoj funkciji među grupama, a ni u preosetljivosti na pojedine alergene (prosečano smanjenje FEV1: I grupa – 32,9 ± 2,4% i II grupa – 31,5 ± 2,2%). **Zaključak.** Nije utvrđena direktna povezanost između stepena specifične i nespecifične bronhijalne hiperreaktivnosti kod bolesnika sa alergijskom astmom.

Ključne reči:
astma; bronhusi, spazam; hipersenzibilnost; alergeni; koža, testovi; histamin; spirometrija.

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways that is clinically characterized by attacks of shortness of breath, especially in the titration phase, followed by wheezing, cough and sputum tough secretions [1, 2].

The cause of asthma is unknown but it is thought that there is a tendency to develop the disease, which is transmitted as an autosomal dominant inheritance; also, numerous external and internal factors are determined (eg. atopy) that can trigger immune reaction in the airways. A specific type of chronic inflammation of the bronchial mucosa with dominant engaging lymphocytes (CD4+, Th2), eosinophils and metahromal cells in the airway mucosa forms the basis for bronchial hyperreactivity, expression and chronicity of the disease. In addition to edema, epithelial damage and increased mucus production, inflammation leads to irreversible morphological changes such as subepithelial fibrosis and hypertrophy of smooth muscle, resulting in the so-called airways remodeling [1–4].

The diagnosis of asthma is based on a history of problems, physical and pathological findings of the lung, pulmonary function tests (spirometry, which confirms the limitation of airflow, which usually registers obstructive disorders of ventilation – reducing the value of forced expiratory volume in one second (FEV1) and relations with the vital capacity (VC) Tiffeneau index – FEV1/VC × 100), skin tests to inhalant allergens and basic indicators of immune status and inflammation in the bronchial tree [eosinophilic leukocytes in peripheral blood, immunoglobulin E in serum and eosinophils in sputum and nitric oxide (NO) in exhaled air). When the spirometry test is normal, and clinical picture indicates asthma, broncho-provocation testing is performed (non-specific bronchial provocation test – histamine, methacholine and others, or specific bronchial provocation test with inhalant allergens, which is less common) [4–12].

The aim of the study was to analyze the correlation between non-specific bronchial hyperreactivity and specific bronchial hyperreactivity in patients with allergic asthma, estimated by bronchial provocation test with inhaled allergens, as well as to assess their relationship.

Methods

The prospective study at the Pulmonology Clinic in the tertiary health care university hospital, the Military Medical Academy, Belgrade, Serbia, during 2014, included 70 male subjects aged 18 to 30 years, who had had for many years asthma symptoms (shortness of breath, difficulty breathing, wheezing, fatigue, night choking and dry or productive cough). All were non-smokers.

All the patients were tested by hypersensitivity skin tests to inhalant allergens (Torlak, Institute of Virology, Vaccines and Sera, Belgrade, Serbia) and nonspecific bronchial provocation test with histamine (Fluka Histamine dichydrochloride, Sigma-Aldrich, Germany). Patch testing was done, a standardized, prick method with dual control (saline solution and histamine). Allergens and dual controls were injected intradermally to produce a small bleb, and the outcome measure was an increase in the size of the wheal after 20 minutes. Allergens needed to be diluted (100–1000 fold) from the concentrations used for skin prick testing. There were skills required to inject correctly and interpret the result.

Spirometry finding in all patients was within normal ranges (SpiroPro, Erich Jaeger GMBH). Chest Radiological findings in all patients were normal and there were no symptoms of respiratory infection over the past 2 months. Nonspecific bronchial hypersensitivity and hiperreactivity were tested with histamine solutions with the help of the device for inhalation (Inhalog 2, Drägerwerk AG Lübeck). The tests were performed by cumulative technique until reaching the threshold of sensitivity (PC_{20}) – provocation concentration of inhaled histamine that led to a drop in FEV1 by 20% compared to baseline. The value of PC_{20} was calculated by algorithmic transformation of the measurement results. The histamine test was estimated as negative if PC_{20} was not reached, even with the concentration of histamine higher than 8 mg/mL of histamine. The value of PC_{20} in the range of 4–7.9 mg/mL, ment slight degree of nonspecific bronchial hypersensitivity [6–12].

On the basis of histamine test results subjects were divided into two groups: the group I, in which there was a slight degree of hypersensitivity (PC_{20} = 4–7.9 mg/mL), and the group II with negative histamine test (PC_{20} = 8 mg/mL).

Specific bronchial provocation tests were performed in all patients, 24 hours after histamine test. The allergens were selected on the basis of the results of skin testing (Torlak, Institute of Virology, Vaccines and Sera, Serbia). The initial concentration of the solution of allergen was 1,000 units of total nitrogen (TNU). The test was considered positive when it led to a drop in FEV1 of 20%, and more activity compared to the initial value. The reactivity was evaluated on the dose of allergen that had a major response of the bronchi, and on the basis of decrease in FEV1 [6, 9–14]. Due to possible late asthmatic reactions, the subjects were being observed for 7 hours after the test.

All attribute variables were presented in the form of the frequency of certain categories, and statistical significance between the individual categories was tested by χ^2 test. Continuous variables were presented as arithmetic means and standard deviations ($\bar{x} \pm SD$). Continuous variables were compared using Student's *t*-test or Mann-Whitney *U*-test. The normality of the data was assessed by using Kolmogorov-Smirnov test. All the analyses were estimated at $p < 0.05$ level of the statistical significance.

Results

Average age, duration of the disease and parameters of pulmonary ventilation in study participents are presented in Table 1. There were statistically significant difference of these parameters between the group with mild degree of hypersensitivity and the group with negative histamine test. The analysis of the average age and disease duration in our patients established that patients in the group II were older in comparison with those in the group I, while the duration of illness was significantly shorter in the group II in comparison with the group I.

In both groups, spirometry findings were within normal values [forced vital capacity – FVC and FEV1 > 80% of predictive value), but there was a statistically significant difference in FEV1 between groups ($p < 0.05$) and FEV1/ FVC index ($p < 0.05$).

The results of skin sensitization to inhaled allergens are shown in Table 2. There were no significant differences in allergens between groups ($p > 0.05$). The most common allergens were mites *Dermatophagoides pterronissinus*, grass pollen and house dust.

The degree of nonspecific bronchial sensitivity, determined by histamine test is shown in Table 3. Statistical analysis showed that there was a significant difference in the percentage fall in FEV1 between the groups I and II ($p < 0.05$). Nonspecific bronchial provocation test with histamine was negative for the whole group II (a significant decline in FEV1 was not caused in any of the patients), while in the group I it was minor (average fall FEV1: 1.44 ± 0.34 L or 27.2 ± 5.7%).

Specific bronchial provocation tests with solutions of inhaled allergens in both groups caused a significant decline in FEV1 (≥ 20%) in all patients individually (the group I: 32.9 ± 2.4%; the group II: 31.5 ± 2.2%; $p = 0.0151$). The results are shown in Table 4.

Discussion

Asthma is a major burden for governments, healthcare providers and patients [15]. The annual costs of the European economy of healthcare and lost productivity due to asthma are estimated as €33,9 billion [16]. This is a disease that is characterized by the variability of the clinical picture, physical and functional status which is caused by chronic inflammation with the presence, also variable, bronchial hypersensitivity and hyperreactivity.

Bronchial hyperresponsiveness and hypersensitivity were tested in patients with clinical suspicion of the existence of asthma, in whom the disorder in pulmonary ventilation was not registered by spirometry test. Positive bronchial provocation test can confirm the diagnosis of bronchial asthma. Standardized method of bronchial challenge with

Table 1

Characteristics of study participants

Parameters	Group I (n = 30)	Group II (n = 40)	*p*
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	
Average age (years)	19.9 ± 1.3	20.8 ± 1.6	0.0116
Disease duration (months)	11.1 ± 1.9	8.2 ± 2.2	< 0.0001
FVC, (L)*	5.24 ± 0.64 (95.0)*	5.36 ± 0.71 (96.8)*	0.4616
FEV1, (L)*	4.36 ± 0.53 (88.2)*	4.67 ± 0.71 (101.8)*	0.0402
FEV1/ FVC	83.7 ± 6.4† (98.3)*	86.7 ± 5.73† (104.5)*	0.0470

Group I – patients with high degree of hypersensitivity to histamine; group II – patients with negative histamine test; FVC – forced vital capacity; FEV1 – forced expiratory volume in one second; †Tiffeneau index – the ratio of FEV1/FVC, expressed as a percentage; *percentage of values obtained in relation to the standard values for this population of patients; $\bar{x}$ – arithmetic mean; SD – standard deviation.

Table 2

Skin tests with inhalant allergens

Allergens	Group I (n = 30)	Group II (n = 40)	*p*
Dermatophagoides pterronissinus	25 (83.3)	34 (85.0)	
Grass pollen	16 (53.3)	21 (52.0)	
House dust	10 (33.3)	20 (50.0)	
Weed pollen	6 (20.0)	11 (27.5)	0.9542
Feathers	5 (16.7)	5 (12.5)	
Tree pollen	5 (16.7)	6 (15.0)	
Linen	1 (3.3)	2 (5.0)	

Group I – patients with high degree of hypersensitivity to histamine; Group II – patients with negative histamine test.

Table 3

Nonspecific bronchial hyperreactivity test results in the study participants

Parameter	Group I			Group II			*p*
	Value	n	%	Value	n	%	
Reactivity degree	Minor (4 mg/mL ≤ PC_{20} ≥ 7.9 mg/mL)	30	100	Insignificant (PC_{20} ≥ 8 mg/mL)	40	100	-
Average fall of FEV1 (L) $\bar{x} \pm SD$	1.44 ± 0.34	30	27.2 ± 5.7	0.68 ± 0.26	32	12.48 ± 4.09	< 0.0001
PC_{20}, $\bar{x} \pm SD$	6.09 ± 1.1	30	100	14.58 ± 6.34	32	75.0	< 0.0001

Group I – patients with high degree of hypersensitivity to histamine; Group II – patients with negative histamine test; PC_{20} – provocation concentration of histamine, which leads to a fall in forced expiratory volume in one second (FEV1) of 20%; $\bar{x}$ – arithmetic mean; SD – standard deviation.

Table 4

Specific bronchial provocation test results in the study participants

Parameters	Group I		Group II		*p*
	n (%)	↓ FEV1 (%)	n (%)	↓ FEV1 (%)	
Allergen					
Dermatophagoides pterronissinus	24 (80.0)	33.6	28 (70.0)	30.8	
Grass pollen	4 (13.3)	28.5	10 (25.0)	34.2	0.7858
House dust	2 (6.6)	33.0	2 (5.0)	28.0	
Total	30 (100)	32.9 ± 2.4*	40	31.5 ± 2.2*	0.0151

Group I – patients with high degree of hypersensitivity to histamine; Group II – patients with negative histamine test; FEV1 – forced expiratory volume in one second; * arthitmetic mean ($\bar{x}$) ± standard deviation (SD).

histamine determined the degree of hypersensitivity in all patients, and this served as a criterion for distribution of patients per groups: moderate and slight degree of hypersensitivity and negative histamine test [6, 8–14, 17]. In this study the analyzed groups were with the slight degree of hypersensitivity and negative histamine test.

The test results of skin sensitization to inhaled allergens showed no difference in the prevalence of allergens tested by groups of respondents. The intensity of skin reaction was used for the selection of allergens which will be used for specific bronchial provocation testing. In our study, the most common allergens were mites *Dermatophagoides pterronissinus*, grass pollen and house dust. In other studies, skin prick test with standard extracts including house dust mites, animal dander, molds, pollens etc. were also performed on patients according to the herbal geography of the local area. The common aeroallergens were house dust mites (88.5%), molds (82.9%), animal dander (79.5%), weeds (77.6%), trees (75.5%) and grass pollen (71.5%) [18]. Bazarbachi et al. [19] found to identify sensitized patients on the eleven allergens, Dermatophagoides pteronyssinus, *Dermatophagoides farinae*, *Blomia*, *Parietaria, grass*, *Salicaceae, oak*, *Oleaceae*, dog, cat, and cockroaches.

Cockroft et al. [20, 21] and Boulet et al. [22] studied 25 patients with asthma and examined the relationship between specific bronchial hypersensitivity, skin hypersensitivity and nonspecific bronchial hypersensitivity to histamine. They concluded that there was a positive correlation between specific hypersensitivity to inhaled allergens and nonspecific hypersensitivity to histamine.

In this study, the specific bronchial provocation test with solutions of different concentrations of allergens caused a significant response in all 70 patients. The analysis of FEV1 decline achieved during the test did not find significant differences between the first and the second group. By analyzing the distribution of the concentration of allergens, the existence of significant differences between the groups with different levels of nonspecific bronchial hypersensitivity was not established.

Examining the effect of inhalation of repeated low doses of allergens in bronchial asthmatics with atopy in 1998, Sulakvelidze et al. [23] reported that despite a lack of significant response to bronchial allergen (FEV1 decline to 5%) in induced sputum after inhalation of allergens, a significant increase in eosinophil number was recorded, as well as the increase in the level of eosinophilic cationic protein (ECP) and an increase in levels of cytokines (interleukins IL-5).

In addition to the studies that presented the results of experiments, in 1996 Djukanovic et al. [24] published the results of the study in which they carried out cytological and histological analysis of inflammatory response development in the bronchus before and after natural exposure of allergic patients with bronchial asthma to grass pollen. They confirmed, as well as some other authors, that exposure to an allergen leads to the induction of inflammation in bronchus with the engagement of T lymphocytes, mast cells and eosinophilic leukocytes. In natural conditions, seasonal allergen exposure of sensitized persons will lead to enhanced secretion of proinflammatory cytokine IL-4 and the mobilization and activation of T lymphocytes and eosinophils [25].

Conclusion

Presented prospective study did not demonstrate existance of a compulsory direct relationship between the degree of specific and non-specific bronchial hyperreactivity in patients with allergic asthma.

Testing specific bronchial hyperreactivity, as a simulation of natural processes, should be performed in selected patients with suspected allergic asthma.

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Radiographic cephalometry analysis of head posture and craniofacial morphology in oral breathing children

Radiografsko-kefalometrijska analiza položaja glave i kraniofacijalne morfologije kod dece koja dišu na usta

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Abstract

Background/Aim. Nasal breathing plays an important role in overall physical growth and mental development, as well as in the growth of the craniofacial complex. Oral breathing over a long period of time, can cause changes in position of the head relative to the cervical spine and jaw relationship. It can cause an open bite and the narrowness of the maxillary arch due to increased pressure of strained face. The aim of this study was to analyze the position of the head and craniofacial morphology in oral breathing children, and compare the values obtained compared with those of the same parameters in nasal brething children. **Methods**. We analyzed the profile cephalometric radiographs of 60 patients who had various orthodontic problems. In the first group there were 30 patients aged 8–14 years, in which oral breathing is confirmed by clinical examination. In the second group there were 30 patients of the same age who had orthodontic problems, but did not show clinical signs of oral breathing. The analyses covered the following: craniocervical angle (NS/OPT), the length of the anterior cranial base (NS), anterior facial height (N-Me), posterior facial height (S-Go), the angle of maxillary prognathism (SNA), angle of mandibular prognathism (SNB), difference between angles SNA and SNB (ANB angle), the angle of the basal planes of the jaws (SpP/MP), cranial base angle (NSB), and the angle of facial convexity (NA/Apg). **Results**. The average value of the craniocervical angle (NS/OPT) was significantly higher in OB children ($p = 0.004$). There were significantly different values of SNA ($p < 0.001$), ANB ($p < 0.001$), NA/APg ($p < 0.001$) and length of the anterior cranial base (NS) ($p = 0.024$) between groups. **Conclusion**. Oral breathing children have pronounced retroflexion of the head in relation to the cervical spine compared to nasal breathing children, and the most prominent characteristics of the craniofacial morphology of skeletal jaw relationship of class II and increased facial convexity.

Key words:
mouth breathing; craniofacial abnormalities; child.

Apstrakt

Uvod/Cilj. Disanje na nos ima važnu ulogu u celokupnom telesnom rastu i psihičkom razvoju, pa i u rastu kraniofacijalnog kompleksa. Kod dece koja dišu na usta u dugom vremenskom periodu može se promeniti položaj glave u odnosu na vratnu kičmu, kao i odnos vilica. Može se javiti otvoren zagrižaj i uskost maksilarnog zubnog luka zbog povećanog pritiska zategnutih obraza. Cilj ove studije bio je da se analizira položaj glave i kraniofacijalna morfologija dece koja dišu na usta i dobijene vrednosti uporede sa vrednostima istih parametara kod dece koja dišu na nos. **Metode**. Analizirani su profilni telerendgen snimci kod ukupno 60 pacijenata koji su imali različite ortodontske probleme. U prvoj grupi je bilo 30 pacijenata starosti 8–14 godina, kod kojih je kliničkim pregledom utvrđeno disanje na usta. U drugoj grupi je bilo 30 pacijenata iste starosti koji su imali ortodontske probleme, ali nisu pokazivali kliničke znake disanja na usta. Analizirani su: kraniocervikalni ugao (NS/OPT), dužina prednje kranijalne baze (NS), prednja visina lica (N–Me), zadnja visina lica (S–Go), ugao maksilarnog prognatizma (SNA), ugao mandibularnog prognatizma (SNB), razlika između uglova SNA i SNB (ugao ANB), ugao osnovnih ravni vilica (SpP/MP), ugao baze lobanje (NSBa) i ugao konveksiteta lica (NA/Apg). **Rezultati**. Prosečna vrednost kraniocervikalnog ugla- (NS/OPT) bila je značajno veća kod dece koja dišu na usta ($p = 0{,}004$). Ustanovljena je značajna razlika u vrednosti uglova (SNA) ($p < 0{,}001$), ANB ($p < 0{,}001$), NA/Apg ($p < 0{,}001$), kao i dužine prednje kranijalne baze ($p = 0{,}024$) između ispitivanih grupa. **Zaključak**. Deca koja dišu na usta imaju izraženiju retrofleksiju glave u odnosu na vratnu kičmu u poređenju sa decom koja dišu na nos, a najupadljivija karakteristika njihove kraniofacijalne morfologije jeste skeletni odnos vilica II klase i povećan konveksitet lica.

Ključne reči:
disanje na usta; kraniofacijalne anomalije; deca.

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Introduction

The head position is associated with the growth and morphology of the craniofacial complex, but also with certain non-physiological and pathological conditions such as respiratory problems and sleep problems. Breathing is the first vital function that can be approached immediately after birth. It is, above all, the nasal function, but during the life, there are shorter or longer periods when there is breathing through the mouth in some pathological (chronic respiratory infection), or physiological (increased need for oxygen during body activity) conditions. Nasal breathing (NB) during the growth period plays an important role in the overall physical growth and mental development, as well as the growth of the craniofacial complex. Breathing is largely determined by the position of the head, mandible and tongue. Breathing through the mouth requires descending mandible and tongue and throwing the head back. Therefore, it is logical that the change of breathing from nasal to oral may lead to a change of the position of the jaws, tongue and head.

Oral breathing (OB) can affect the form of the jaws, and it has been shown that it leads to the so-called ''adenoid face'', which is characterized by a narrow face, proclination maxillary incisors, lips apart at rest, retroclined mandibular incisors, and increased anterior facial height [1–3].

Children, who breathe through their mouth for a long period of time can change the position of the head relative to the cervical spine, as well as the relationship between the upper and lower jaw [4]. The anterior face height can be increased [5] and an open bite can appear [4, 5] as well as the narrowness of the maxillary arch due to increased pressure of strained face.

The position of the head is connected to the cervical spine, and the position (posture) of the entire body, is under the control of the conditioned and unconditioned reflexes. No conditioned reflexes are formed on the basis of the sense of sight, sense of balance nor a sense of proprioceptive organs and muscles of the body. Conditioned reflexes develop under the influence of environmental factors, and, therefore, posture of each individual is different.

The head posture is assessed on the basis of the craniocervical angle forming the main plane of the anterior cranial base (NS) and tangent odontoid process (OPT) passing through the most inferior and posterior point on the second cervical vertebra corpus [6]. It was found that in children aged 7–13 years, without diseases of muscles and joints, and obstruction of the upper airways, the average value of this angle is 94.6° [7]. Higher values indicate retroflexion or extension, and less anteflexion of the head in relation to the cervical spine.

It is considered that nasal obstruction which causes retroflexion of the head is a consequence of this obstruction compensation [8]. This is confirmed by studies which have noted that there has been a reduction of craniocervical angulation after interventions such as tonsillectomy, adenoidectomy, rapid maxillary expansion (RME) or cortisone therapy for children with asthma and chronic rhinitis [3, 9, 10].

Examining changes in the head position after RME in girls who had to breathe through their mouths, it was found that after RME the capacity of nasopharyngeal tract increased, leading to a significant change in the value of the craniocervical angle that reflected the position of the head relative to the cervical spine [11].

Many studies have shown that there is a connection between head posture, craniofacial morphology and obstruction of the upper airways [12–15]. It was found that narrow and long faces in persons with reduced nasopharynx correspond to the position of head of extensions to the cervical spine, while broad faces in persons with well-developed nasopharynx correspond to the flexion of the head from the spine. Oral breathing, caused by artificial obstruction of the nasal passages, leads to changes in head position so that it comes to extensions [16].

Examining the position of the head in OB children, Antonino et al. [4] concluded that oral breathing leads to an extension of the head in relation to the cervical spine, as well as changes in the craniofacial morphology. They also concluded that the change of breathing from oral to nasal, if occurs during early adolescence, may lead to normalization of craniofacial dimensions during further growth.

Comparing craniofacial morphology, head posture and hyoid bone position with different breathing patterns it has been found that the maxilla is more retrognathic, and palatal plane has a posterior rotation in patients who breath through their mouth. No significant differences are found in the hyoid bone position between the two groups of patients [5].

The aim of this study was to analyze the position of the head and craniofacial morphology in children who breathe through their mouths and to compare the values obtained with those of the same parameters in children who breathe through their nose.

Methods

We analyzed the profile cephalometric radiographs of 60 patients of the Department of Orthodontics, Faculty of Medicine, University of Pristina, with the headquarters in Kosovska Mitrovica.

The sample was divided into two groups of patients. The first group consisted of 30 children aged 8–14 years (16 girls and 14 boys), in which the clinical examination confirmed oral breathing. When a mirror was put in front of the mouth of these children, there was a condensation of exhaled air at the surface of it. Also, when these children were given a sip of water and were instructed to keep it in their mouths and not to swallow it, they could not keep it longer than 10 seconds. In clinical examination with these patients, the existence of "facies adenoidea" is confirmed, and it is characterized by a narrow face, proclination maxillary incisors and lips apart at rest. In the second group there were 30 children of the same age (19 girls and 11 boys), which had different orthodontic problems, but showed no clinical signs of mouth breathing. For each patient lateral cephalogram was made at standard shooting conditions on the appliance brand "Siemens" output of 90 KV and the exposure of 1 s (standard shooting conditions imply that each participant shot in the standing position, with the head oriented so that the Frank-

fort plane is parallel with the floor). Distance from the source of X-rays to the film was 150 cm. Mid-sagittal plane of the patient's head was parallel to cassette with the film. X-ray film cassette was in distance of 15 cm from the mean sagittal plane of the patient's head. Central X-ray falls in the middle of the opening of the outer skin of the ear canal. At the time of recording the teeth were in centric occlusion and lips relaxed and at rest. Each lateral cephalogram had to satisfy that, in addition to other structures, the first two cervical vertebrae are clearly visible. The parents of all patients gave informed consent for their participation in the study.

All the lateral cephalograms were analysed manually. The corresponding craniometric points and planes were labeled 3 linear and 7 angular measurements were hand made (Figures 1 and 2). The following points were labeled: 1) nasion (N) – the most anterior point of the frontonasal suture in the frontonasal suture; 2) sella (S) – the midpoint o the pituitary fossa; 3) basion (Ba) – median point of the anterior margin of the foramen magnum; 4) spina nasalis anterior (Sna) – the tip of the bony anterior nasal spine of the maxilla; 5) spina nasalis posterior (Snp) – the tip of the bony posterior nasal spine; 6 subspinale (A) – the most posterior point on the anterior contour of the upper alveolar process; 7) supramentale (B) – deepest point on the anterior contour of the lower alveolar process; 8) menton (M) – the most inferior point on the symphysis of the mandible; 9) pogonion (Pg) – the most forward point on the anterior surface of the chin; 10) gonion (Go) – the constructed point of the intersection of the ramus plane and the mandibular plane; 11) most inferior and posterior point on the second cervical vertebra corpus – (CV2ip).

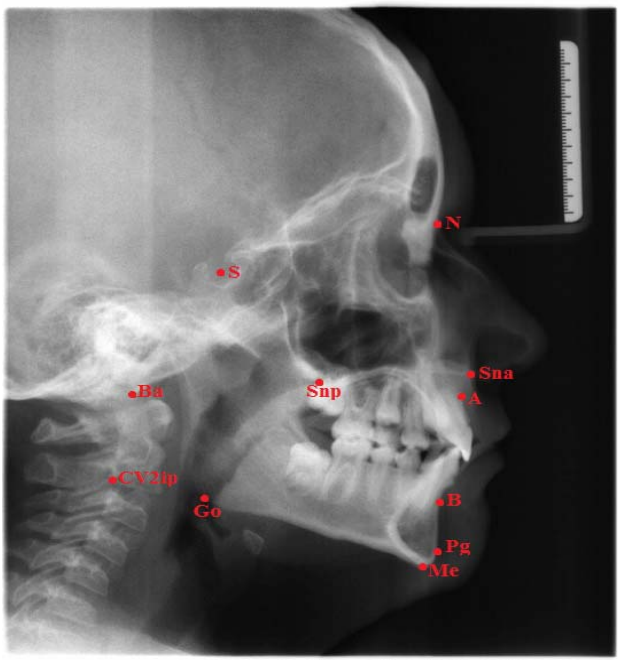

Fig. 1 – The cephalometric and craniocervical points.

The following linear measures were analyzed: 1) anterior cranial base lenght – (N-S); 2) anterior facial height – (N-Me); 3) posterior facial height – (S-Go);

The following angles were analyzed: 1) craniocervical angle – (NS/OPT); head position in relation to the second cervical vertebra, intersection of NS with odontoid process tangent through CV2ip point – (OPT); 2) angle of maxillary prognathism; formed by the connection of the sella, nasion, and A point – (SNA); 3) angle of mandibular prognathism; formed by the connection of the sella, nasion – (SNB) and B point; 4) difference between angles SNA and SNB (ANB); indicating sagittal relation of maxilla and mandible to each other; 5) basal plane angle (SpP/MP); formed by basal plane of the maxilla (SpP) and mandible (MP); indicating vertical relation of maxilla and mandible to each other; 6) angle of basis cranii; formed by the line joining nasion to sella to basion – (NSBa); 7) facial convexity angle; formed by NA and APg line – (NA/Apg).

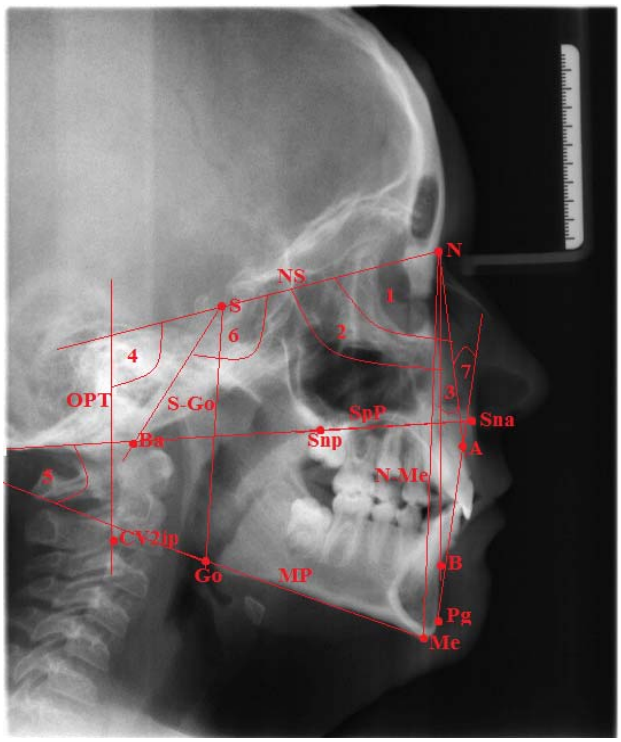

Fig. 2 – The cephalometric angular (1-SNA, 2-SNB, 3-ANB, 4-NS/OPT, 5-SpP/MP, 6-NSBa, 7-NA/APg) and linear (N-Me, S-Go, NS) parameters.

SNA° – angle of maxillary prognathism; SNB – angle of mandibulary prognathism; ANB – difference between angles SNA and SNB; NS/OPT – craniocervicale angle; NS – anterior cranial base lenght; SpP/MP – basal plane angle; NSBa – angle of basis cranii; Na-Apg – facial convexity angle; N–Me – anterior facial height; S-Go – posterior facial height.

The values of angular measure are expressed in degrees, and linear in millimeters.

Craniocervical angulation was evaluated on the basis of the value of the craniocervical angle NS/OPT. Other variables examined were indicators of craniofacial morphology.

The analysis of primary data was conducted by using descriptive statistical methods and methods for testing statistical hypotheses. Descriptive statistical methods included measure of central tendency (mean and median) and measures of variability (standard deviation, and range). Testing statistical hypotheses was performed by using *t*-test and Mann-Whitney test. Data analysis was done by using Software pac-

kage SPSS 21 Differences between groups were considered statistically significant at a level of $p < 0.05$.

Results

The results of the study are shown in Table 1. It can be seen that there were differences in the values of the examined parameters between the two study groups. The average value of the craniocervical angle (NS/OPT) in children who breathed through their mouth was 101.5° which was significantly higher than the average value of this angle in children who breathed through their nose ($p = 0.004$). Significantly different values were also found in maxillary prognathism angle (SNA) ($p < 0.001$) and ANB ($p < 0.001$). Anterior (N-Me) and posterior (S-Go) face height, and basal plane angle of the jaws (SpP/MP) did not differ significantly regardless of breathing through the nose or mouth. The length of the anterior cranial base (NS) was significantly higher in patients who breathed through their mouth ($p = 0.024$), while the values of the angle of the cranial base (NSB) did not differ significantly between the two groups of patients. The facial convexity angle (NA/APg) was significantly higher in patients who breathed through their mouth ($p < 0.001$).

Disscusion

The mouth breathing in a large number of children is a bad habit, which may occur due to the disturbed anatomical relationships and bad features of circumoral muscles. It is usually a consequence of frequent respiratory infections. On the other hand, the mouth breathing causes a change in the balance of pressure on the jaw and teeth, and the reasons for its occurence are orthodontic anomalies such as the narrowness of the maxillary arch, open bite and distal position of the mandible because of an extreme rotation down and back.

In this study, it was assumed that there was a difference in craniocervical angulation and craniofacial morphology between OB and NB pearsons, as other authors have found in their studies [1–4, 11, 17].

The research confirmed that in the OB children there is a difference in the position of the head to the cervical spine in relation to the NB children [4]. The difference is reflected in significantly higher average value of the craniocervical angle NS/OPT ($p = 0.004$) in the OB children. Increasing of this angle points to the increased extension of the head in relation to the cervical spine, which is probably a compensation for nasal obstruction [8].

The value of the maxillary prognathism angle (SNA) is significantly higher in children who breathe through their mouths, with its average value of 81.6°, implying mild retrognathism of upper jaw [5], which could be explained by pressing the soft tissue due to the extension of the head and limiting growth in advance [18].

The values of the angle of mandibular prognathism did not differ significantly between the two study groups of children, which is not in accordance with the findings of some previous studies that speak in favor of a reduced SNB angle and length of the mandible in people who breathe through their mouths [19, 20].

The difference in angles of the maxillary and mandibular prognathism, the angle of ANB, was also significantly higher in the OB children. This finding suggests that in the OB children, mainly, there is a relationship of the skeletal jaw of class II, which is consistent with findings from the previous studies [4, 20].

The results of our study indicate that the angle of the basal planes of the upper and lower jaw (SpP/MP), is not significantly different regardless whether a person breathes

Table 1

Variables of craniofacial morphology and craniocervical angulation in patients with oral breathing (OB) and nasal breathing (NB)

Variables	NB group (n = 30)	OB group (n = 30)	*p*
SNA°, $\bar{x} \pm SD$	79 ± 3	81.6 ± 2	< 0.001
SNB°, $\bar{x} \pm SD$	75.9 ± 2.8	75.9 ± 1.7	0.856
ANB°, median (range)	3 (2–4)	5.6 (3–9.2)	< 0.001
NS/OPT °, $\bar{x} \pm SD$	93.6 ± 10.3	101.5 ± 10.1	0.004
N-Me (mm), $\bar{x} \pm SD$	103.9 ± 6.7	103 ± 7.5	0.619
S-Go (mm), $\bar{x} \pm SD$	66 ± 4.9	65 ± 6	0.505
SpP/Mp °, $\bar{x} \pm SD$	28 ± 4.5	26.8 ± 3.9	0.244
N-S (mm), $\bar{x} \pm SD$	65.9 ± 5.2	63.3 ± 3.4	0.024
NSBa°, $\bar{x} \pm SD$	131.6 ± 4.5	132.4 ± 4.6	0.491
NA/APg°, median (range)	5.2 (1.2–11)	10.6 (5.3–15.5)	< 0.001

SNA° – angle of maxillary prognathism; SNB° – angle of mandibulary prognathism; ANB° – difference between angles SNA and SNB; NS/OPT ° – craniocervicale angle; NS – anterior cranial base lenght; OPT – odontoid process tangent through CV2 ip point; CV2ip – most inferior and posterior point on the second cervical vertebra corpus; N–Me – anterior facial height; S-Go – posterior facial height; SpP/Mp – basal plane angle; NSBa – angle of basis cranii; NA/Apg ° – facial convexity angle;
x – arithmetic mean;
SD – standard deviation.

through the nose or the mouth. In accordance with these results are values of anterior (N-Me) and the posterior (S - Go) face height in the OB children, which do not differ significantly in relation to the values in the NB children. Munoz and Orta [21] have found increased anterior facial height in the OB children. The values of these three parameters (SpP/MP, N-Me and S-Go) suggest that oral breathing does not lead to significant changes in the vertical dimension of the face. In contrast to this, Antonino et al. [4] in their analysis of the position of the head in the OB, have found significantly larger angle of the basal planes of jaw in OB children. Based on the increased value of this parameter, as well as the ANB, the same author suggests that people who breathe through the mouth are predominantly dolichofacial, with skeletal jaw relation of the class II.

The length of the anterior cranial base (N-S) was significantly higher in the OB children. This finding differs from the results of Ang et al. [22], and Shrivastava and Thomas [23] who found no significant difference in the length of the anterior cranial base, regardless someone breathes through the nose or mouth.

The value of cranial base angle (NSB) was not significantly different between the two study groups in our study. Similar results have been reported by Ang et al. [22] and Antonino et al. [4]. In contrast, Solow and Tallgen [24] and Solow and Greve [25] have found that the increased craniocervical angle is followed by the bigger angle of the cranial base.

The facial convexity (NA/APg) was significantly higher in OB children, which can be linked to the fact that in these children, skeletal jaw relationship of class II, characterized by a convex profile was dominant.

Different results in the literature have been discussed by Viveros [26] who believes that although there were many researches that have attempted to resolve the influence of breathing patterns on the facial growth, the direct relation between obstruction of the respiratory tract and facial malformations is not established. The author suggests, that in order to explain these dependences, the genetic and environmental influences should be considered. The author, also suggests that well-controlled studies on large population should be carried out in order to clarify the connection between facial growth and breathing through the nose or mouth.

Conclusion

The long-term oral breathing, especially if it appears in the period of growth, can lead to changes in head position and disorders in the growth of the craniofacial complex. Children who breathe through their mouths have pronounced retroflexion of the head in relation to the cervical spine. The most striking characteristic of craniofacial morphology of children who breathe through their mouth is skeletal jaw relationship of class II and increased facial convexity. The vertical craniofacial morphology parameters were not significantly changed.

It is necessary to reveal and remove the cause of oral breathing in its early stage, and thus create conditions for modifying breathing from oral to nasal that can prevent adverse effects on the growth of craniofacial structures.

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Psammoma bodies as signs of choroid plexus ageing – a morphometric analysis

Psamomatozna telašca kao pokazatelji starenja horoidnog pleksusa – morfometrijska analiza

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Abstract

Background/Aim. Psammoma bodies (PB) are regarded as benign consequences of ageing in choroid plexus stroma. The aim of this study was to assess the morphometric characteristics of psammoma bodies of all four choroid plexuses during the ageing process. Our intention was to find the possible relations between psammoma bodies and choroid plexus and blood vessels parameters. **Methods**. This study was conducted on the material taken from 15 cadavers during routine autopsies. Tissue samples were collected from both lateral, third and forth ventricles' choroid plexus. Slices were stained with Mallory trichrome stains. In each image, we analyzed morphometrically the epithelium, blood vessels present and all the psammoma bodies. **Results**. With age, right choroid plexus surface density decreases ($p < 0.05$), while the psammoma bodies volume density increases ($p < 0.05$). A decrease in the blood vessels volume density was observed in the third ventricle's choroid plexus ($p < 0.05$), as well as an age-related decrease in the psammoma bodies perimeter ($p < 0.01$). Not associated with ageing, the increase in psammoma bodies perimeter and volume density predicts a decrease in choroid plexus surface density ($p < 0.05$ and $p < 0.001$, respectively). There was a decrease in the volume density of blood vessels with age and with the increase in Feret's diameter of psammoma bodies, ($p < 0.001$ and $p < 0.05$, respectively). **Conclusion**. We want to point out that there is an association between ageing and increased size and volume density of psammoma bodies. More important is the fact that psammoma bodies' presence and their morphometric characteristics are good predictors of changes occurring on the level of choroid plexus structure and vascularization, which may have crucial effects on the choroid plexus physiology.

Key words:
choroid plexus; cell aging; image cytometry; blood vessels.

Apstrakt

Uvod/Cilj. Psamomatozna telašca smatraju se benignom posledicom starenja u stromi horoidnog pleksusa. Cilj rada bio je da se utvrde morfometrijske karakteristike psamomatoznih telašaca u sva četiri horoidna pleksusa, tokom procesa starenja. Osim toga, namera je bila da se pronađe povezanost između parametara psamomatoznih telašaca i parametara horoidnog pleksusa i krvnih sudova. **Metode**. Ova studija urađena je na materijalu uzetom sa 15 kadavera tokom rutinske autopsije. Tkivni uzorci uzimani su sa horoidnih pleksusa iz obe lateralne, treće i četvrte moždane komore. Dobijeni preseci bojeni su Mallory *trichrome* metodom. Na dobijenim slikama, morfometrijski je analiziran epitel, krvni sudovi i sva prisutna psamomatozna telašca. **Rezultati**. Sa starenjem, smanjuje se površinska gustina horoidnog pleksusa ($p < 0.05$), ali raste zapreminska gustina psamomatoznih telašaca ($p < 0.05$). Smanjenje zapreminske gustine krvnih sudova uočeno je u horoidnom pleksusu treće moždane komore ($p < 0.05$), kao i smanjenje perimetra psamomatoznih telašaca ($p < 0.01$). Povećanje perimetra i zapreminske gustine psamomatoznih telašaca daje mogućnost predviđanja pada površinske gustine horoidnog pleksusa ($p < 0.05$ i $p < 0.001$), bez obzira na starost. Sa starenjem i porastom Feretovog dijametra psamomatoznih telašaca, smanjuje se zapreminska gustina krvnih sudova ($p < 0.001$ i $p < 0.05$). **Zaključak**. Možemo istaći da postoji međusobna povezanost između starenja i povećane veličine i zapreminske gustine psamomatoznih telašaca. Što je još važnije, prisustvo psamomatoznih telašaca i njihove morfometrijske karakteristike su dobri prediktori promena na horoidnom pleksusu i u njegovoj vaskularizaciji, što može imate vrlo značajne efekte na fiziologiju horoidnog pleksusa.

Ključne reči:
pleksus, horoidni; ćelija, starenje; morfometrija; krvni sudovi.

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Introduction

Choroid plexus (CP) is a leaf-like structure, highly vascularized with fenestrated capillaries and venules, located within both lateral (LV), the third (V3) and the fourth cerebral ventricle (V4) [1]. The role of CP is complex, beyond simple secretion of cerebrospinal fluid. Its epithelium shows enzymatic activity, transports molecules in both ways acting as a selective blood-brain barrier, and takes a role in immunoreactivity [2]. Besides flattening of the CP epithelium and shortened choroidal villi [3–5], ageing of the CP is characterized with calcification, cyst formation, psammoma bodies (PBs) formation and iron deposition [6]. Cerebral microvasculature changes with age as well, showing an increase in the cross-sectional area of the capillary wall, number and length per unit volume of capillaries, due to endothelium cells elongation, perivascular gliofibrillar proliferation and basement membrane thickness, while the number of endothelial cells decreases [4, 7, 8]. As the result, the main physiologic changes in the aged are decreased cerebrospinal fluid secretion and more than two times diminished clearance of various molecules, endogenous and exogenous, from the central nervous system [5, 9].

Psammoma bodies are regarded as benign consequences of ageing in choroid plexus stroma. On the other hand, they are seen in numerous tumorous formations of other tissues, mostly with papillary structure [10–15], but never in the view of senescence. In malignant tumors PBs are associated with better prognosis (vascular thrombosis, calcification and tumor necrosis) [16], while in the CP, their presence is correlated with epithelium atrophy and, therefore, reduced physiological functioning [17]. Nevertheless, recent studies suggest that in case of thyroid papillary carcinoma, the presence of either intratumorous, or extratumorous PBs, is associated with aggressiveness [12, 18]. There are no definite theories of the PBs formation mechanism, neither in the CP, nor in the other tissues. Even though there is such an extreme difference between the PBs in CP and other tissues, in relation to normal ageing process versus malignant pathologic conditions, some similarities exist: fenestrated capillaries, a characteristic of both the CP and some malignancies associated with the PBs, as a consequence of augmented angiogenesis, ease the entrance of noxious substances, as well as leukocytes [19] or even nanobacteria [20, 21]. A proposed mechanism is that the presence of these agents, or inflammation itself, may induce stromal reaction and the PBs formation [22].

The aim of this study was to assess the morphology and the number of the PBs in the CP during the ageing process. Furthermore, we tested the role of age, sex and the PBs morphology and number in predicting the changes in morphology and structure of the CP of all four cerebral ventricles.

Methods

This study was conducted on the material taken from 15 cadavers, 8 males and 7 females, during routine autopsies performed at the Department of Forensic Medicine, Faculty of Medicine, Niš, Serbia. It was approved by the Ethics Committee of the University of Niš, Faculty of Medicine. Neither of the cadavers included had been previously diagnosed a nervous system disease, nor any abnormalities or brain damage had been observed at the autopsy. Tissue samples were collected from both lateral, third and forth ventricles' CP. Cadavers' age ranged from 35 to 84 years (average 61.8 ± 15.0 years). Tissue obtained was fixated in 10% buffered formalin, and embedded into paraffin blocks. Afterwards, slices, 5 μm thick, were stained with Mallory trichrome stains.

In each case, 10 fields of vision, randomly selected, were analyzed under a light microscope (Leica DM2500) and photographed under the 10x times lens magnification with digital camera (Leica DFC420, resolution 2592 × 1944 pixels) mounted on the third microscope ocular. Morphometric analysis was performed using Image J image processing and analysis software (version 1.49, National Institutes of Health, USA). After spatial calibration using an objective micrometar, in each image, we have analyzed the epithelium and the blood vessels present in order to quantify the surface density of choroid plexus (SDCP), as well as the volume density of the blood vessels (VDBV). Beside counting all the PBs, each was measured giving us the following measures: average total area per case (APB), average perimeter (PPB), average Feret's diameter (FDPB), numeric (NDPB) and volume density of PBs (VDPB). A multipurpose test system M168 (d = 82.0μm, Lt = 6888.1μm, At = 978365.9μm^2) was used for the stereologycal analysis, and the calculation of SDCP, VDBV, NDPB and VDPB [23].

The data analysis was performed by Statistical Package for Social Sciences (SPSS 16.0; Chicago, IL, USA). The normality of the data distribution was tested using a contingent on distributional characteristics (i.e. skewness, kurtoasis, presence of extreme values, Shapiro-Wilk test). Baseline characteristics are presented as means with standard deviation (SDs). Data deviating heavily from normal distribution was displayed as median and interquartile range. A non-parametric methods, Mood's median test for independent samples was used to determine the differences in observed measures between age groups. Univariate linear regression was performed in order to determine the relevance of age, sex and morphometric parameters of PBsin predicting the changes in choroid plexus. A *p*-value less than 0.05 was considered to be a measure of statistical significance.

Results

Histological analysis

Tissue slices obtained represented choroidal villi, as well as the fibrovascular core without or with small sections of the epithelium visible. As it can be seen in Figure 1, numerous amorphous calcifications could be observed in cross and longitudinally sectioned choroidal villi. Psammoma bodies analyzed were present in both forms: immature PBs with amorphous core and mature lamellar PBs. As a result of the ageing process, especially in the old cases, there were cystic formations containing the PBs. Psammoma bodies were not observed in five cases: four samples of the forth

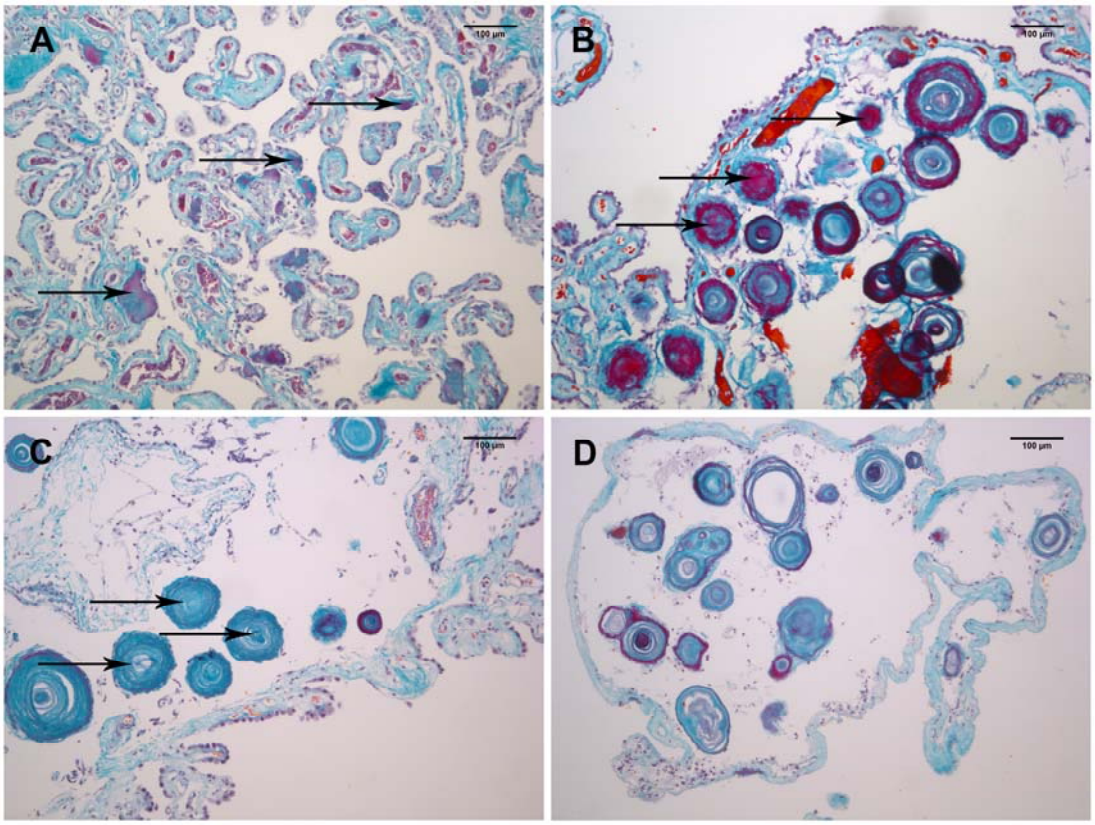

Fig. 1 – Psammoma bodies in choroid plexus (Mallory trichrome stain, × 10): A) amorphous calcifications; B) immature psammoma bodies; C) mature laminated psammoma bodies; D) cyst with psammoma bodies.

ventricle's choroid plexus (aged 35, 44, 59 and 69) and one sample of the third ventricle's choroid plexus (aged 44). These samples were excluded from the morphometric study of PBs.

Morphometric analysis

In order to analyze the ageing characteristics of choroid plexus, cases were divided into three age groups: Group I (35–50 years), Group II (51–70 years) and Group III (71–84 years). Due to the significant deviation from normal distribution, morphometric data are shown as median and interquartile range.

Left and right lateral cerebral ventricles' choroid plexus morphometric analysis

The results of the lateral ventricles' choroid plexus morphometric study are presented in Table 1. PBs diameter ranged from 145 μm to 357 μm. As it can be seen, there were some significant differences between left and right-side choroid plexus, but only in the young and middle-aged. Right lateral ventricle's choroid plexus was characterized with larger numeric density of in the first age group ($p < 0.001$), while in the second one the PBs became larger (area, perimeter and Feret's diameter) ($p < 0.001$) compared to the left side. In the oldest age group, such differences were not observed. With age, right choroid plexus surface density decreased ($p < 0.05$), while the PB volume density increased ($p < 0.05$).

Third and fourth cerebral ventricles' choroid plexus morphometric analysis

The results of the third and fourth ventricles' choroid plexus analysis are presented in Tables 2 and 3, respectively. The size range of the PBs in V3 was similar to the LV (149–340 μm), while in the V4 the PBs as small as 99 μm in diameter were found. A decrease in the blood vessels volume density was observed in the third ventricle's choroid plexus ($p < 0.05$), as well as an age-related decrease in PB perimeter ($p < 0.01$). There were no statistically significant changes during ageing in the choroid plexus of the forth ventricle during age.

The association of age, sex and psammoma bodies morphometric parameters with changes in the choroid plexus

There was no difference in the distribution of sexes among age groups ($\chi^2 = 5.640$, $p = 0.060$). We performed univariate linear regression in order to determine the relevance of age, sexand PBs morphometric parameters (APB, PPB, FDPB, NVPB and VDPB), as independent variables, in predicting the changes in the choroid plexus. For each of the independent variables (SDCP, VDBV), a separate model was created (Table 4).

A total of 50.9% of SDCP variance could be explained with these independent factors ($F = 13.213$, $p < 0.001$). The following variables were shown to independently predict SDCP: sex and three of the PBs dimensions (APB, PPB,

Table 1

Morphometric analysis of lateral ventricles' choroid plexus and psammoma bodies (PBs) across three age groups

Morphometric parameters	Left lateral ventricle's choroid plexus [Right lateral ventricle's choroid plexus]			
	Group I (35–50 years)	Group II (51–70 years)	Group III (71–84 years)	χ^2 (*p*)
SDCP (1/mm)	12.40 (8.08–14.92) [11.17 (8.85–16.29)]	14.08 (8.83–15.46) [12.89 (10.99–18.48)]	9.38 (6.46–11.86) [8.48 (7.36–9.99)]	2.143 (0.343) 6.964 (0.031)
Left vs. Right (*p*)	0.897	0.738	0.897	
VDBV (mm^3)	13.29 (9.32–16.25) [13.43 (9.92–19.90)]	12.92 (8.31–15.47) [11.32 (8.88–16.68)]	9.40 (7.19–10.40) [8.56 (7.65–12.05)]	2.143 (0.343) 3.750 (0.153)
Left vs. Right (*p*)	0.738	0.897	0.738	
APB (mm^2)	47.71 (29.37–60.60) [40.84 (29.70–50.14)]	41.16 (27.13–69.60) [58.71 (38.25–64.45)]	39.50 (37.96–49.22) [51.37 (44.55–61.79)]	0.536 (0.765) 3.750 (0.153)
Left vs. Right (*p*)	0.897	0.000	0.500	
PPB (mm)	0.75 (0.58–0.84) [0.54 (0.60–0.77)]	0.70 (0.54–0.89) [0.82 (0.67–0.86)]	0.71 (0.67–0.77) [0.77 (0.72–0.87)]	0.536 (0.765) 3.750 (0.153)
Left vs. Right (*p*)	0.897	0.000	0.738	
FDPB (mm)	0.25 (0.20–0.29) [0.23 (0.20–0.26)]	0.24 (0.19–0.30) [0.28 (0.23–0.29)]	0.24 (0.23–0.26) [0.26 (0.25–0.30)]	0.536 (0.765) 3.750 (0.153)
Left vs. Right (*p*)	0.897	0.000	0.738	
NDPB ($1/mm^3$)	13.43 (9.95–20.10) [14.32 (10.03–32.77)]	27.15 (9.42–36.46) [9.23 (7.61–18.41)]	19.95 (15.98–25.80) [19.07 (14.44–26.39)]	2.143 (0.343) 3.750 (0.153)
Left vs. Right (*p*)	0.000	0.738	0.500	
VDPB (mm^3)	4.10 (2.21–5.05) [4.10 (2.81–6.14)]	6.26 (2.79–8.53) [3.82 (1.42–6.48)]	6.62 (4.30–7.90) [6.45 (6.27–7.46)]	2.143 (0.343) 8.571 (0.014)
Left vs. Right (*p*)	0.738	0.500	1.000	

Results are given as median (interquartile range)

SDCP – surface density of choroid plexus; VDBV – volume density of blood vessels; APB – average area of PBs; PPB – average perimeter of PBs; FDPB – average Feret's diameter of PBs; NDPB – numeric density of PBs; VDPB – volume density of PBs.

Table 2

Morphometric analysis of the third ventricle's choroid plexus and psammoma bodies (PBs) across three age groups

Morphometric parameters	Group I (35–50 years)	Group II (51–70 years)	Group III (71–84 years)	χ^2 (p)
SDCP (1/mm)	16.81 (10.83–19.63)	19.25 (14.92–22.37)	13.53 (10.31–17.33)	0.400 (0.819)
VDBV (mm^3)	11.23 (10.63–14.69)	8.64 (7.79–11.84)	8.45 (8.36–11.09)	6.000 (0.050)
APB (mm^2)	35.16 (26.31–49.37)	34.32 (16.37–61.05)	24.12 (21.57–27.11)	3.000 (0.223)
PPB (mm)	0.64 (0.58–0.73)	0.61 (0.44–0.83)	0.54 (0.50–0.55)	9.200 (0.010)
FDPB (mm)	0.22 (0.20–0.25)	0.22 (0.15–0.29)	0.19 (0.18–0.20)	3.000 (0.223)
NDPB ($1/mm^3$)	9.87 (9.21–10.41)	6.58 (0.92–12.58)	20.60 (7.56–28.53)	3.600 (0.165)
VDPB (mm^3)	1.97 (1.55–2.47)	0.52 (0.19–1.67)	2.86 (0.82–3.69)	3.000 (0.223)

Results are given as median (interquartile range)

SDCP – surface density of choroid plexus; VDBV – volume density of blood vessels; APB – average area of PBs; PPB – average perimeter of PBs; FDPB – average Feret's diameter of PBs; NDPB – numeric density of PBs; VDPB – volume density of PBs.

Table 3

Morphometric analysis of the fourth ventricle's choroid plexus and psammoma bodies (PBs) across three age groups

Morphometric parameters	Group I (35–50 years)	Group II (51–70 years)	Group III (71–84 years)	χ^2 (p)
SDCP (1/mm)	19.45 (15.91–19.45)	19.37 (14.98–19.37)	17.82 (12.76–19.60)	2.396 (0.302)
VDVB	10.21 (8.64–10.21)	8.68 (7.75–8.68)	7.21 (6.30–12.12)	0.782 (0.676)
APB (mm^2)	19.40 (10.68–19.40)	28.54 (11.39–28.54)	42.10 (8.18–49.58)	0.782 (0.676)
PPB (mm)	0.43 (0.38–0.43)	0.60 (0.38–0.60)	0.71 (0.32–0.79)	0.782 (0.676)
FDPB (mm)	0.16 (0.14–0.16)	0.21 (0.14–0.21)	0.24 (0.11–0.29)	0.782 (0.676)
NDPB ($1/mm^3$)	8.15 (0.81–8.15)	3.48 (2.30–348)	2.43 (0.58–5.72)	2.396 (0.302)
VDPB	2.10 (0.07–2.10)	0.74 (0.12–0.74)	0.18 (0.00–0.29)	2.396 (0.302)

Results are given as median (interquartile range)

SDCP – surface density of choroid plexus; VDBV – volume density of blood vessels; APB – average area of PBs; PPB – average perimeter of PBs; FDPB – average Feret's diameter of PBs; NDPB – numeric density of PBs; VDPB – volume density of PBs.

Table 4

Univariate linear regression of choroid plexus parameters in function of age, sex, location and psammoma bodies (PBs) characteristics

Parameters		Unstandardized coefficients		Standardized coefficients			Model		
		B	SE	Beta	*t*	*p*	F	*p*	Adjusted r^2
SDCP (1/mm)	Constant	18.853	2.230		8.454	0.000	13.213	0.000	0.509
	Sex (male)	2.720	1.024	0.283	2.656	0.010			
	Age	-0.044	0.034	-0.138	-1.298	0.200			
	APB	0.143	0.066	0.632	2.175	0.034			
	PPB	-12.256	5.754	-0.627	-2.130	0.038			
	VDPB	-1.110	0.189	-0.628	-5.858	0.000			
VDVB	Constant	16.371	1.902		8.607	0.000	6.758	0.001	0.226
	Age	-0.097	0.028	-0.043	-3.393	0.001			
	PPB	47.587	21.584	3.276	2.205	0.032			
	FDPB	-134.779	63.448	-3.166	-2.124	0.038			

SDCP – surface density of choroid plexus; VDBV – volume density of blood vessels; APB – average area of PBs; PPB – average perimeter of PBs; FDPB – average Feret's diameter of PBs; VDPB – volume density of PBs.

VDPB). We found SDCP to be lower in males ($p < 0.01$). Surprisingly, the increase in APB predictedan increase in SDCP ($p < 0.05$), opposite to the PPB and VDPB which were inversely correlated to SDCP ($p < 0.05$ and $p < 0.001$, respectively).

In the case of the VDBV as the dependent variable, 22.6% of its variance was explained by the model (F = 6.758, $p < 0.001$) including age and the PBs' perimeter and Feret's diameter. All of these variables were shown to be statistically significant as independent predictors. Higher PPB was associated with higher VDBV ($p < 0.05$). One the other hand, with age and the increase in Feret's diameter of the PBs, there was a decrease in blood vessels volume density ($p < 0.001$ and $p < 0.05$, respectively).

Discussion

Psammoma bodies have never been observed in prenatal tissue samples, nevertheless, by the end of the 1st year, small ones start to concentrate around the vascular stock. Logically, in older specimens, larger ones are located centrally, often in cystical formations. On the other hand, peripherally, in choroidal villi beneath the CP epithelium, the PBs are significantly smaller [24]. The incidence of PBs findings on computed tomography (CT) scans rises from 0.5% to 86% from the 1st to the 8th decade [25]. Psammoma bodies consist of collagen whorls, 50–150 μm in diameter, but often even larger than 300 μm. Lamellar structure is the result of irregularcalcium deposition and reflects the time needed for a PB to be formed. Therefore, the mineral component is mostly calcium (phosphate and hydroxyapatite), but iron, magnesium, zinc and manganese are also present [26, 27]. Studies on the organic ingredients prove the incorporation of light-chain amyloid [17], associated with Alzheimer's disease. The maturity of the PBs may be evaluated by the presence/absence of the amorphous core. It is suggested that the immature PBs arise from the amorphous calcifications. Further deposition of connective fibers leads to the lamification and maturation of the PBs. Non-related to the age, larger and more mature PBs are associated with more intense changes on the CP epithelium.The presence of large PBs is realted to more prominent changes in the CP epithelium: extreme flattening, presence of cysts, larger vacuoles often deforming the cell and dislocating the nucleus [22, 28].

The presence of numerous PBs and the PBs with higher average area is not age-related [28]. In our study, we have found that the PBs volume density increases with age in elderly humans (beyond 70 years) in the right LV. Besides, there was a progressive decrease in the PBs perimeter during ageing in the V3. In accordance with the morphometric findings on the PBs, there was a decrease in surface density of the CP in the right LV which may be explained by the destruction of choroidal villi following the increased PBs formation. A decrease in blood vessels volume density in the V3 was noted as well, due to the thickening of the vessels walls [8] and, therefore, diminishing their caliber. Interestingly, there were significant differences between left and right lateral ventricles' CP, in terms of the PBs size and numeric density, which was also reported in the literature [25]. If we take into account the hypothesis of the circulating agents inducting connective tissue activation [22], differences in the blood flow may result in the various speed and intensity of the PBs formation. These differences were noted in the first two age groups, suggesting that it is relevant only in the view of age at which new PBs start to form and mature. As the whole process progresses, these differences disappear.

Our findings suggest that the CP degeneration due to the shortening of the choroidal villi [4] is not age-related, but is well correlated with the PBs formation. Other factors, such as sex, contribute to the prediction of the CP size. We have found surface density of the CP to be smaller in male specimens. The impact of sex hormones on the CP and cerebrospinal fluid is well documented, describing the sex differences in circadian rhythm signalling, the CP barrier function, its metabolism and stem cell differentiation [29]. Volume density and perimeter of the PBs are found to be strongly correlated

with a decrease in the SDCP. Bigger PBs (their perimeter) and more densely packed, seem to be a good predictor of the CP impairment. On the other hand, the decrease in the density of blood vessels seems to be a direct consequence of ageing. Age-related changes in the CP (probably the thickening of the basal membrane and the surrounding increased stromal reaction [8, 22]), in combination with the PBs of the large FDPB, may cause the diminishing of blood supply into the CP.

Conclusion

As a summary, we want to point out there is an association between ageing and increased size and volume density of psammoma bodies. More important is the fact that the PBs presence and their morphometric characteristics are good predictors of changes occurring on the level of choroid plexus structure and vascularization, which may have crucial effects on the CP physiology.

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The changes of oxidative stress and endothelial function biomarkers after 6 weeks of aerobic physical training in patients with stable ischemic coronary disease

Promena biomarkera oksidativnog stresa i funkcije endotela posle šestonedeljnog aerobnog fizičkog treninga kod bolesnika sa stabilnom ishemijskom bolesti srca

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Abstract

Background/Aim. Exercise-based cardiac rehabilitation improves endothelial function, reduces cardiac mortality and anginal symptoms in patients with established cardiovascular disease. We evaluated the changes of oxidative stress and endothelial function biomarkers after 6 weeks of aerobic physical training in patients with stable ischemic coronary disease (IHD) participating in a cardiovascular rehabilitation exercise program. **Methods**. Thirty-five patients with stable IHD engaged in cardiovascular rehabilitation program with the regular aerobic physical activity during 6 weeks were consecutively included together with 37 control, age and sex-matched, IHD patients with a sedentary lifestyle. Clinical data about anthropometric and cardiovascular parameters and laboratory data: serum cell adhesion molecules intracellular (sICAM-1) and vascular (sVCAM-1), reactive carbonyl derivatives (RCD), lipid peroxidation products malondialdehyde (MDA) and nitric oxide (NO) concentration were determined at the beginning and after 6 weeks of aerobic training (45 minutes of continuous exercise up to 80% of maximal heart rate, 3 times a week). **Results**. The baseline characteristics of examined groups were similar according to age, gender, and cardiovascular risk profiles. The regular aerobic physical activity induced significant reduction of body mass index, blood pressure, heart rate, triglycerides, RCD (1.27 ± 0.48 μmol/g proteins vs. 1.04 ± 0.22 μmol/g proteins), sVCAM-1 [100.4, interquartile range (IQR)(78.4–118.3) ng/mL vs. 80.0 IQR(68.5–97.2 ng/mL)] and increasing of NO (64.72 ± 16.06 nmol/mg proteins vs. 74.38 ± 18.57 nmol/mg proteins) and HDL cholesterol ($p < 0.05$), which was not seen in sedentary control RCD (1.16 ± 0.25 interquartile range vs. 1.12 ± 0.14 interquartile range), sVCAM-1 [92.2 IQR (73.6–106.8 ng/mL) vs. 91.3 IQR (73.0–105.3 ng/mL) and NO (68.5 ± 17.9 nmol/mg vs. 65.7 ± 19.6). The values of sICAM-1 were lower in exercise training group baseline without significant changes during observation [80.74 IQR (54.92–97.3) vs. 80.36 IQR (68.1–95.3)] compared to the control [86.35 IQR(57.32–95.8) vs. 84.65 IQR(55.67–93.8. In the exercise training group, values of sVCAM-1 and RCD were significantly lower and NO higher at the end of the study compared to those in the sedentary control. **Conclusion**. Regular physical activity induced reduction of oxidatively modified proteins and vascular cells adhesion molecules alongside with increased NO bioavailability and favorable changes in HDL cholesterol and triglycerides.

Key words:
myocardial ischemia; oxidative stress; endothelium, vascular; exercise; treatment outcome.

Apstrakt

Uvod/Cilj. Redovna fizička aktivnost u sklopu kardiovaskularne rehabiltacije popravlja endotelnu funkciju, redukuje kardiovaskularni mortalitet i anginozne tegobe bolesnika sa kardiovaskularnim oboljenjem. U radu su analizirane promene parametara oksidativnog stresa i endotelne funkcije nakon sprovedenog redovnog aerobnog fizičkog treninga tokom šest nedelja kod bolesnika sa stabilnom formom koronarne bolesti (KB) uključenih u program kardiovaskularne rehabilitacije. **Metode**. Analizirano je 35 uzastopnih bolesnika sa stabilnom formom KB, uključenih u program kardiovaskularne rehabilitacije sa redovnom aerobnom fizičkom aktivnošću tokom šest nedelja, zajedno sa 37 kontrolnih bolesnika sa KB, uparenih prema polu i starosti, sa sedentarnim načinom života. Anamnestički podaci o komorbiditetima, faktorima rizika od kardiovaskularnih bolesti, primenjenoj terapiji, klinički podaci o antropometrijskim i kardiovaskularnim parametrima kao i laboratorijske

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analize: adhezioni molekuli, derivati oksidativne modifikacije proteina (RCD), derivati lipidne peroksidacije malondialdehid (MDA) i azot monoksid (NO) određivani su na početku i nakon šest nedelja aerobnog treninga. Trening se sastojao od 45 minuta kontinuirane fizičke aktivnosti (do 80% maksimalne srčane frekvencije), tri puta nedeljno. **Rezultati**. Karakteristike grupa na početku studije bile su slične u pogledu polne i starosne strukture, kao i prisustva faktora rizika od kardiovaskularnih bolesti. Redovna aerobna fizička aktivnost dovodila je do značajne redukcije indeksa telesne mase, krvnog pritiska, srčane frekvencije, koncentracije triglicerida, RCD (1,27 ± 0,48 μmol/g proteins naspram 1,04 ± 0,22 μmol/g proteins), sVCAM-1 [100.4 (IQR) (78,4–118,3) ng/mL naspram 80,0 IQR (68,5–97,2 μmol/g proteins)] i povećanja NO (64,72 ± 16,06 μmol/g proteins prema 74,38 ± 18,57) i HDL holesterola ($p < 0,05$), što nije registrovano u sedentarnoj kontroli: RCD (1,16 ± 0,25 naspram 1,12 ± 0,14), sVCAM-1 [92,2 IQR (73,6–106,8) naspram 91,3 IQR(73,0–105,3)] i NO (68,5 ± 17,9 naspram 65,7 ± 19,6). Serumske vrednosti intracelularnog adhezionog molekula sICAM-1 su startno bile niže u grupi sa primenjenim treningom bez značajne promene tokom praćenja [80,74 IQR (54,92–97,3) *vs* 80,36 IQR (68,1–95,3)] u odnosu na kontrolnu grupu [86,35 IQR (57,32–95,8)] *vs* 84,65 IQR (55,67–93,8). U grupi sa fizičkim treningom vrednosti sVCAM-1 i RCD su bile značajno niže a NO više na kraju studije u poređenju sa kontrolnom grupom. **Zaključak**. Redovan fizički trening indukuje smanjenje intenziteta oksidativnog stresa i ćelijskih adhezionih molekula uz porast bioraspoloživog NO i povoljne promene HDL holesterola i triglicerida.

Ključne reči:
miokard, ishemija; stres, oksidativni; endotel krvnih sudova; vežbanje; lečenje, ishod.

Introduction

Stable ischemic heart disease (IHD) is characterized by systemic endothelial dysfunction. Besides from pharmacological interventions, exercise training improves endothelium-dependent vasodilatation in coronary blood flow and shows positive effects on regression of coronary atherosclerosis and prevention of restenosis. These effects were mediated through stabilization of atherosclerotic lesions and favorable changes in serum lipoproteins in patients with IHD [1]. Exercise has positive effects on an arterial endothelial function by an increased endothelial nitric oxide synthase (eNOS) protein expression and thus increased concentrations of bioavailable nitric oxide (NO) [2].

Exercise training is the major component of cardiac rehabilitation. It has positive effects on the psychosocial well-being and quality of life. Regular exercise improves cardiovascular risk factors' profile and a long-term prognosis in patients with IHD [3]. Results from other studies show that low maximal aerobic capacity is associated with an increased rate of cardiac events [4]. Despite all these benefits, a great proportion of adults in developed countries is physically inactive (about 70% of all Americans), which represents a great pool of individuals at a risk to develop cardiovascular diseases [5].

Increased oxidative stress in endothelium is also a fundamental pathophysiological mechanism of IHD development. The redox signaling pathways have an important role in regulating the cardiac function and vascular tone by reducing NO bioavailability and increased quenching of NO by superoxide [6]. Exercise training plays a positive role in virtually all redox aspects of cardiac and vascular pathophysiology. Regular physical activity of moderate intensity has antioxidant properties and improves endothelial function. It has evolved as an accepted therapy to improve endothelial function. However, the molecular mechanisms by which exercise training improves redox homeostasis in cardiovascular diseases remain unknown and need further investigation [7].

The aim of this study was to evaluate the changes of oxidative stress and endothelial function biomarkers after 6 weeks of aerobic physical training in patients with stable IHD participating in a cardiovascular rehabilitation exercise program.

Methods

The study included 72 patients with stable IHD divided into 2 groups. Exercise training group consisted of 35 patients who spent 21 days under supervising cardiovascular rehabilitation in the medical centre and 21 days of planned physical training at home. They were recruited for the study in a consecutive manner from a cohort of IHD patients included in a cardiovascular rehabilitation program. The control group consisted of 37 age and sex-matched patients with stable IHD who had usual everyday physical activity at home without any other physical activity or physical training 6 weeks before the examination. The control participants were chosen from a cohort of dyslipidemic and hypertensive patients with stable IHD treated in the outpatient Department of Lipid Metabolism and Hypertension, according to the age range obtained from the exercise training group.

The diagnosis of IHD was confirmed by medical documentation of significant coronary stenosis, stenting or bypass procedures or myocardial infarction. The patients were included if they had no anginal symptoms or had stable angina (functional classification of angina: Canadian Society of Cardiology class II / III) diagnosed with the onset of anginal chest pain provoked by physical or mental stress, which disappears after rest or sublingual nitro-glycerine application [8].

The study excluded patients with acute and chronic inflammatory diseases, liver diseases, recent trauma, mild to severe anemia, patients with anti-inflammatory medication (except acetyl salicylic acid), vitamins and antioxidants. Exclusion also comprised those with the acute coronary syndrome within last 3 months, poorly controlled congestive heart failure, signs of myocardial ischemia on electrocardiogram (ECG) at stage 1 of the exercise tolerance test (Bruce protocol), or presence of ECG abnormalities such as left bundle branch block, presence of pacemaker and poorly controlled diabetes or thyroid disease. Patients with non provoked angina's pain in rest were excluded.

The clinical characteristics of patients involved: the presence of concomitant diseases, cardiovascular risk factors, used therapy, anthropometric (data waist and hip circumference, body mass index and waist/hip ratio). The blood pressure was determined according to American Heart Association procedure (the average value from the 3 measurements after 5 minutes resting was adopted) [9].

All patients signed the informed consent to participate in the study and the local Ethical Review Board approved the study.

All the patients had a therapy for secondary prevention of IHD according to the European Society of Cardiology (ESC) guidelines and all cardiac-related medication doses were kept constant during the study. During the study, all patients were required to apply the recommended hypolipemic diet and to avoid any change in their usual housework.

Exercise training protocol

During 6 weeks all patients in the exercise group had 45 minutes of continuous aerobic physical activity using the treadmill, ergo bicycle or walking 3 times a week. The intensity of the physical activity was limited up to 80% of maximal heart rate which was obtained by pre-study ergo test for every patient.

Blood sampling and laboratory measurements

Blood samples were obtained from all patients in the exercise training and the control group at baseline and after 6 weeks. Venous blood samples were taken from the cubical vein in the morning, after 12 h of fasting, before the regular therapy was taken. The following parameters were laboratory determined in obtained samples: total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high- density lipoprotein cholesterol (HDL-C) and triglycerides (TG). In the exercise training group, blood samples were collected at least 24 h after the last physical exercise in order to avoid the immediate effects of exercise. After centrifugation, aliquots were frozen at -80°C until assayed.

Endothelial function

Nitric oxide (NO) release was determined spectrophotometrically by measuring the accumulation of its stable degradation products nitrite and nitrate. Total nitrites were then determined spectrophotometrically by using the Navarro-Gonzalvez et al. [10] (1998), reaction based on Griess reaction. Total nitrate and nitrite concentrations were given in nmol/mg protein.

Cell adhesion molecules, intracellular (CAM-1) and vascular (VCAM-1) were detected by enzyme-linked immunosorbentassay (ELISA), using a commercial test (Beckman Coulter Inc.) on a Bio Systems – ELISA reader. Results were expressed in ng/ml [serum VCAM-1 (sVCAM-1) measuring range: 0-250 ng/mL, sensitivity: 7.4 ng/mL), and sICAM-1 (measuring range: 0-160 ng/mL, sensitivity: 1 ng/mL).

Oxidative stress

Lipid peroxidation intensity was monitored by measuring serum malondialdehyde (MDA), as one of the end products of lipid peroxidation (method by Andreeva et al. [11]). MDA on high temperature in low pH environment, with the addition of Fe^{2+} turns to thiobarbiturate acid and colors the suspension pink. Chromogen absorption was detected at 523 nm. Levels of serum MDA were presented in μmol/L.

Determination of oxidatively modified proteins was done by spectrophotometric measurement of carbonyl group content in amino acids residues. The carbonyl content was determined by colorimetric reaction with 2,4-dinitrophenylhydrazine (DNPH) and expressed as μmol/g plasma proteins [12]. Protein concentration was determined by the Lowry et al. [13] method.

Statistical analysis

Continuous data with normal distribution were expressed as the mean ± standard deviation (SD) or median (interquartile range, IQR) for skewed data. Categorical data were presented as numbers and percentages. Differences between two groups were compared by Student's *t*-test and Mann-Whitney *U* test for continuous variables and $\chi 2$ and Fisher test for categorical variables. Statistical analyses were done by software package SPSS 16.0.

Results

The baseline characteristics of examined groups were similar according to age, gender and cardiovascular risk profiles (proportions of smokers, the prevalence of hypertension, dyslipidemia, diabetes mellitus, obesity, family history for coronary artery diseases (CAD). The therapy for secondary prevention of IHD was similar in both groups and was kept constant during 6 weeks (Table 1).

Data are presented as the mean ± SD or n (%); MI – myocardial infarction; PTCA – percutaneous transluminal coronary angioplasty; CAB – coronary artery bypass; CAD – coronary artery disease; ACEI – angiotensin-converting-enzyme inhibitors; ARB – angiotensin receptor blockers; NS – nonsignificant.

The effects of 6 weeks cardiovascular rehabilitation program were visible in a significant reduction of body mass index (BMI), systolic and diastolic blood pressure, heart rate and triglycerides as well as increasing of HDL cholesterol ($p < 0.05$) which was not seen in the group with a sedentary lifestyle (Table 2).

Regular, aerobic, moderate exercise training during 6 weeks induces favorable increase of NO, reduction of RCD and sVCAM-1 level compared with its initial level which is not seen in the control group. The values of sICAM-1 were lower in the exercise training group at the start and at the end of the study without significant changes during the period of observation. In the exercise training group, values of sVCAM-1 and RCD were significantly lower and NO significantly higher at the end of the study compared to the sedentary control group (Table 3).

Discussion

There is a consistent evidence that any type of regular rhythmic physical exercise reduces blood pressure and decrease heart rate independently of weight loss, dietary habits

Table 1

Baseline characteristics of patients

Parameters	Exercise training group	Control group	*p*
Male/female, n	15/20	18/19	NS
Age (years), $\bar{x} \pm SD$	57.4 ± 5.7	59.3 ± 6.8	NS
MI, n (%)	23 (65.7)	26 (70.2)	NS
CAB grafting, n (%)	5 (14.2)	4 (10.8)	NS
PTCA, n (%)	7 (20.0)	7 (19)	NS
Duration of CAD (years), $\bar{x} \pm SD$	4.9 ± 3.8	6.8 ± 5.2	< 0.05
Risk factors for CAD, n (%)			
smokers	14 (40.0)	16 (43.2)	NS
elevated blood pressure	30 (85.7)	30 (81.1)	NS
lipid disorders	15 (42.8)	15 (40.5)	NS
diabetes mellitus	12 (34.2)	13 (35.1)	NS
obesity	11 (31.4)	10 (27.0)	NS
family history for CAD	15 (42.8)	15 (40.5)	NS
Evidence based therapy, n (%)			
beta blockers	30 (85.7)	28 (75.7)	NS
ACEI / ARB	31 (88.5)	33 (89.2)	NS
calcium channel blockers	25 (71.4)	27 (72.9)	NS
statins	31 (88.5)	32 (86.4)	NS
acetylsalicylic acid	33 (94.2)	35 (94.6)	NS
nitrates	20 (57.1)	20 (54.1)	NS

MI – myocardial infarction; PTCA – percutaneous transluminal coronary angioplasty; CAB – coronary artery bypass; CAD – coronary artery disease; ACEI – angiotensin-converting-enzyme inhibitors; ARB – angiotensin receptor blockers; NS – nonsignificant. $\bar{x}$ – arithmetic mean; SD – standard deviation;

Table 2

Clinical and biochemical data of patients included in the study

Parameters	Exercise training group		Control group	
	Δ change	% change	Δ change	% change
BMI (kg/m^2)	-1.5*	-5.05	-1.5	-5.70$^{\&}$
WC (cm)	-1.2	-1.26	+0.6	+0.68
WHR	-0.01	-1.14	-0.01	-1.12
sBP (mmHg)	-7.7$^{\&}$	-5.37	-4.7	-3.39
dBP start (mmHg)	-7.4$^{\&}$	-8.20	-2.6	-2.98
HR (/min)	-6.3$^{\&}$	-8.02	+1.3	+1.71
TC (mmol/L)	-0.2	-3.70	-0.2	-3.77
LDL-C (mmol/L)	-0.3	-9.09	-0.2	-5.56
HDL-C (mmol/L)	+0.23$^{\&}$	+23.47	-0.01	-1.00
TG (mmol/L)	-0.2$^{\&}$	-11.24	+0.1	+5.88

Data are presented as Δ change from baseline or % change from baseline; BMI – body mass index; WC – waist circumference, WHR – waist/hip ratio; sBP – systolic blood pressure; dBP – diastolic blood pressure; HR – heart rate; TC – total cholesterol; LDL-C – low density lipoprotein cholesterol; HDL-C – high density lipoprotein cholesterol; TG – triglycerides;
***$p < 0.05$ vs initial values.**

Table 3

Carbonyl contents, lipid peroxydation and markers of endothelial disfunction

Parameters	Exercise training group		Control group	
	before	after	before	after
RCD (μmol/g plasma proteins), $\bar{x} \pm SD$	1.27 ± 0.48	1.04 ± 0.22†	1.16 ± 0.25	1.12 ± 0.14
MDA (μmol/L), $\bar{x} \pm SD$	14.09 ± 6.65	12.84 ± 3.25	15.12 ± 6.15	13.79 ± 5.23
sVCAM (ng/mL), median (IQR)	100.49 (78.42–118.3)	80.08 (68.5–97.2)†*	92.26 (73.64–106.8)	91.33 (73.01–105.3)
sICAM-1 (ng/mL), median (IQR)	80.74 (54.92–97.3)*	80.36 (68.1–95.3)*	86.35 (57.32–95.8)	84.65 (55.67–93.8)
NO (nmol/mg prot.), $\bar{x} \pm SD$	64.72 ± 16.06	74.38 ± 18.57†*	68.5 ± 17.9	65.7 ± 19.6

$\bar{x}$ – arithmetic mean; SD – standard deviation; IQR – interquartile rang; MDA – malondyaldechyde; RCD – reactive carbonyl derivatives; NO – nitric oxyde; sVCAM-1 – soluble vascular cell adhesion molecule-1, sICAM-1 soluble intercellular adhesion molecule-1; *$p < 0.05$ vs control; †$p < 0.05$ vs initial values.

or smoking compared to sedentary ones [14–16]. Aerobic exercise (30–40 minutes at 65% of VO_2 max) three times a week showed effects on vascular function too. It significantly reduces augmentation index (an index of arterial stiffness which measures the reflected wave at the aorta), improves carotid artery compliance, and can restore vascular endothelial function in adults [17, 18].

Considering the similar age, gender distribution, cardiovascular risk factors profile and therapy in both groups of patients at the start of the study, the significant reduction of BMI, systolic and diastolic blood pressure, heart rate and triglycerides as well as increasing of HDL cholesterol could be of pathogenetic importance in the oxidative stress reduction and endothelial function improvement after 6 weeks of regular aerobic exercise training implementation.

Significant reduction of triglyceride concentration and raising of HDL cholesterol level in the group with physical training are in the concordance with data obtained by other authors in similar settings [19]. Volaklis et al. [20] showed that exercise training program during 16 weeks significantly reduced a total cholesterol and triglycerides without altering LDL cholesterol level in patients with CAD.

The cardiovascular risk factor management is widely recognized as a priority in the secondary prevention programs [21, 22]. The regular physical activity and its favorable effects on some cardiovascular risk factors such as blood pressure, dyslipidemia and obesity are important especially in stable asymptomatic patients since it improves the prognosis of this severe disease in which absence of symptoms implies no benignity, as it was presented in this study.

Reactions such as cell injury, adhesion, inflammation, and oxidative stress occur not only at the early stage of risk but persist throughout the process of atherosclerosis. The increased oxidant stress is common to these processes, characterized by the excessive generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and reduced antioxidant capacity. There is an accumulating evidence from prospective studies for a predictive role of elevated circulating levels of sICAM-1 in initially healthy people, and of sVCAM-1 in patients at high risk or with overt CAD. In those reports that quantify atherosclerosis, sVCAM-1 seems to be more specific for atherosclerosis than other markers. The serum level of sVCAM-1 appears to correlate with the extent of atherosclerosis and might allow for the detection of early stages of atherosclerosis [23]. Thus, adhesion molecules especially VCAM-1 might be useful for clinical risk prediction in IHD patients and serve as therapeutic targets during a cardiovascular rehabilitation program, which is supported by the finding of its significant reduction after 6 weeks of exercise training protocol in conducted study. Similar significant decreasing of VCAM-1 was presented in the study by Lim et al. [24] in elderly obese women after 12 weeks of a healthy life exercise program.

Early therapeutic intervention based on an aerobic exercise program is able to prevent progression and manifestation of the clinical sequelae of atherosclerosis which is also shown by other authors [24, 25]. The continuous or intermittent aerobic exercise training leads to a significant decrease of serum VCAM-1 and ICAM-1 in patients with New York (NYHA) class II–III chronic heart failure and with a left ventricular ejection fraction of 35–55% after 10 weeks [26]. This further spreads the spectrum of patients who benefit from a physical training. This observation could be applied not only in atherosclerotic cardiovascular disease patients but also in healthy individuals with professionally increased cardiovascular risk such as night shift workers. The intermittent exercise at 10-min bouts (30 min per day), 3 days a week during 10 weeks induces significant decreasing of VCAM-1 and plasma concentrations of some serum's elastases in night shift workers [27]. The common observation in all studies which registered decreasing of CAMs was a reduction of BMI and percent of body fat [19, 24, 26, 27] as seen in conducted study.

The large systematic review about the influence of physical activity on key biomarkers in atherosclerosis suggests that effects on atherosclerotic process may depend on the type, duration, and intensity of physical activity. The cell adhesion molecules seemed to be affected by aerobic exercise while resistance training showed no effect [28]. According to literature data, the effects of aerobic physical training are visible after 4 weeks of aerobic training and persist during the training period of 1 year [29, 30].

Decreasing of reactive carbonyl derivatives but not MDA indicates a specific antioxidative pattern of physical training beyond effects on lipids and endothelial function. The effects of physical exercise training program on blood pressure reduction independently of used antihypertensive treatment could be distinctive antihypertensive mechanism mediated by reactivation of different signaling protein molecules and improving the mitochondrial dysfunction with additional effects on subsequent coronary event prevention [6]. This is in line with novel observations that distinct from the NO pathway different vasoregulative factors improve vasomotion [31]. Further studies confirmed the beneficial effects of exercise on vascular vasomotor function independently of markers of inflammation and oxidative stress and were interrelated with improved exercise capacity [32].

The other important effect of the regular physical training program is increasing concentration of NO. Besides its effects on vasodilatation, it improves the efficiency of myocardial O_2 consumption and produces cardioprotection. These responses are mediated, at least in part, by protein S-nitrosylation, a redox-dependent reversible protein posttranslational modification that involves attachment of a NO moiety to a protein sulphydryl group, further protecting these thiol groups from irreversible oxidative/nitrosative modifications [33, 34] which are also shown in this study.

Conclusion

The exercise training is important as a non-pharmacological tool in treating hypertension, lipid disorders and endothelial dysfunction in selected motivated patients with stable coronary artery disease. Regular physical activity induces the reduction of oxidatively modified proteins and vascular cells adhesion molecules alongside with increased NO bioavailability and favorable changes in HDL cholesterol and triglycerides.

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Identification of *Candida* spp. in the oral cavity in patients with malignant diseases

Identifikacija vrsta gljivica iz roda *Candida* u usnoj duplji bolesnika sa malignim oboljenjem

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Abstract

Background/Aim. Oral candidiasis frequently causes discomfort in patients treated for malignant diseases, acting as well as a potential source of systemic infection. This disease may present itself through different clinical manifestations of both acute or chronic type. The aim of this study was to identify different *Candida species* from oral cavities of patients suffering from malignant diseases. **Methods.** Thirty patients admitted to the hospital for diagnostics/treatment of malignant diseases were included in this investigation. All subjects had visible changes of oral mucosa in the form of pseudomembranes and inflammation corresponding to oral candidiasis. Control group included 30 non-hospitalized patients diagnosed with candidiasis. Diagnosis of oral candidiasis was confirmed in all patients by microbiological analysis of tongue swabs. For microbiota identification, three different tests were used: germination test, fungal growth test on corn meal agar, and biochemical identification with commercially available ID 32 C kit (bio-Merieux, Marcy-l´Etoile, France). **Results.** Out of 30 isolates collected from hospitalized patients, 90% was related to *Candida albicans*, 7% was identified as *Candida kefyr*, and 3% as *Candida famata.* In samples collected from non-hospitalized controls, we isolated *Candida albicans* in 90% of the cases, in 7% *Candida kefyr*, while in 3% we identified *Candida glabrata.* **Conclusion.** Based on this investigation, oral candidiasis in patients treated with radiotherapy and chemotherapy is mainly caused by *Candida albicans*. It is to be expected that *Candida albicans* will remain the most significant causative agent of oral candidasis, although we must bear in mind the possibility of other pathogenic species.

Key words:
candida; candida albicans; mouth; neoplasms; microbiological techniques; candidiasis, oral.

Apstrakt

Uvod/Cilj. Oralna kandidijaza čest je uzročnik nelagodnosti kod bolesnika na terapiji zbog malignih bolesti, a može biti i potencijalni izvor sistemskih infekcija. Bolest se može manifestovati kroz različite kliničke slike, koje mogu poprimiti akutni ili hroničan tok. Cilj ovog rada bio je da se identifikuju *Candida* iz usne duplje bolesnika sa malignim bolestima. **Metode.** U istraživanje je bilo uključeno 30 bolesnika hospitalizovanih zbog maligne bolesti. Svi ispitanici imali su promene na oralnoj sluzokoži, sa pseudomembranama i upalom, odnosno s kliničkim izgledom oralne kandidijaze. Kontrolnu grupu ispitanika sačinjavalo je 30 nehospitalizovanih bolesnika koji su imali kliničku sliku kandidijaze. Kod svih ispitanika sprovedena je mikrobiološka obrada brisa oralne sluzokože sa jezika, koja je potvrdila dijagnozu oralne kandidijaze. U svrhu identifikacije korišćena su tri testa: test klijanja, test rasta kvasaca na kukuruznom agaru i biohemijska identifikacija pomoću komercijalnog testa ID 32 C (*bio-Merieux, Marcy-l´Etoile*, France). **Rezultati.** Od ukupno 30 izolata sakupljenih od 30 hospitalizovanih bolesnika, kod 90% radilo se o vrsti *Candida albicans*, 7% identifikovano je kao *Candida kefyr*, a 3% kao *Candida famata.* U uzorcima sakupljenim od 30 nehospitalizovanih osoba, u 90% bila je izolovana *Candida albicans*, u 7% *Candida kefyr*, a u 3% *Candida glabrata.* **Zaključak.** Na osnovu ovog istraživanja potvrđeno je da je oralna kandidijaza kod bolesnika na radioterapiji i/ili hemoterapiji zbog maligne bolesti najčešće uzrokovana gljivicom *Candida albicans*. Može se očekivati da će *Candida albicans* i dalje biti najznačajniji uzročnik oralne kandidijaze, iako su mogući i drugi uzročnici infekcije.

Ključne reči:
candida; candida albicans; usta; neoplazme; mikrobiološke tehnike; kandidijaza, oralna.

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Introduction

Candida species (*Candida* spp.) are constitutive members of oral flora. Nearly half of the healthy adult population has these fungi on the mucosal surface without experiencing any symptoms since the immune system controls its excessive growth and development of the disease [1, 2]. However, in cases of some local and systemic predisposing factors, there is a possibility of oral candidiasis development. The local predisposing factors are changes in salivary gland function, use of antibiotics and corticosteroid drugs, carbohydrate-rich diet, changes in oral epithelium, dentures, or excessive tobacco use. Systemic predisposing factors include changes in hormonal status, iron, folic acid and vitamin B12 deficiencies, use of antibiotics, malignant diseases and immune suppression of different origins [3–5]. Oral candidiasis may be manifested through different clinical signs and symptoms, as acute pseudomembranous, acute atrophic candidiasis, chronic atrophic candidiasis, glossitis and angular cheilitis [4].

Oral candidiasis is the most common opportunistic infection in patients with malignant diseases [6]. Cytotoxic therapy and radiotherapy are both important predisposing factors in its development. Patient's defense mechanisms which have already been weakened by the main disease may be further depleted by cytotoxic and radiation therapy [6, 7]. As a consequence of cytotoxic therapy, oral candidiasis occurs in 30–70% of the patients [8]. Side effects of head and neck radiotherapies, such as dry mouth and thick sticky saliva, are coupled with an increased colonization of oral mucosa with *Candida*, especially *Candida albicans (C. albicans)*. Such milieu favors the development of oral candidiasis in 17% to 52.5% of the patients [6, 7, 9]. Fungi belonging to *Candida* spp. are responsible for 75% of all fungal infections in patients with malignancies. According to the literature, *C. albicans* is isolated in 70–80% of all patients, while *Candida glabrata (C. glabrata)* and *Candida tropicalis (C. tropicalis)* appear in 5–8% of the cases. Recent research shows an increase in trend of non-*albicans*candidal infections (*C. glabrata*, *C. parapsilosis and C. tropicalis*), while the share of *C. albicans* decreased [9].

Development of oral candidiasis in patients treated with radiotherapy and chemotherapy presents a significant factor for the systemic infection and candidaemia which may act as a direct cause of death [10–12]. The European Organization for Research and Treatment of Cancer – International Antimicrobial Therapy Group published the data of oral cavity being the source of 23% of the microorganisms isolated from the blood of neutropenic patients with carcinoma [13].

The purpose of this study was to determine the *Candida* species responsible for the development of the disease in patients under cytotoxic therapy and/or radiotherapy for treatment of a malignant disease.

Methods

This research included thirty patients hospitalized at the Clinic of the Internal Medicine and Clinic of Radiology and Oncology, Clinical Hospital Center Rijeka. Table 1 presents demographic and clinical characteristics of hospitalized patients. At the time of sampling, 19 of them were subjected to cytostatic therapy, while 11 of them went through radiotherapy. All subjects had visible changes in the oral mucosa with pseudomembranes whose clinical appearance corresponded to oral candidiasis. All subjects were microbiologically tested in order to confirm the diagnosis. Patients did not use any antifungal drugs for at least one month before sampling procedures.

The control group was made by 30 patients from the Clinic of Dental Medicine of the same hospital center. All of them had a fully developed scope of clinical signs and symptoms corresponding to oral candidiasis and did not suffer from any malignancy.

Both groups were introduced to the purpose of the research upon the inclusion and by signing the Informed consent agreed to participate. The research had been approved by the Ethics Committee of the Clinical Hospital Center Rijeka.

Cultivation and identification of Candida

Subjects were taken the oral mucosa swabs from the tongue region with a sterile cotton swab (Copan, Zagreb, Croatia). Immediately upon collection, the material was striked out on solid Sabouraud dextrose agar plates (PanreacQuimica, Cultimed, Spain) and incubated at 37°C for 72 h. Distinctive colonies sized 2–3 mm, with smooth and shiny surface and clean margins, white to cream in color, and with typical yeast smell, were transferred and multiplied on new solid medium plates. Positive strains were identified by standard mycological methods, by germ tube production, chlamydospore development in the microculture in cornmeal Tween 80 (Difco, Detroit, USA) and API ID 32 *Candida* identification kit (bio-Merieux, Marcy-l'Etoile, France). The germ-tube test involved the induction of hyphal outgrowths from yeast cultured in rabbit serum for 3 h at 37°C. Microscopic slides were examined under light microscope. This test was used for *C. albicans* identification. Chlamydospore production was also associated with *C. albicans*. *C. albicans* produced thick-walled, dormant growth forms induced *in vitro* by culture agar supplemented with Tween 80. The inoculated area was covered with the cover slip and the agar incubated at 22°C for 72 h. Under such conditions, cornmeal agar also included a characteristic filamentous growth which could aid in the identification of *C. albicans*. The API ID 32 C system consists of a single use disposable plastic strip with 32 wells containing substrates for 29 assimilation tests (carbohydrates, organic acids, and amino acids), one susceptibility test (cycloheximide), one colorimetric test (esculin), and one negative control. The yeast identification procedures were conducted in accordance with the manufacturer's instructions. One day-cultures and sterile distilled water were used to prepare the suspensions with final turbidity equivalent to McFarland #2. Five drops of this suspension were then dispensed to ampoules of C medium provided by the manufacturer and homogenized to prepare an even dispersion of inoculum. The inoculum suspensions were used to inoculate the wells. The systems were incubated at 30°C for 48 h. The results were visually examined and transformed into numerical bio-codes. At the end, the isolates were identified by ID 32 Analytical Profile Index.

Table 1

Age, gender, type of malignancy and type of therapy in hospitalized group

Hospitalized patients	Gender	Age (years)	Type of malignancy	Type of therapy		
				surgery	chemotherapy	radiotherapy
1	m	73	Non-Hodgkin lymphoma	–	+	–
2	f	57	Leukemia	–	+	–
3	m	71	Colon cancer	+	+	–
4	f	51	Ovarian cancer	+	+	–
5	m	73	Oral cancer	+	–	+
6	f	55	Breast cancer	+	–	+
7	m	51	Colon cancer	+	+	–
8	m	71	Prostate cancer	+	+	–
9	f	69	Thyroid cancer	+	–	+
10	f	31	Leukemia	–	+	–
11	m	52	Colon cancer	+	+	–
12	m	64	Oral cancer	+	–	+
13	f	51	Breast cancer	+	–	+
14	f	49	Breast cancer	+	–	+
15	f	66	Thyroid cancer	+	–	+
16	f	65	Uterine cancer	+	–	+
17	f	71	Oral cancer	+	–	+
18	m	66	Prostate cancer	+	+	–
19	f	78	Breast cancer	+	–	+
20	f	59	Leukemia	–	+	–
21	f	64	Breast cancer	+	+	–
22	f	69	Colon cancer	+	+	–
23	f	49	Non-Hodgkin lymphoma	–	+	–
24	m	70	Oral cancer	+	–	+
25	f	72	Breast cancer	+	+	–
26	m	58	Gastric cancer	+	+	–
27	f	65	Leukemia	–	+	–
28	f	61	Breast cancer	+	+	–
29	f	54	Colon cancer	+	+	–
30	m	64	Prostate cancer	+	+	–

m – male; f – female.

Statistical analysis

Statistical analysis was performed using the Statistica 12.7 software (StatSoft, Inc., Tulsa, OK, USA)

The Kolmogorov-Smirnov normality test was applied to our data. The Student-*t* test was used to analyze the age difference between groups while the χ^2 test was used to compare the genders. Fisher's exact test was used to analyze oral candida distribution in hospitalized and non-hospitalized group of patients.

Statistically significant difference was set to $p < 0.05$.

Results

The hospitalized group of patients suffering from oral candidiasis (n = 30) included 17 women and 13 men. Chemotherapeutic protocol for treatment of leukemia was assigned for 4 patients, 2 for treatment of lymphoma, 3 for breast cancer, 6 for malignancies of digestive organs, 3 for prostate cancer and 1 for ovarian cancer. A total of 4 subjects was treated with irradiation therapy for breast cancer, 4 for oral cancer, 2 for thyroid cancer, and 1 for uterine cancer. All patients had pronounced inflammatory changes on the oral mucosa with pseudomembranes. Microbiological analysis proved oral candidiasis in all patients. Average patient age was 61.23 ± 9.07 years (Table 2).

The control group of patients with oral candidiasis (n = 30) included 11 males and 19 females. Denture stomatitis was diagnosed in 14 patients, 13 had acute atrophic candidiasis, and 3 patients developed acute pseudomembranous candidiasis. Clinical diagnosis of oral candidiasis was confirmed in all patients through microbiological analysis. Average patient age in this group was similar to the study group (Table 2). The distribution of different species in both groups is presented in table 3. Of 30 isolates, collected from 30 patients, *C. albicans* was detected in 27 (90%), two (7%) isolates

Table 2

Clinical characteristics of hospitalized and non-hospitalized group of patients

Parameter	Patients		*p*-value
	Hospitalized	Non-hospitalized	
Total number of patients	30	30	
Gender (m/f), n	13/17	11/19	0.60*
Age (years), $\bar{x} \pm SD$	61.2 ± 9.0	58.7 ± 11.6	0.05**

m – male; f – female; $\bar{x}$ – mean value; SD – standard deviation.
***Student-*t* test; **χ^2 test.**

Table 3

Oral candida distribution in hospitalized and non-hospitalized group of patients

Candida spp.	Hospitalized patients (n = 30)	Non-hospitalized patients (n = 30)	*p*-value
	n (%)	n (%)	
Candida albicans	27 (90)	27 (90)	1**
*Candida kefyr**	2 (7)	2 (7)	
Candida famata *	1 (3)	1 (3)	
Candida glabrata *	0 (0)	0 (0)	

***Categories pooled for data analysis because of the small number in cells; **Fisher's exact test.**

were identified as *Candida kefyr (C. kefyr)*, and one (3%) as *Candida famata (C. famata).* When analyzing 30 isolates collected from non-hospitalized patients, the main isolate was *C. albicans* in 27 (90%) cases, in two (7%) samples *C. kefyr*, and in one (3%) *C. glabrata.*

Discussion

Fungal infections pose a significant source of complications in patients suffering from malignancies. During the last 50 years their numbers increased, they frequently develop in early stages of the disease, and are caused by the species which had not been previously considered as pathogenic [14–16]. Cytotoxic and radiation therapies are important predisposing factors in the development of oral candidiasis. While the organism had already been weakened by the principal disease, its defense mechanisms are further depleted by those treatment modalities [6, 17]. In addition, a consequence of these therapies is frequently oral mucositis, which is mainly caused by their direct cytotoxic effect on mucosal cells as well as a negative effect of long-standing inflammation due to inadequate immune reaction to fungal infection [18]. It is estimated that 30–70% of the patients on cytotoxic therapy develop oral candidiasis. Moreover, negative side-effects of radiation therapy in the head and neck regions, such as dry mouth and thick sticky saliva, are coupled with an increase in colonization with *Candida* spp, especially *C. albicans*. Such a milieu favors the development of oral candidiasis in 17% to 52.5% of the patients [8, 19–21]. In all our test subjects, clinical examination and microbiological analysis proved oral candidiasis.

Most superficial fungal infections of the oropharyngeal region and digestive system are caused by *C. albicans* [22]. In immunologically challenged patients, this fungus may cause an invasive infection through damage and ulcerations of the mucosal surfaces. Disseminated type of the infection may appear in cases of neutropenia, hematologic malignancies, and in patients on high dose-regimens of antimicrobial and cytotoxic therapy [23, 24]. In this research, *C. albicans* was the most common cause of the infection, and these results correspond to the majority of other investigations. Nicolatuo-Galitis et al. [19] investigated patients in irradiation therapy; a total of 61 patients participated, and pseudomembranous candidiasis developed in 31 patients. The most commonly isolated fungus was *C. albicans* (84%), followed by *C. tropicalis* (9%), *C. glabrata* (3.4%), *Candida krusei* (1.2%) and *Candida holmii* (1.2%). Similar results were obtained by Swoboda-Kopec et al. [25]. The main causative agent was *C. albicans*, while of non-*albicans Candida* significant role in infection was played by *C. glabrata, C. kruisei, C. tropicalis, C. parapsilosis* and *C. kefyr*. The cases where *C. albicans* is isolated as the main causative agent range from 51–93% [25, 26]. These results are in line with our results since in our patients 90% of the infections were caused by *C. albicans*.

To a lesser extent (6%) we were able to isolate *C. kefyr* in our patients. It is well known that *C. kefyr* may cause superficial infections. Besides, some cases of fungaemia caused by this fungus have been described in hospitalized patients both from departments of surgery and oncology [27]. *C. famata* is a saprophyte which rarely causes infections in humans, however, there are case reports of fungaemia and peritonitis in hospitalized patients. In addition, it may cause urogenital infections and deep fungal infections of heart or lung tissue with the subsequent development of sepsis. In our investigation, one of the subjects was positive on *C. famata* infection [28].

C. glabrata had been considered as a harmless member of the oral flora; however, it has recently been identified as a significant causative agent of infections in immunologically compromised patients. In patients submitted to irradiation therapy, *C. glabrata* is regarded as an important cause of oropharyngeal candidiasis. The probability of *C. glabrata*-caused infection of the oral mucosa is significantly increased if the previous fungal infection was treated with fluconazole or ketoconazole [29, 30]. In hospitalized patients who participated in this research, no *C. glabrata* isolate has been detected, but we have isolated this fungus from the one of the control patients' oral mucosa.

Conclusion

This study confirmed that oral candidiasis in patients with malignant diseases treated with irradiation and chemotherapy is mostly caused by *C. albicans*. It is to be expected that *C. albicans* will remain the most significant causative agent of oral candidiasis, although other species should also be taken into account (such as *C. kefyr* and *C. famata*).

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Relative frequency of immature CD34+/CD90+ subset in peripheral blood following mobilization correlates closely and inversely with the absolute count of harvested stem cells in multiple myeloma patients

Relativna učestalost nezrelog podtipa ćelija CD34+/CD90+ u perifernoj krvi posle mobilizacije je u tesnoj i obrnutoj korelaciji sa apsolutnim brojem matičnih ćelija u afereznom produktu kod bolesnika sa multiplim mijelomom

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Abstract

Background/Aim. Stem cells (SCs) guarantee complete/long-term bone marrow (BM) repopulation after SC-transplants. The aim of the study was to evaluate absolute count of total SCs (determined by ISHAGE-sequential-gating protocol – SC^{ish}) and relative frequency of immature $CD34^+/CD90^+$ ($CD90^+SC^{ish}$) subset in peripheral blood (PB) as predictive factors of mobilization and apheresis product (AP) quality. **Methods**. Mobilization included chemotherapy and granulocyte-growth-factor (G-CSF). Harvesting was performed by Spectra-Optia-IDL-system. The SCs^{ish} were determined as a constitutional part of $CD34^+$ cells in the "stem-cell-region" using FC-500 flow-cytometer. In this study, the original ISHAGE-sequential-gating protocol was modified by introduction of anti-CD90-PE monoclonal-antibody into the analysis of CD90 expression on SC^{ish} ($CD90^+SC^{ish}$). The results were presented as a percentage of SC^{ish} per nucleated-cell count, absolute SC^{ish} count in μL of the PB or the AP, percentage of the $CD90^+SC^{ish}$ expressed to SC^{ish} and absolute $CD90^+SC^{ish}$ count in μL of the PB or the AP. **Results**. The absolute count of total SC^{ish} and $CD90^+SC^{ish}$ was significantly higher ($p = 0.0007$ and $p = 0.0266$, respectively) in the AP than in the PB samples. The $CD90^+SC^{ish}$/total SC^{ish} indexes from PB were higher than indexes from the AP ($p = 0.039$). The relative frequency of $CD90^+SC^{ish}$ showed a highly significant inverse correlation with the absolute count of total SC^{ish} in both, the PB and AP ($p = 0.0003$ and $p = 0.0013$ respectively). The relative frequency of $CD90^+SC^{ish}$ from the PB also showed a significant ($p = 0.0002$) inverse relationship with total SC^{ish} count in the AP. Patients with less than 10% $CD90^+SC^{ish}$ in the PB had evidently higher ($p = 0.0025$) total SC^{ish} count in the AP. **Conclusion**. We speculate that lower $CD90^+SC^{ish}$ yield in the AP is not a consequence of an inferior collection efficacy, but most likely a result of several still not fully resolved immature SC cytomorphological/biophysical features. Therefore, following the mobilization by chemotherapy G-CSF, some logical questions appear – whether we should follow the absolute count of total SC^{ish}, or, whether we should test for relative frequency of $CD90^+SC^{ish}$ prior to harvesting. To reach the final conclusions, it is essential to conduct further controlled and larger investigations concerning the correlation of circulating and harvested SCs with patients' hematopoietic recovery.

Key words:
stem cells; hematopoietic stem cell transplantation; bone marrow; flow cytometry; multiple myeloma; antineoplastic combined chemotherapy protocols.

Apstrakt

Uvod/Cilj. Matične ćelije (MĆ) obezbeđuju kompletnu/dugotrajnu repopulaciju kostne srži (KS) posle transplantacije. Cilj ove studije bila je procena apsolutnog broja ukupnih MĆ (utvrđena protokolom „ISHAGE-sequential-gating" – Stem-Cell^{ish} [SC^{ish}]) i relativne učestalosti primitivnih podipova $CD34^+/CD90^+$ ($CD90^+SC^{ish}$) u perifernoj krvi (PK)

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kao prediktora efikasnosti mobilizacije i pokazatelja kvaliteta afereznog produkta (AP). **Metode.** Mobilizacija je postignuta hemioterapijom/faktor-rasta-granulocitopoeze (G-CSF). Prikupljanje je izvedeno pomoću sistema Spectra-Optia-IDL. Ćelije SCs^{ish} determinisane su kao konstitutivni deo CD34^{+} u regiji-matičnih-ćelija („stem-cell-region“) upotrebom protočnog citometra FC-500. U ovoj studiji, originalni protokol „ISHAGE-sequential-gating” modifikovan je uvođenjem monoklonskog antitela anti-CD90-PE radi analize ekspresije antigena CD90 na ćelijama SC^{ish} (CD90^{+}SC^{ish}). Rezultati su prikazani kao procenat ćelija SC^{ish} u odnosu na broj nukleisanih ćelija, apsolutni broj SC^{ish} u μL PK ili AP, procenat ćelija CD90^{+}SC^{ish} izražen u odnosu na SC^{ish} i apsolutni broj CD90^{+}SC^{ish} u μL PK ili AP. **Rezultati**. Apsolutni broj ukupnih ćelija SC^{ish} i CD90^{+}SC^{ish} bio je značajno (p = 0,0007 i p = 0,0266) veći u uzorcima AP nasuprot PK. Indeks CD90^{+}SC^{ish}/ukupne SC^{ish} u uzorku PK je bio veći od indeksa u uzorku AP (p = 0,039). Relativna učestalost CD90^{+}SC^{ish} pokazala je vrlo značajnu inverznu korelaciju sa apsolutnim brojem ukupnih SC^{ish} u PK i AP (p = 0.0003 i p = 0,0013). Relativna učestalost ćelija CD90^{+}SC^{ish} u PK takođe je pokazala značajnu (p = 0,0002) inverznu korelaciju sa apsolutnim brojem ukupnih ćelija SC^{ish}. Bolesnici sa manje od 10% CD90^{+}SC^{ish} u PK su imali značajno (p = 0,0025) veći apsolutni broj ukupnih SC^{ish} ćelija u AP. **Zaključak**. Smatramo da niži prinos CD90^{+}SC^{ish} u AP nije prouzrokovan manje efikasnim prikupljanjem, već je najverovatnije posledica različitih, još uvek samo delimično razjašnjenih, citomorfoloških/biofizičkih osobina manje zrelih MĆ. Zato, posle mobilizacije hemoterapijom/G-CSF nameću se logična pitanja - da li bi trebalo pratiti apsolutni broj ukupnih SC^{ish} ćelija ili je celishodnije testirati relativnu učestalost CD90^{+}SC^{ish} pre sprovođenja afereznog prikupljanja MĆ. Za donošenje definitivnih zaključaka neophodna su buduća kontrolisana i sveobuhvatnija istraživanja MĆ, u vezi sa utvrđivanjem korelacije cirkulišućih i priku-pljenih ćelija sa hematopoetskim oporavkom bolesnika.

Ključne reči:
ćelije, matične; transplantacija hematopoeznih matičnih ćelija; kostna srž; citometrija, protočna; multipli mijelom; lečenje kombinovanjem antineoplastika, protokoli.

Introduction

The “cytopoiesis” – defined as *in vivo* cell development and expansion – is a multi-cyclic event in which a spectrum of mature cells is produced from a small number of stem-cells (SCs). SCs could be characterized as cells having well-balanced self-renewal, differentiation and proliferative capacity, as well as potential for plasticity, that is an ability to “switch” into other cell lineages. The SCs guarantee steady-state homeostasis in various “tissue-generating” (e.g. hematopoietic) systems [1, 2].

High-dose chemotherapy followed by allogeneic or autologous SC-transplants is considered as standard treatment for some malignant and few immune-mediated diseases (e.g. multiple sclerosis) [1–3]. The use of SCs for organ repair (damaged myocardium, liver, pancreas, etc) opens new perspectives in regenerative medicine [1, 3]. For transplants, bone marrow (BM) has been the primary SC-source, in which approximately 2–4% of total nucleated cells (TNCs) express the CD34 antigen [4, 5]. The CD34^{+} cells were recognized in peripheral blood (PB), but in extremely low ratio in the “steady-state” hematopoiesis: 0.01–0.05% of the TNCs [1, 4]. Mobilization by chemotherapy and recombinant human granulocyte-colony-stimulating factor (rHuG-CSF) radically increases the count of circulating CD34^{+} cells numbers in patients and healthy donors [1]. However, just a small fraction of double positive (CD45^{+}CD34^{+}) cells, with typical size and specific intracellular granularity/complexity – according to the International Society for Hematotherapy and Graft Engineering (ISHAGE) protocol – represents “true” SCs (or SC^{ish}) [6–10]. Moreover, immature or more primitive hematopoietic progenitors (PHPs) bare antigen CD90 (Thy-1), a 25 to 35 kDa molecule, which is also expressed by 1–4% of human fetal liver cells, umbilical cord blood (UCB), BM and several PB cells. PHPs are responsible for complete and long-term BM repopulation with durable or late hematopoietic reconstitution – and are limited within the immature CD34^{+}/CD90^{+} subset (or CD90^{+}SC^{ish}) [7, 10–12]. As confirmed, the CD34^{+}/CD90^{+} cells infused most appropriately predict platelet (Plt) recovery after the SC-transplants [11]. The CD34^{+}/CD90^{+} subset is also heterogeneous: evidently enriched in PHPs, but contains some less primitive lineage committed progenitors (LCPs). However, the majority of LCPs exist in an additional CD34$^{+/}$CD90^{-} (or CD90^{-}SC^{ish}) cell population [7].

Traditional sources of the SCs are the BM and PB. The UCB has been used as an alternative source since the late 1980s [1, 13]. Damages caused by the chemotherapy (applied prior to autologous SC-transplants) could be an important limiting factor of the SC-mobilization. Regularly, the count of total CD34^{+} cells in PB of healthy donors is higher than in mobilized non-Hodgkin lymphoma patients [12]. However, after mobilization in PB of these patients the CD34^{+} cell population is more immature, since they have a higher CD90 expression [12]. That could be a significant factor that influenced marrow repopulation, with special impact of late and durable hematopoietic reconstitution following SC-transplants [11]. This preclinical study aimed to evaluate absolute count of total SC^{ish} (including the CD90^{+}SC^{ish} and CD90^{-}SC^{ish} subsets) and relative frequency of CD90^{+}SC^{ish} in the PB, as predictive factors of the mobilization efficacy and of the apheresis product (AP) quality.

Methods

In this pilot study the importance of our own novel predictive factors of the efficacy of the SC mobilization (absolute count of total SC^{ish} and relative frequency of CD90^{+}SC^{ish}) using apheresis system Spectra-Optia IDL-system (Terumo-BCT, USA) were evaluated. Cell harvesting in a comparatively homogeneous (considering pre-transplant chemotherapy, mobilization protocol and conditioning regi-

men) category of multiple myeloma patients (n = 12) were performed. Patients were aged 26–62 years; male/female ratio was 1.3 : 1. The study was performed according to the guidelines of the Declaration of Helsinki Principles and Good Clinical Practice and was approved by the local institutional Ethic board.

Cell harvesting technology

Standardized apheresis procedures – processing two patients' total blood volumes with equal quantity (200 mL) of the AP – were performed. The mobilization protocol included chemotherapy (cyclophosphamide 2–4g/m^2 and etoposide 400–800 mg/m^2) with rHuG-CSF (12–16 μg/kgbm/day). Citrate-containing anticoagulant (Acid-Citrate-Dextrose, with 2.2% citrate concentration – ACD formula A, USP) was applied, at the same ACD : whole blood ratio (1 : 10) for all procedures. Additional patients' systemic or the AP heparinization was not performed. Vascular access was obtained across central venous catheter applied into subclavian or jugular and occasionally femoral veins. In this study, cell collections were performed when the absolute count of CD34$^+$ in the patient's PB was $\geq 19.4 \pm 5$ / μL and the relative frequency of CD90$^+$SC^{ish} was $\geq 9.3 \pm 12.2$%, respectively. The patients tolerated performed apheresis procedures well, without severe adverse effects. The adverse event of apheresis was considered as severe if it was life-threatening or leads to irreversible consequence with organ failure.

Cell quantifications techniques

The TNC, mononuclear cell (MNC), Plt and red blood cell (RBC) numbers in the patients' PB and/or AP samples were quantified using Advia-2120 blood counter (Bayer, Germany). The following monoclonal antibodies (mAbs) were used for the flow cytometric determination of CD45, CD34 and CD90 antigens/markers: anti-CD45-ECD, Immunotech, France; anti-CD34-FITC (class III antibody), BD Pharmingen, USA; anti-CD90-PE, Miltenyi Biotec GmbH, Germany). The samples were analyzed on FC-500 flow cytometer (Beckman-Coulter, FL, USA).

The SCs^{ish} were determined as a constitutional part of CD34$^+$ cells in the „stem cell-region" of the ISHAGE sequential gating protocol [5–9]. In our study, the original ISHAGE protocol was modified by introduction of anti-CD90-PE mAb into the analysis of CD90 expression on SC^{ish}. Briefly, the SC^{ish} were first gated on CD45 vs Side Scatter dot plot in order to separate the CD45$^+$ WBC from RBCs, Plts and other debris. From the primary gate on the CD45$^+$ events, the CD34$^+$ cells were identified on the CD34 *vs* Side Scatter dot plot by their expression of CD34 and characteristic light scatter properties. From the second gate on the CD34$^+$ events, the SC^{ish} were back-gated on CD45 vs Side Scatter dot plot in order to separate "true" CD34$^+$ or SC^{ish} with low CD45 fluorescence and low side scatter, from nonspecifically stained events – lymphocytes (CD45^{high}), monocytes (CD45^{high}) and higher Side Scatter) and granulocytes (high Side Scatter). In the next step, on the Forward Scatter vs. Side Scatter dot plot, gated on events with low CD45 expression and low side scatter, the SC^{ish} were identified by their size slightly larger than small lymphocytes and uniformly low side scatter. Finally, on the CD90 vs. Side Scatter dot plot, the selected „true" CD34$^+$ (SC^{ish}) cells were analyzed for CD90 expression. Cell viability was estimated on the basis of the 7-aminoaktinomicin D (7-AAD) flow cytometric assay (Immunotech, France) [5, 7, 9].

The results obtained in this study were presented as a percentage of the SC^{ish} *per* TNC count, absolute SC^{ish} count in μL of the PB and AP, percentage of the CD90$^+$SC^{ish} expressed in SC^{ish} and absolute CD90$^+$SC^{ish} count in μL of PB and AP (calculated on the basis of CD90$^+$SC^{ish} percentage).

For autologous SC-transplants, cryopreservation was performed according to our original five-step controlled-rate freezing protocol (with compensation of the released fusion heat), using dimethyl sulfoxide (DMSO; 10% final concentration) by Planer 560-16 equipment (Planer Products Ltd, UK), as it was earlier described [13–15].

Statistical analysis

Descriptive data of the SC investigations were expressed as the mean value ± standard error of mean (SEM) for each of the parameters examined. Statistical analyses were performed using GraphPad Prism 5 software. Differences were considered as statistically significant if *p* value was less than 0.05.

Results

Enrichment of total SC^{ish} (absolute count) and the CD90$^+$SC^{ish} (relative frequency) cells in the apheresis product

In this study, 12 patients subjected to autologous SC-transplant within the treatment of multiple myeloma were included. We initially assessed the quality of AP by comparing the absolute counts of targeted cells in the PB samples after mobilization with their yield in the AP. As expected, higher absolute count of total SC^{ish} in the AP compared with the PB samples with very high statistical significance (2487 ± 2678 *vs* 137.6 ± 129.7; $p = 0.0007$; Figure 1A) was found. Similarly, the absolute count of CD90$^+$SC^{ish} was also significantly higher in the AP (40.7 ± 42.8 *vs* 14.4 ± 6.7; $p = 0.0266$; Figure 1B).

The collection efficiency for the CD90$^+$SC^{ish} is lower than for the CD90$^-$SC^{ish} cells

Next, we assessed the harvesting efficiency of CD90$^+$SC^{ish} by comparing of the CD90$^+$SC^{ish} yield with the total SC^{ish} yield in the AP. With that purpose, we calculated the CD90$^+$SC^{ish} / total SC^{ish} index by dividing the absolute count of CD90$^+$SC^{ish} with the absolute count of total SC^{ish} and multiplied with 100, for both, the PB samples, as well as for the AP samples.

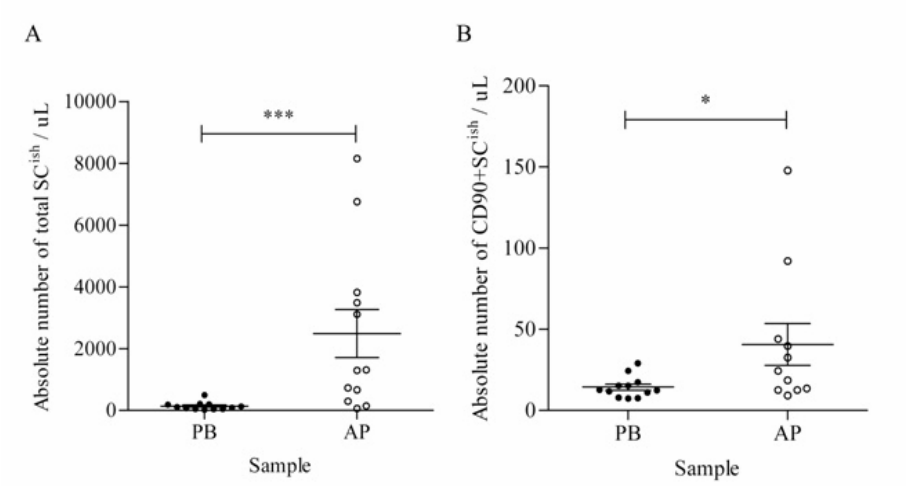

Fig. 1 – Comparison of (A) total SC^{ish} and (B) $CD90^{+}SC^{ish}$ absolute counts between peripheral blood (PB) and apheresis product (AP) samples showing significantly higher counts in the AP samples (*$p < 0.05$; *** $p < 0.001$, respectively). (mean ± SEM); Mann Whitney test).
SEM – standard error of the mean.

We found that, despite considerable positive correlation between these two indexes (Spearman r = 0.6503; p = 0.0221; Figure 2A), the $CD90^{+}SC^{ish}$/total SC^{ish} indexes from the PB samples were significantly higher compared to the indexes from the AP samples (18.4 ± 12.9 *vs* 9.3 ± 11.7; p = 0.0392; Figure 2B). This finding indicates much higher $CD90^{+}SC^{ish}$/total SC^{ish} proportion in the PB samples.

The relative frequency of $CD90^{+}SC^{ish}$ correlates inversely with total SC^{ish} in mobilized peripheral blood and apheresis product

The relative frequency of $CD90^{+}SC^{ish}$ showed highly significant inverse relationship with the absolute count of total SC^{ish} in both, the PB (Spearman's r = -0.8652; p = 0.0003; Figure 3A), and in the AP samples (Spearman r = -0.8140; p = 0.0013; Figure 3B).

Interestingly, the relative frequency of $CD90^{+}SC^{ish}$ from the PB samples also showed highly significant inverse relationship with the absolute counts of total SC^{ish} in the AP samples (Spearman's r = -0.8722; p = 0.0002; Figure 4A). In addition, we found that the patients with less than 10% of relative frequency of the $CD90^{+}SC^{ish}$ in PB, had significantly higher total SC^{ish} [since the higher appearance of mature SC^{ish} ($CD90^{-}SC^{ish}$) subset] in the AP when compared with the patients with more than 10% of $CD90^{+}SC^{ish}$ in their PB samples (5067 ± 2249 *vs* 644 ± 513.1; p = 0.0025; Figure 4B).

Discussion

Although significantly progress in conventional medications has improved the prognosis of hematological malignancies, SC-transplant remains the most effective approach in obtaining disease free long-term survival of patients.

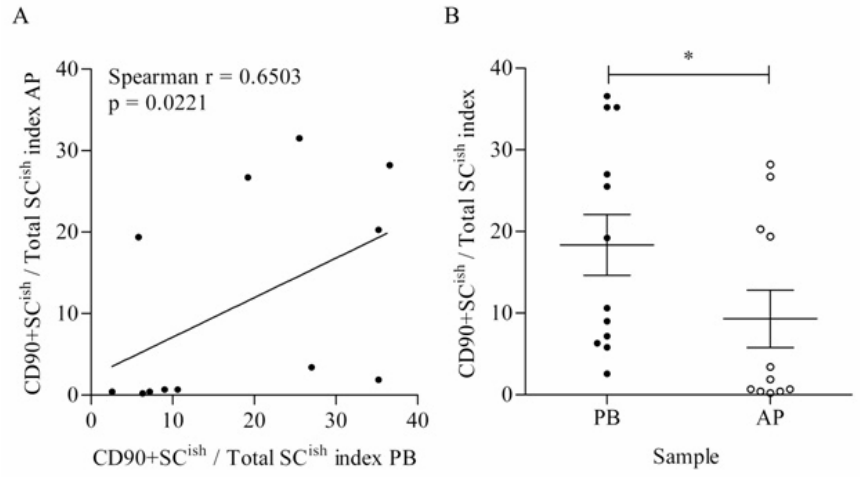

Fig. 2 – (A) Correlation of peripheral blood (PB) and apheresis product (AP) $CD90^{+}SC^{ish}$/total SC^{ish} indexes showing significant positive relationship (Spearman's correlation test); (B) Comparison between PB and AP $CD90^{+}SC^{ish}$/total SC^{ish} indexes showing significantly higher values in the PB samples (*$p < 0.05$; mean ± SEM; Mann Whitney test).
SEM – standard error of the mean.

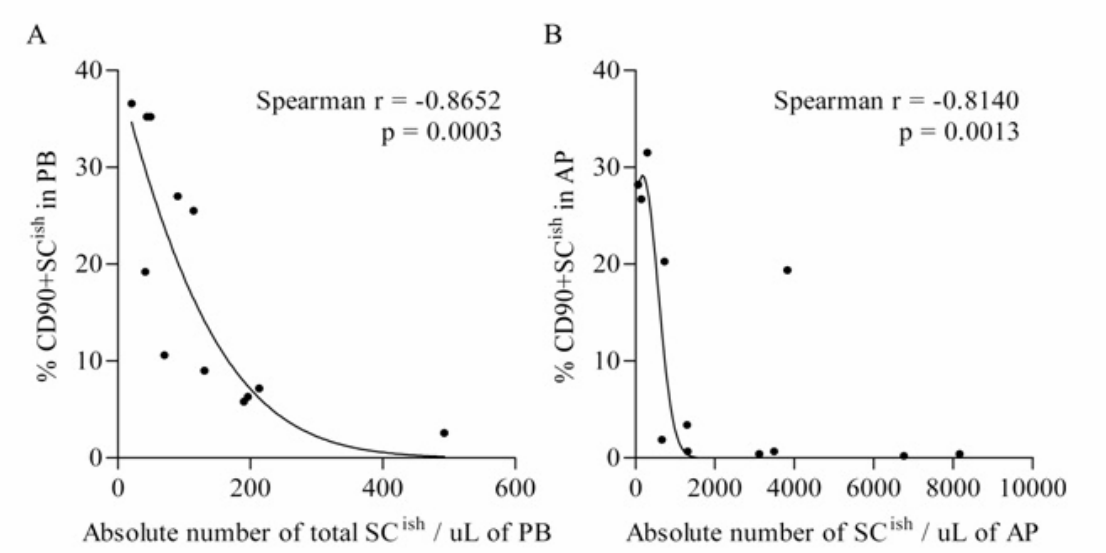

Fig. 3 – Correlation between relative frequency of $CD90^{+}SC^{ish}$ and absolute counts of total SC^{ish} in (A) peripheral blood (PB) and (B) apheresis product (AP) samples showing significant inverse relationship (Spearman's correlation test).

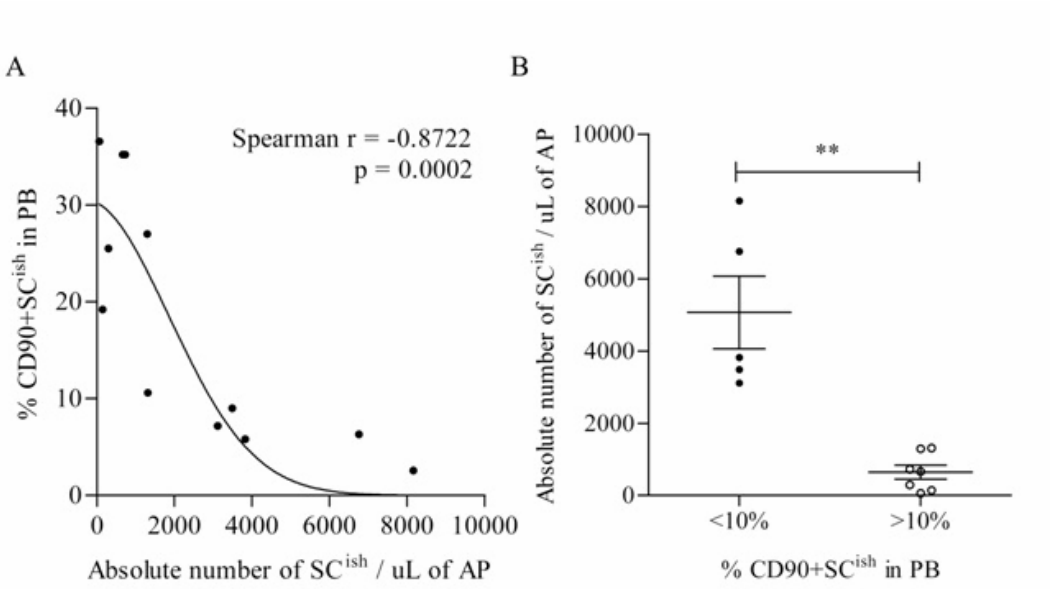

Fig. 4 – (A) Correlation between relative frequency of $CD90^{+}SC^{ish}$ and absolute counts of total SC^{ish} in apheresis product (AP) samples showing significant inverse relationship (Spearman's correlation test). (B) Comparison of the SC^{ish} absolute counts in the AP samples between the patients with less than 10% relative frequency of the $CD90^{+}SC^{ish}$ and patients with more than 10% of the $CD90^{+}SC^{ish}$ in their peripheral blood (PB) samples, showing significantly higher SC^{ish} absolute counts in the group of patients with less than 10% of $CD90^{+}SC^{ish}$ in PB ($p < 0.01$; mean ± SEM; Mann Whitney test). SEM – standard error of the mean.**

However, high-dose treatment incorporates risks of conditioning-regimen related morbidity/mortality; accordingly it is limited for relatively younger patients in better clinical condition. This age limitation is regrettable since SC-transplant candidates in multiple myeloma are frequently older than 60 or even 65 years [1].

The earliest SC collections from PB were accomplished in "steady state hematopoiesis" – by numerous procedures since late 1970s, and following cryopreservation was needed [1, 2, 16]. The cell harvesting is the same for allogeneic donors as for autologous patients. Vascular access, as mentioned, is regularly realized using central/venous (jugular, subclavian or femoral) catheter.

In the course of cell harvesting, selection of the best collection system and determination of optimal timing for apheresis, are the most critical events. The most recent SC software design incorporates the Intermediate density layer (IDL) system using Spectra-Optia device [1].

There are several advantages of PB as a source of the SCs as compared to the BM: absence of general anesthesia and multiple bone aspirations, higher $CD34^{+}$ yield in the AP and earlier hematopoietic reconstitution, as well as shortened

hospital stays and reduced transplant related morbidity. Commonly, the SC-engraftment is defined as the first of three days with neutrophil count greater than 0.5×10^9/L [1] and Plt count exceeding 20×10^9/L (without transfusion support for 7 consecutive days) [17].

Due to the reasons mentioned above, the number of patients treated by PB derived allogeneic, especially autologous SC-transplants is increasing worldwide [1, 2]. Nowadays, the PB derived SCs are used for approximately 80% of allogeneic and practically for all autologous SC-transplants [1, 4].

Enumerated $CD34^+$ cell dose by flow cytometry is routinely performed to optimize timing of the PB stem cell collections and assess engraftment potential of the AP. Moreover, the immature $CD34^+/CD90^+$ cells or PHPs have a capacity to initiate long-term hematopoiesis *ex vivo*, and according to some data, they are mobilized into blood to a maximum level a few days earlier than the peak mobilization of the total $CD34^+$ cells [18]. As mentioned, the $CD34^+/CD90^+$ cells in the AP were a better predictor of Plt recovery than the total dose of the $CD34^+$ cells, and $80x10^4$/kg of the $CD34^+/CD90^+$ cells is the minimal essential dose capable of durable long-term engraftment [11]. Therefore, the measurement of the $CD34^+/CD90^+$ cells is helpful for the evaluation of the grafts quality in the PB autologous SC-transplant.

There is an increasing interest in evaluation of the responsibility of the $CD34^+$ subsets for complete and long-term repopulation, as a marker of cell harvest quality [1, 10–12]. In our initial clinical study, as a possible and potentially more objective collection predictor, the $CD34^+/CD90^+$ subset evaluation was also recommended [19]. Precisely, on the basis of examination of the immature SC antigens and 7-amino-actinomycin D (7-AAD) viability, we suggested that the $CD34^+/CD33^-$, $CD34^+/CD38^-$, $CD34^+/DR^-$, $CD34^+/CD90^+$ cells could be superior predictive factor over circulating the total $CD34^+$ count for an optimized collection-timing and outcome of autologous SC-transplant [19]. Other authors also confirmed that engraftment and hematopoietic recovery are not necessarily associated with cell dose of the total $CD34^+$ cells [10]. Thus, the $CD34^+/CD90^+$ cells could be a useful quality marker for the AP [10, 19], especially for prediction of the Plt recovery (reduced hazard of the prolonged thrombocytopenia) [11]. Consequently, the $CD34^+/CD90^+$ cells could be a practical predictive factor for complete and durable hematopoietic reconstitution [10, 11]. The quantity of the mature $CD34^+$ subsets in the AP correlates obviously with engraftment rapidity [1, 2]. However, data concerning the potential of the immature $CD34^+$ subsets (such as $CD90^+SC^{ish}$) for long-term marrow repopulation are still not completely clarified.

Our previous preclinical research (using standardized cell harvesting protocols) confirmed that Spectra-Optia resulted with superior collection efficiency compared to Cobe-Spectra for the $CD34^+$ cells ($CE2[\%]_{CD34^+}$) [20]. The $CE2[\%]_{CD34^+}$ was calculated on the basis of the $CD34^+$ count in the AP versus their number in processed whole blood [20]. We estimated that the "predictive-value" of circulating total $CD34^+$ cells for the SC harvesting could be enhanced or improved by determination of the relative frequency of $CD90^+SC^{ish}$ in patients' PB.

In this study the relative frequency of $CD90^+SC^{ish}$ demonstrated inverse correlation with the absolute count of total SC^{ish} in both, the PB and AP. Accordingly, on the basis of the inverse correlation between the PB and the AP counts of $CD90^+SC^{ish}$ observed in present study, it is possible that the level of the collection efficiency of both existing (Cobe-Spectra and Spectra-Optia) apheresis systems is inferior for the immature ($CE[\%]_{CD90+SC^{ish}}$) as compared to the mature ($CE2[\%]_{CD34^+}$) cells from mobilized PB. However, we believe that the lower $CD90^+SC^{ish}$ yield is not a consequence of inferior $CE[\%]_{CD90+SC^{ish}}$, but possibly a result of several still not fully resolved immature SC features, such as cytomorphological and biophysical (intracellular granulation, cell-density, etc) parameters. Using the „dye-efflux" method for SC-sorting, Radley et al. [21] defined more precisely ultrastructural characteristics of the most primitive SCs in murine BM and provided a basis for studying the structural changes that occur with progressive activation and differentiation of immature hematopoietic SCs (HSCs) toward their progeny of lesser proliferative capacity. The structural changes could likely have effect on the density and consequently "centrifugation-properties" of these cells. Sharma et al. [22] showed that the $CD90^+$ cells were predominantly found within the subpopulation of the SCs with low CD34 and very low CD45 antigen expression, in the patients with hematological malignancies. They also showed that those $CD90^+CD34^{dim}CD45^{very\ dim}$ cells were the smallest among all other examined subpopulations (including $CD133^+$ and $CD117^+$ CSs with the different CD45 antigen expression) with an electronic volume of 87.1–143.7 μm^3 corresponding to a diameter of 5.5–6.5 μm. In the review article, Kucia et al. [23] postulate that the fraction of the SCs, small in their size, could be easily lost during collection/isolation protocols based on gradient or centrifugation. Our finding of significantly lower enrichment index for $CD90^+SC^{ish}$, compared with enrichment index of the total SC^{ish}, is in accordance with the aforementioned earlier investigations.

In this study an intriguing observation is the inverse relationship between relative frequencies of the $CD90^+SC^{ish}$ and absolute counts of the total SC^{ish} in PB, suggesting that continual increase in absolute counts of the total SC^{ish}, during the G-CSF-induced mobilization, is not followed by an appropriate increase in the $CD90^+$ cells. The G-CSF acting – either as an endogenously produced after chemotherapy or as exogenous or administered factor (rHuG-CSF), or a combination of both – is considered to be an initial signal for HSC mobilization, explained by several proposed pathways [24]. Nevertheless, there are data describing the superior effects of some new agents, such as mozobil or plerixafor (which is an antagonist of the alpha chemokine receptor CXCR4) in mobilizing the immature HSC, capable for long-term repopulation (LT-HSC) [25].

Conclusion

We speculate that the inferior $CD90^+SC^{ish}$ yield in the AP is not a consequence of the inferior collection efficacy – but most likely a result of several still not fully understo-

od/clarified immature SC cytomorphological and/or biophysical features. Thus, when the chemotherapy/G-CSF is used for mobilization, there are some logical questions to ask- whether we should follow the absolute count of the total SC^{ish}, or, whether we should test for relative frequency of $CD90^{+}SC^{ish}$ prior to apheresis.

To reach the final conclusions, it is essential to conduct further controlled and larger SC-investigations (our clinical study has been already initiated) concerning the correlation between circulating and harvested SCs, and patients' hematopoietic recovery.

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CASE REPORTS

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Isolated metastasis of lung cancer to carpal bones

Izolovana metastaza karcinoma pluća u kostima ručja

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Abstract

Introduction. Lung cancer often gives metastases in the bone system, of which the wrist accounts for 0.1% and the bones of the wrist are primarily affected in only 17% of cases. We presented a patient with the delayed diagnosis and a rare localization of isolated metastases of lung cancer to carpal bones which ended with upper arm amputation. **Case report.** A 56-year-old, a laborer, healthy, smoker, coughing for decades with occasional expectoration, hoarseness, during the last 3 months felt pain in his right wrist. He denied trauma. Physical examination led to the diagnosis of tendovaginitis of the hand. He visited a physiatrist and began treatment. After the therapy, symptoms were partially decreased and later began to worsen with symptoms of the median nerve compression. The neurologist diagnosed it as the carpal tunnel syndrome. The patient's condition worsened and he was sent to the Emergency Center of the Clinical Center of Vojvodina, Novi Sad, Serbia with the diagnosis of arthritis of the wrist. The final diagnosis of lung adenocarcinoma with isolated metastasis to bone tissue was made with a biopsy of the tumor and examination by an oncologist. Primary tumor localization was diagnosed with a computed tomography (CT) scan and skeletal scintigraphy. The patient underwent upper arm amputation and was sent to an oncologist. **Conclusion.** Carefully taken anamnesis, detailed general and local examination, and frequent monitoring of patients could help make a correct diagnosis of this rare localization of the lung cancer, before the spreading process and the occurrence of severe complications.

Key words:
bone neoplasms; neoplasm metastasis wrist joint; carpal bones; lung neoplasms; diagnosis; treatment outcome.

Apstrakt

Uvod. Karcinom pluća obično daje metastaze u skeletni sistem, od kojih 0,1% u predeo ručnog zgloba, a od tog broja samo 17% otpada na kosti ručja. Prikazan je bolesnik sa odloženom dijagnozom izolovane metastaze karcinoma pluća retke lokalizacije u kostima ručja, sa nadlakatnom amputacijom kao krajnjim ishodom. **Prikaz bolesnika.** Bolesnik starosti 56 godina, fizički radnik, zdrav, "teški pušač", sa kašljem tokom decenija uz povremeno iskašljavanje i promuklost, poslednja tri meseca žalio se na bol u desnom ručnom zglobu. Bolesnik je negirao traumu. Na osnovu kliničkog pregleda postavljena je dijagnoza zapaljenja tetiva šake (dijagnoza fizijatra) i odmah je započeta fizikalna terapija. Nakon završetka fizikalne terapije, simptomi su se delimično smirili, ali je ubrzo došlo do pogoršanja stanja u smislu pojave simptoma kompresije medijalnog živca (*nervus medijanus*). Bolesnik je poslat na dalje lečenje neurologu, koji je postavio dijagnozu sindroma karpalnog tunela. Stanje bolesnika se pogoršavalo zbog čega je upućen u Urgentni centar Kliničkog Centra Vojvodine, Novi Sad, sa uputnom dijagnozom zapaljenja ručnog zgloba. Konačna dijagnoza izolovane metastaze adenokarcinoma pluća postavljena je biopsijom i pregledom onkologa. Primarna lokalizacija tumora pluća dijagnostikovana je kompjuterizovanom tomografijom i scintigrafijom skeleta. Načinjena je visoka amputacija ruke i bolesnik je poslat na dalje lečenje onkologu. **Zaključak.** Pažljivo uzeta anamneza, detaljan opšti i lokalni klinički pregled i učestalije kontrole bolesnika mogu pomoći u postavljanju tačne dijagnoze tumora pluća, pre širenja procesa i nastanka težih komplikacija.

Ključne reči:
kosti, neoplazme; neoplasme, metastaze ručje, zglob; karpusne kosti; pluća, neoplazme; dijagnoza; lečenje, ishod.

Introduction

Lung cancer gives metastases in the bone system, of which the wrist accounts for 0.1% [1]. In only a few cases metastasis of lung cancer in the bones of the wrist was presented as the first sign of the disease and the only metastasis [2–5]. Initial infiltration of semilunar bone was shown in only one case [6]. First signs are related to other more common pathologies of the hands [7]. Lost time until the diagnosis is made is significant [2–6]. We reported a patient with an undiagnosed,

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rare localization of isolated metastases of lung cancer to carpal bones which ended with upper arm amputation.

Case report

A 56-year-old man, a laborer, heavy smoker, last 3 months felt pain in his right wrist. Firstly, he visited a physiatrist who began treatment, with the diagnosis tendovaginitis of hand and fingers. Symptoms were initially decreased and then began to worsen. Radicular symptomatology of the median nerve appeared and then a neurologist was included. The pain became stronger, redness and swelling of the wrist appeared. After re-examining, the bacterial arthritis of the wrist was suspected, and the patient was sent to the Emergency Center of the Clinical Center of Vojvodina, Novi Sad, Serbia.

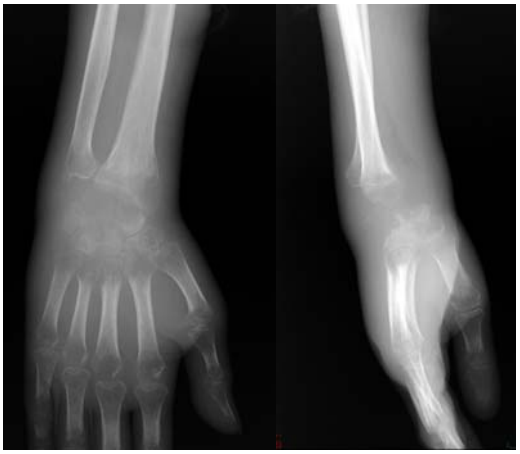
Clinical examination of the patient showed a local swelling and hyperemia of the wrist. On neurological examination, there was a partial loss of motor functions of the median nerve.

Native X-ray of the wrist (Figure 1), showed a marked osteoporosis of carpal and metacarpal bones with almost complete destruction of the upper row of carpal bones (most prominent in semilunar and navicular bone) and with a volar subluxation of the wrist. X-ray of lungs was in the referent range. Computed tomography (CT) scan of the wrist was not made initially.

The laboratory findings showed hyperkalemia [5.6 mmol/L normal range (NR) 3.5–5.5 mmol/L], hyperglycemia [7.6 mmol/L (NR 3.3–5.5 mmol/L)] and increased values of C-reactive protein (CRP) – 59.6 mg/L (NR < 8 mg/L). Kidney and liver function were preserved, as well as the hemostatic mechanism of the blood.

After a short preparation of the patient, the wrist incision was done. Expected pus was not obtained but there was gray-lardy tissue that permeated the wrist and carpal bones with their destruction. A biopsy of the tumor was done with external fixation of hand and forearm (Figure 2).

The CT scan of the chest demonstrated irregular nodular lesions in the right lung (Figure 3A and B) and a subpleural nodule in the S6 segment of the left lung, (Figure 3C and D). Isolated lymph nodes were seen in mediastinum enlarged to 20 mm. Skeletal scintigraphy of the whole body showed



A) B)

Fig. 1 – X-ray the right wrist and forearm: A) Anteroposterior view – diffuse osteoporosis bones of the hand and distal forearm. Bones of the wrist infiltrated, almost completaly destruction of the navicular and semilunar bone. Capitate bone partially destructed; B) Lateral view – volar subluxation of the wrist.

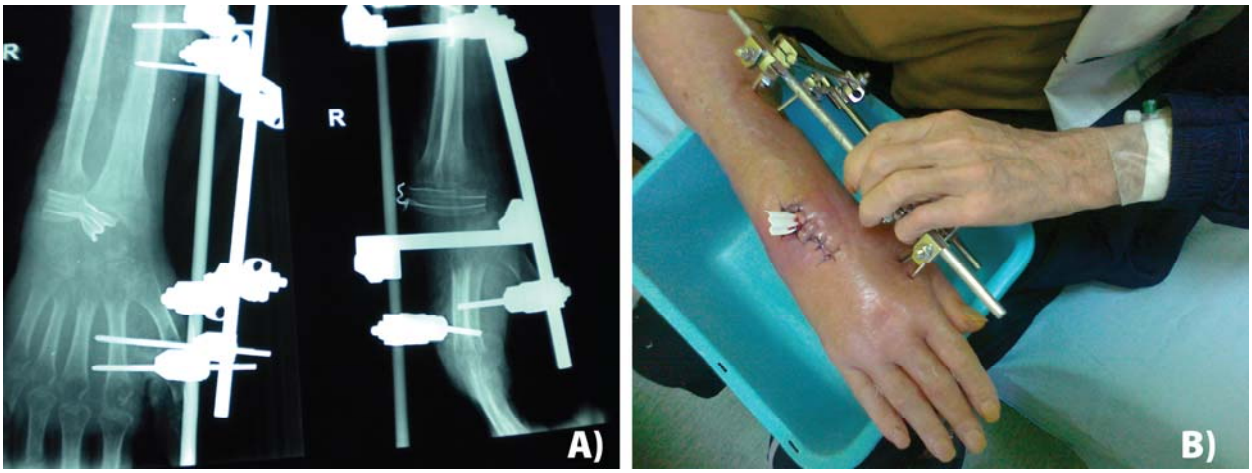


Fig. 2 – A) Postoperative anteroposterior and lateral radiographies of the right wrist show devastated wrist, passive rubber drain is situated in the tumor tissue. Wrist is stabilased with external fixation; B) Postoperative local clinical picture shows swelling of the hand and wrist with hyperemia, minimal bleeding around the drainage.

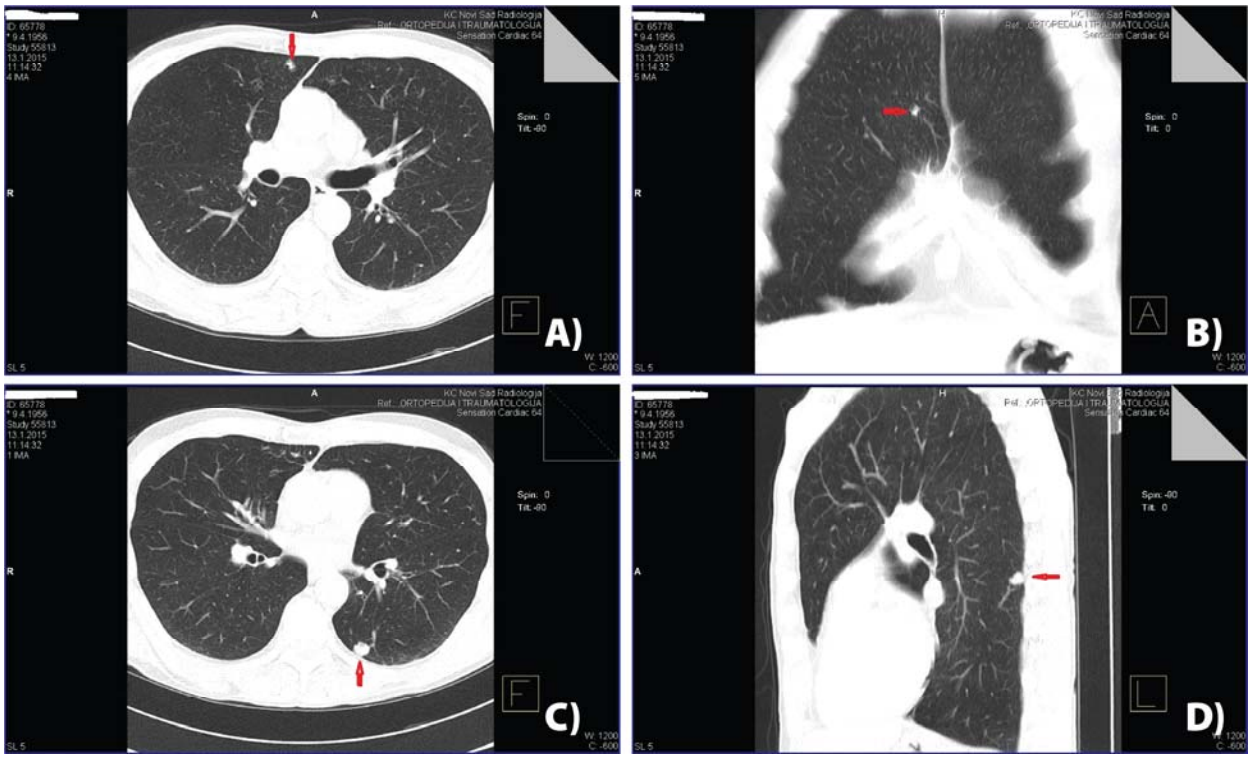


Fig. 3 – Computed tomography (CT) appearance of tumors in the lungs. No invasion of the bronchi, main blood vessels nor bone structures. No effusion in the pleural space.
A) and B) – Axial and frontal appearance of the tumor in the S3 segment of the right lung, dimensions 8 mm, partialy calcificated (see the arrow under A).
C) and D) – Axial and frontal appearance of the tumor in the S6 segment of the left lung, subpleural situated, dimensions 13 × 12 mm (arrow).

diffusely increased bone metabolic activity in the region of the right wrist. Other scintigraphy was normal. After diagnostic procedures, the patient was sent to an oncologist.

Histopathological findings of the tumor mass from the wrist (Figure 4) showed fragments of bone tissue with the tumor. The tumor cells were cylindrical, with moderately vesicular nuclei and many visible mitoses. The diagnosis of adenocarcinoma with metastasis to bone tissue was initially made by the pathologist and a definitive diagnosis of primary lung carcinoma was set up by the oncologist, based on the anamnesis, clinical examination, radiological and histopathological findings.

Before the treatment of cancer, there was an acceleration of tumor growth in the carpal region with the deteriorati-

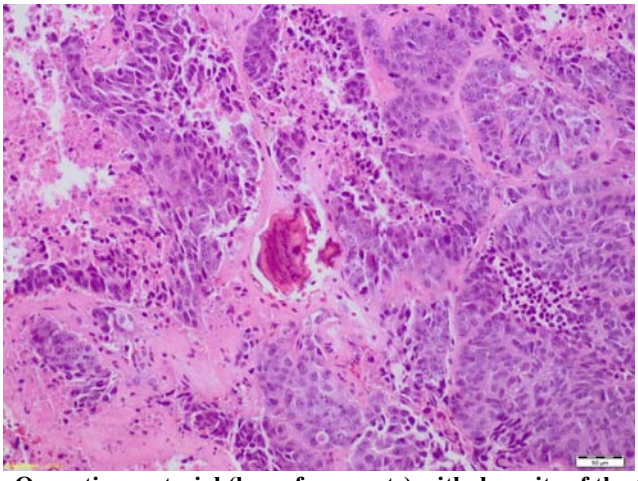


Fig. 4 – Operative material (bone fragments) with deposits of the tumor.
Tumor tissue is arranged into solid groups and small adenoid structures. Tumor cells have polygonal and cylindrical shape, with large nuclei with visible abundant mitotic figures. On immunohistochemical analysis tumor cells are cytokeratin AE1/AE3 (CKAE1/AE3) positive and thyroid transcription factor-1 (TTF1) positive. The diagnosis of metastatic adenocarcinoma was made, with most probable primary lung tumor.

on of the general condition of the patient (Figure 5). After removal of the external fixation and partial healing of wounds from the biopsy, an attempt to perform magnetic resonance imaging (MRI) of the hand and forearm was made to evaluate the extent of changes, but the patient could not withstand the procedure. The patient underwent upper arm amputation (Figure 6A and B) and his further treatment was continued by the oncologist.

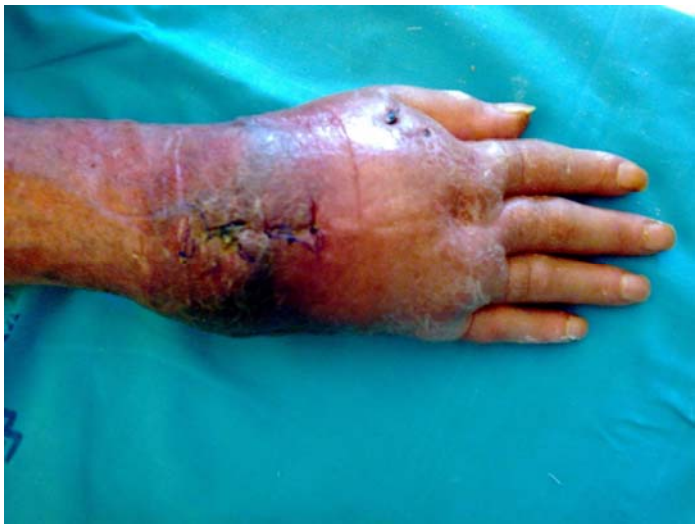


Fig. 5 – Local finding after removal of the external fixation, two weeks after the biopsy, shows accelerated growth of tumor, skin necrosis, and exulceration to place the incision.

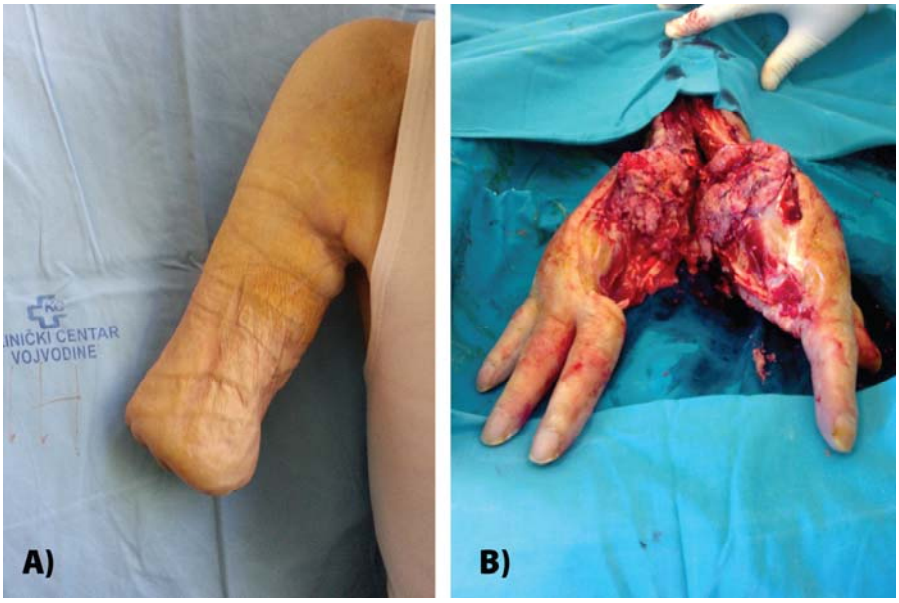


Fig. 6 – A) Upper arm amputation; B) Sagital section through an amputated hand shows completelly destruction of the carpal bones and distal radius and ulna. The tumor penetrates skin on the dorsum of the wrist.

Discussion

The primary metastases to the hand are extremely rare and include 0.1% of all bone metastases [2]. They are most frequently produced by carcinomas of the lung, breast, and kidney from which the metastasis of lung cancer is present in about half of the cases [8]. Handley [9] first described metastasis of the breast cancer in the metacarpal bone. Flynn et al. [10] showed at 257 cases that the most commonly affected was the third finger of the dominant hand, then thumb and other fingers. Hsu et al. [7] state that the carpal bones metastases are present in 17% of all cases of bone metastases in the hand.

Cancer metastasis to trapezium was described by Asencio et al. [2], Rinonapoli et al. [3], Lederer et al. [4], Song and Yao [5] and Gaston et al. [11]. Nissenbaum et al. [12] showed metastasis of the lung cancer in the hamate bone. Metastasis of the lung cancer in the scaphoid bone was shown earlier by Ioia et al. [13]. According to available literature, isolated metastatic bronchial carcinoma invading the lunate bone was described only by Abrahams [6]. He described the lytic changes of the ulnar part of semilunar bone and loss of bone mass in a 59-year-old female patient with a quarterly pain wrist. A biopsy of

the bone confirmed that this was a metastatic squamous cell carcinoma of the lung.

The time from the onset of symptoms and signs, and the diagnosis can be a few weeks, months to a year or longer [3, 4, 7]. In our case, time lost was about three months.

Craigen et al. [14] describe the metastasis of gastric cancer in the hamate bone of dominant right hand, while Flynn et al. [10] showed at 257 cases that metastases most commonly affected the third finger of the dominant hand, then the thumb and other fingers. The unilateral lesion in their study was present in 74% of cases. In our case the right, dominant hand was also involved.

Smoking and alcohol abuse, as bad predictor factors, was pointed out in earlier studies [3, 4]. Our patient was a decades-long heavy smoker.

After the X-ray confirmation of the destruction or loss of subchondral bone mass of the carpal bones, open, or CT-guided aspiration biopsy with pathohistological verification has been done in many studies [4, 10, 13]. In our case, neither a physician nor a neurologist did a radiography of the hand, which was a diagnostic failure. We suppose that some radiographic changes of carpal bones could be observed earlier if the X-rays were done in a timely manner. Our patient did not lose weight, unlike the Rinonapoli et al. [3] case, so we did not suspect a tumor growth. The general state of the patient's health, laboratory findings and clinal examination pointed to the inflammatory process in the wrist. We assumed that neurological symptoms were the consequence of the local spread of infection and joint capsule stretching. Local, general and laboratory signs of inflammation with strong pain and carpal instability indicated decompression, toilette, and stabilization of the wrist [15]. X-ray of the wrist showed most prominent destruction in semilunar and navicular bone and because of that, we assumed that primary affection of tumor cells was there.

Changes in both lungs were observed by CT examinations of the chest and abdomen. CT scan of the brain was not done because the patient showed no neurological deficits. Although expecting a larger number of bone metastases, bone scintigraphy showed changes only in the region of the right wrist, indicating that it was a single metastasis of lung cancer. Involvement of carpal bones as the primary sign of malignant disease, except in Rinonapoli et al. [3] report, was described by Lederer et al. [4] and Song and Jao [5]. The most recent, metastasis of lung cancer in the hand, but in the distal phalanx of the finger before the diagnosis of lung cancer, was described by Unsal et al. [16].

Radiation therapy, as the only treatment method, is described in the work of Flynn et al. [13]. They showed metastasis of lung cancer in the wrist as a part of multiple skeletal metastases in the 78-year-old female patient. Bone resection with capsule interposition in place of excision as a way of treating the initial trapezium destruction by lung cancer without metastases spread into surrounding soft tissue of the joint was described by Gaston at al. [11]. Amputation of the hand in the 37-year-old patient to reduce pain due to metastases in hamate and capitate bone was described by Craigen and Chesney [14], while forearm amputation due to isolated metastasis of lung cancer in trapezoidal, trapezium and scaphoid bone, in the 74-years-old male patient described Rinonapoli et al. [3]. They conclud that earlier diagnosis would allow more therapeutic choices. In our case, there was a massive extension of the tumor outside the carpal bones into the subcutaneous tissue where tumor exulceration occurred, so we could not consider the reconstructive surgery. Amputation remained the only surgical option. As the local findings in our patient rapidly deteriorated in terms of rapid acceleration of tumor growth, bearing in mind the good general condition, we decided to take the upper arm amputation. Survival in patients with metastatic carcinoma of the carpus is between four months and one year [3, 4, 13, 14]. As the wrist represents a rare place of primary tumor manifestations, pain is often attributed to benign changes such as tenosynovitis and entensitis, especially when there are nonspecific laboratory findings, and initial radiography shows no osteolytic changes. The patient is often treated by a physiatrist, thus losing precious time for the diagnosis and therapy. After a certain time of unsuccessful therapy, the attention is turned to more detailed diagnostics.

The described case was not easy to diagnose because all signs of the disease were general as well as the rarity of the carpal bone metastasis.

Conclusion

Every long-lasting pain and swelling in the hand require a special attention of the physician. Setting up an early accurate diagnosis, before the spreading of the possible malignant tumor process in the wrist gives time for the adequate surgical intervention and allows the efficient total treatment of the patients avoiding serious complications such as upper arm amputation, which was shown in this case report.

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Bisphosphonate related osteonecrosis of the maxilla – A case report

Bisfosfonatima uzrokovana osteonekroza gornje vilice

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Abstract

Introduction. Bisphosphonates are a group of medications which have an important role in the treatment of some bone diseases. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a side effect of intravenous bisphosphonate therapy. The mechanism of action by which they may cause osteonecrosis is questionable. BRONJ is defined by the American Association of Oral and Maxillofacial Surgeons (AAOMS) and classified into four stages (0-3). Treatment of BRONJ depends on the stage of disease and includes conservative treatment (stage 0 and 1) and surgical treatment (surgical debridement in stage 2 and sequestrectomy in stage 3). **Case report.** We presented a patient who had breast cancer, with stage 3 of bisphosphonate-related osteonecrosis of the upper jaw after zoledronic acid therapy for diffuse metastasis of the vertebrae. Before the treatment with zoledronic acid the patient was treated by a dentist. The osteonecrosis of the upper jaw started a year and a half after the start of zoledronic acid therapy and after tooth 24 extraction. She was treated by an oral surgeon at the beginning according to the protocol of AAOMS. The patient was sent to a maxillofacial surgeon due to the disease progression, and after computed tomography (CT) examination resection of the upper jaw was done. **Conclusion.** BRONJ is a condition with the specific clinical presentation, and it can be very serious for the patient, therefore it is necessary to emphasize the importance of screening. The doctors in different specialties (oncologist, dentist, oral surgeon and maxillofacial surgeon) must cooperate and control the patients under treatment with bisphosphonates before the therapy starts, as well as during and after it, in order to prevent, recognize on time and treat properly this complication.

Key words:
bisphosphonate-associated osteonecrosis of the jaws; diagnosis; drug therapy; oral surgical procedures; treatment outcome.

Apstrakt

Uvod. Bisfosfonati spadaju u grupu lekova koja ima značajno mesto u lečenju nekih bolesti kostiju. Bisfosfonatima uzrokovana osteonekroza vilica (BRONJ) je retka komplikacija intravenske bisfosfonatne terapije. Mehanizam kojim bisfosfonati uzrokuju osteonekrozu još uvek nije u potpunosti razjašnjen. BRONJ je definisan od strane Američkog udruženja maksilofacijalnih i oralnih hirurga (AAOMC) i klasifikovan u četiri stadijuma (0-3). Tretman zavisi od stadijuma bolesti i podrazumeva konzervativni tretman (u stadijumima 0 i 1), hirurški debridman (u stadijumu 2) i sekvestrektomiju (u stadijumu 3). **Prikaz bolesnika**. U radu je prikazana bolesnica kojoj je operisan karcinom dojke, u fazi 3 BRONJ-a gornje vilice nakon terapije zolendroičnom kiselinom zbog difuznih metastaza na kičmenim pršljenovima. Pre početka terapije bisfosfonatima bolesnica je bila podvrgnuta stomatološkom tretmanu. Osteonekroza gornje vilice počela je godinu i po dana nakon početka terapije bisfosfonatima, a nakon ekstrakcije zuba 24. Bolesnica je na početku bila lečena od strane oralnog hirurga prema protokolu AAOMS. Zbog dalje progresije bolesti bolesnica je upućena maksilofacijalnom hirurgu. Nakon sprovedene dijagnostike kompjuterizovanom tomografijom, isplanirana je resekcija gornje vilice. **Zaključak.** BRONJ je stanje sa specifičnom kliničkom slikom, koje može imati ozbiljne posledice za bolesnike zbog čega se mora istaći značaj *screening*-a. Lekari različitih specijalnosti (onkolog, stomatolog, oralni i maksilofacijalni hirurg) moraju sarađivati i kontrolisati bolesnike koji su na terapiji bisfosfonatima, kako pre, tako tokom i nakon terapije, u cilju sprečavanja, blagovremenog prepoznavanja i pravilnog lečenja ove komplikacije.

Ključne reči:
osteonekroza vilica, uzrokovana bisfosfonatima; dijagnoza; lečenje lekovima; hirurgija, oralna, procedure; lečenje, ishod.

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Introduction

Bisphosphonates are a group of antiresorptive medications that act specifically on osteoclasts. They have an important role in maintaining bone density and strength in some bone diseases (osteoporosis, Paget's disease, multiple myeloma, bone metastasis of a malignant tumor). Bisphosphonate-related osteonecrosis of the jaw (BRONJ) presents avascular osteonecrosis of the jaw. It was first time described in 2003 by Marx [1] who reported series of 36 cases of BRONJ in patients with malignant tumors and later in 2004 Ruggerio et al. [2] described series of 63 cases in which most of the patients were cancer patients. Case studies and reviews have been reported since, and treatment guidelines have been published.

The mechanism of action by which bisphosphonates may cause osteonecrosis of the jaw is questionable: it can include bisphosphonate-related apoptosis of osteoclasts, antiangiogenic effect and toxic effect. It is considered that the bone remodeling is depressed, which is the cause of poor healing of post-extraction wounds [3]. Some authors consider that other cancer therapies medications (steroids, chemotherapeutic agents) can influence the development of BRONJ. The global incidence of BRONJ is 0.94% [3, 4].

The American Association of Oral and Maxillofacial Surgeons (AAOMS) defined BRONJ and it is considered that the patient has BRONJ if following characteristics are present [5]: current or previous treatment with antiresorptive or antiangiogenic agents; exposed bone or bone that can be probed through an intraoral or extraoral fistula in maxillofacial region that persists more than 8 weeks; no history of radiation therapy to the jaws or obvious metastatic disease to the jaws.

The categorization of BRONJ into 4 stages (0-III) was accepted in 2009 [5]: Stage 0 – (none-exposed bone). In this stage, patients have no clinical evidence of necrotic bone but have symptoms like: odontalgia nonodontogenic origin, pain in the body of the mandible or in maxillary sinus, and disturbance of neurosensory function. Clinical findings include: loosening of teeth not caused by chronic periodontal disease and periapical fistula not associated with pulpal necrosis. Radiologically alveolar bone loss or resorption, regions of osteosclerosis and thickening of the periodontal ligament can be present; Stage 1 – Patients in this stage have exposed or necrotic bone or fistula, but they are asymptomatic and there is no evidence of infection; Stage 2 – Patients in this stage have the exposed or necrotic bone or fistula with the evidence of infection; Stage 3 – This stage includes patients with the exposed and necrotic bone, with extending of bone exposition beyond the region of the alveolar bone, with the existance of pathologic fracture, extra-oral fistula, oroantral or oronasal communication and osteolysis.

Surgical treatment is recommended only for the stage III.

In this case report, we presented a case of BRONJ advances of the upper jaw. There is no standard therapy in such advanced cases which require a partial osteotomy of the jaw, like in our case.

Case report

A 56-year-old female underwent total mastectomy on the left for the breast cancer (ductal carcinoma) in June 2005. After the surgery, she received a chemotherapy according to the breast cancer therapy protocol: fluorouracil, epirubicin and cyclophosphamide given 3-weekly for 6 cycles. She was operated in September 2007 due to local recurrence. Since 2005 she was treated with hormonotherapy: tamoxifen until November 2007 and anastrozole until November 2007 further. She also underwent laparoscopic ovariectomy in November 2007. Diffuse metastases of the vertebrae in the thoracic and lumbar part were diagnosed after ovariectomy in November 2007. At the same time, she started intravenous zoledronic acid (Zometa®; Novartis) 4 mg every 28 days. Before the start of zoledronic acid treatment the patient had been treated by the dentist and for repairing all carious teeth. The patient was regularly checked by an oncologist and physiatrist, the bone scintigraphy and bone densitometry (DEXA) test were done and they indicated stable findings on the bones. In March 2009 the patient felt pain in the region of upper left first premolar; an oncologist did not advise dental treatment, after 6 months the tooth was extracted because of severe pain. After the tooth extraction necrosis of bone around the alveoli in the region of extracted tooth appeared. An oral surgeon made orthopantomography (OPT) (Figure 1) and there were alveolar bone loss and regions of osteosclerosis in the radiographic findings. BRONJ was diagnosed based on clinical and radiographic findings. The patient was treated by the oral surgeon according to the protocol of the

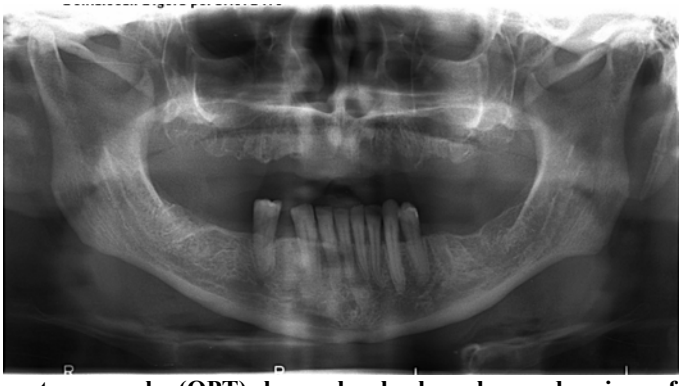

Fig. 1 – Orthopantomography (OPT) shows alveolar bone loss and regions of osteosclerosis.

American Association of Oral and Maxillofacial Surgeon: at the beginning in stage 1 and 2 she was treated with antibacterial mouth rinse and antibiotics and later in 2011 with surgical debridement. Because of the spreading of the process to the other jaw, the patient decided to stop therapy with zoledronic acid in 2012. She was sent to a maxillofacial surgeon in June 2015. Clinical finding showed that the patient had the exposed necrotic bone in the region of alveolar ridge of both sides of the upper jaw and hard palate, and also regions of osteolysis. The patient had extremely bad breath which resulted in social disorder and the occurrence of depression. The computed tomography (CT) was done and radiological findings showed disturbed architecture of the bone structure in terms of demineralization, discontinuity of the corticocalix with numerous sequestrations in the region of both maxillary alveolar process and palatine bone, the changed mucous membrane of both maxillary sinuses, nasal hall and ethmoidal cell to the left (Figure 2). The stage 3 of BRONJ was diagnosed (Figure 3). According to the Naranjo Adverse Drug Reaction Probability Scale [6] this condition was estimated as a definitive adverse drug reaction (score 9). The patient was operated in October 2015. The sequestrectomy was done, which included resection of the both alveolar ridge of maxilla and part of the hard palate (Figures 4 and 5), and reconstruction with an obturator (Figure 6). Because of local status, the primary reconstruction with micro vascular flap was not plan-

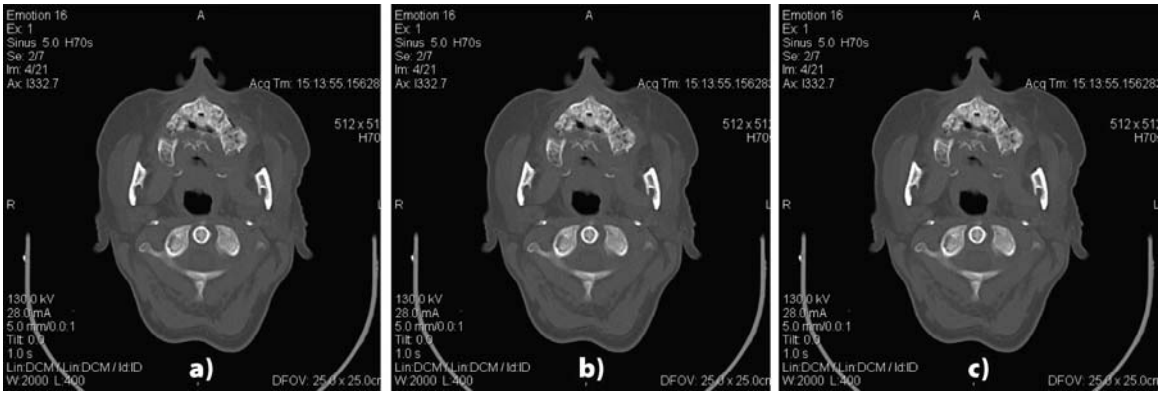

Fig. 2 a–c – Computed tomography (CT) of the midface shows demineralization, discontinuity of the corticocalix with numerous sequestrations in the region of both maxillary alveolar process and palatine bone.

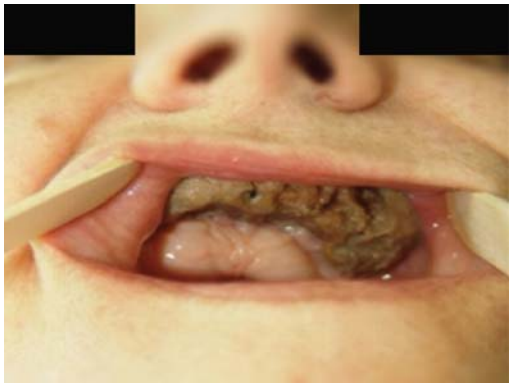

Fig. 3 – Clinical finding.

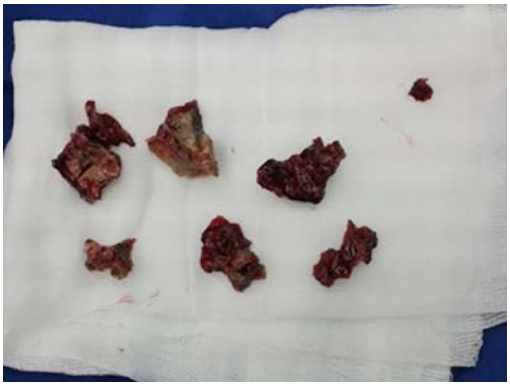

Fig. 4 – Resected parts of the upper jaw.

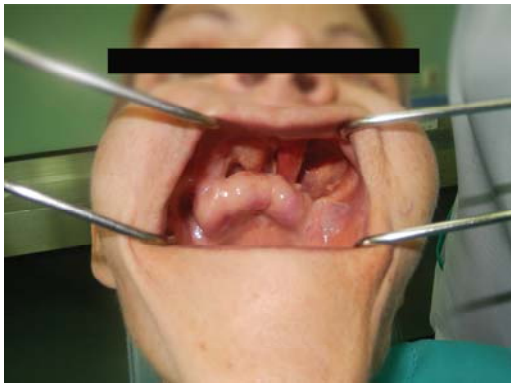

Fig. 5 – Postoperative defect of upper jaw.

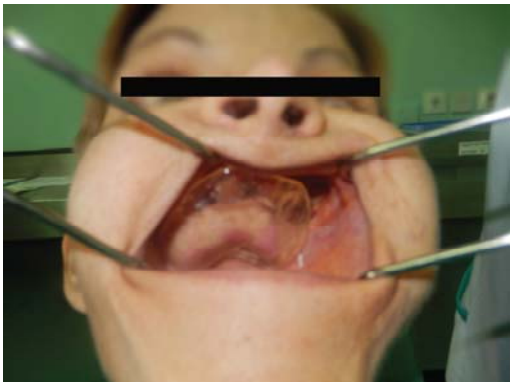

Fig. 6 – Postoperative defect with obturator.

ned. The operation was without complications, and the postoperative course was regular. Histopathological findings confirmed the existence of a bone necrosis and bacterial colonies, as well as a mixed inflammatory infiltrate. Definitive prosthodontic rehabilitation was delayed until the complete healing of the wound due to the instability of dental prostheses, according to the opinion of a prosthetic. The patient was extremely satisfied because bad breath after surgery disappeared.

Discussion

Bisphosphonates are antiresorptive drugs which can be used intravenous (zoledronate and ibandronate) for the treatment of bone metastasis in breast cancer, prostate cancer or lung cancer, and for the treatment of lytic lesions in multiple myeloma and orally in the treatment of osteoporosis, osteopenia, Paget's disease and osteogenesis imperfecta [3, 7]. The pathogenesis of bisphosphonate-related osteonecrosis is not completely clarified and there are different theories for example, that the unique BRONJ localisation entirely to the jaws is connected with modified bone remodeling or oversuppression of the bone resorption; inhibition of angiogenesis; constant microtrauma in the mouth; suppression of the immunity; deficiency of the vitamin D; inflammation or infection [2, 7].

Bisphosphonates inhibit osteoclast differentiation and increase apoptosis which leads to the decrease of bone resorption and remodeling. The predisposition of the jaws for BRONJ can be explained with an increased remodeling rate compared to the other bones in the body. Inflammation and infection is an important component of BRONJ. Many different studies identified bacteria in combination with fungi and viruses on the exposed bone of the jaw. The leading hypothesis in the pathophysiology of BRONJ is inhibition of angiogenesis because it leads to avascular necrosis of the bone. Studies in cancer patients treated with zoledronic acid support this theory [these patients had decreased vascular circulating endothelial growth factor (VCGF)]. Osteoclasts are primary target cells for the bisphosphonates (they bind to the hydroxyapatite in bone), but soft tissue toxicity was noted, too. Bisphosphonates are renally excreted, and their concentration in extraosseal tissue is minimal [8].

BRONJ has the low frequency of occurrence (the risk among cancer patients treated with zoledronate is 1%, and among osteoporosis patients 0.21%). Duration of antiresorptive therapy is an important risk factor of BRONJ and the incidence is higher in patients who had been longer treated with bisphosphonates. The patients with osteoporosis have 100 times smaller risk for developing BRONJ than the cancer patients [9].

The major risk factor for developing of BRONJ is surgery in maxillofacial region and tooth extraction, which is in 52–61% of patient precipitating event. The risk of BRONJ developing in patients on antiresorptive therapy and those with dental implant placement or endodontic procedure is not reported. Pre-existence of periodontal disease is an important risk factor of BRONJ [5, 8, 10].

BRONJ is more likely to develop in the mandible (73%) than in maxilla (22.5%). It can be found in both jaws (4.5%) but that is very rarely [10]. In our case, we presented BRONJ of maxilla which is not so often. The female population has a higher prevalence of BRONJ but it is connected with diseases that are treated with bisphosphonates (breast cancer, osteoporosis). Corticosteroids and antiangiogenic agents are associated with an increased risk of BRONJ [11, 12].

This condition requires a multi-disciplinary approach. Early dental screening and appropriate dental therapy before the initiation of bisphosphonate therapy decreased risk of BRONJ. Screening should include the oral examination and radigraphics to identify acute infections or places where infection can be developed in order to prevent deterioration when the therapy start [4]. A patient must be educated about dental care and must be informed that the risk of BRONJ is much lower if dental preventive measures are implemented before the antiresorptive therapies start. Since 2009, we have a staging system for BRONJ which includes four stages [5]. In this paper, the patient with BRONJ in stage 3 was presented, with a wide area of osteonecrosis in the alveolar bone of maxilla on the both side, hard palate and sinus floor, osteolysis, the presence of infection and unpleasant breath.

The treatment plan depends on the stage of the disease [5, 13–16]. Patients in stage 0 require conservative treatment and treatament of local factors (caries and periodontal disease). Antibiotics and analgetics can be used if it is indicated. Those patients require frequent controls as to follow the further course of the disease. Patients in stage 1 are medically treated with antimicrobial rinses (chlorhexidine). The patients in stage 2 are treated with antimicrobial rinses in combination with antibiotics (quinolones, metronidazole, clindamycin, doxycycline and erythromycin) because of the risk of bacterial colonization of the exposed bone. Operative therapy can be done in order to reduce the volume of necrotic bone and it can be an addition to the antibiotic therapy. The patients in stage 3 require the combination of antibiotic and surgical therapy which includes debridement and even resection of the bone and reconstruction with a reconstruction plate or obturator. A bisphosphonate-related osteonecrosis can develop in the other bones when transplanted in the oral region [16], that is the reason why bone reconstruction in the patient with stage 3 of BRONJ can be risky and why reconstruction with the reconstructive plate and obturator is safer.

Conclusion

BRONJ is a condition with specific clinical picture and presentation. Although it is associated with taking antiresorptive drugs it has relatively obscure pathogenesis. The consequences of these complications can be very serious for the patient, therefore it is necessary to emphasize the importance of screening for the risk of developing of BRONJ. Radical surgical treatment should be performed in cases when medical therapy is no longer capable to stop the development of this complication. Radical resection is necessary in these cases, reconstruction may include free vascular bone grafts which pose a higher risk, and reconstruction with reconstructive plates and obturators which is safer in case of BRONJ. The emphasis, however, must be placed on prevention and on a multidisciplinary approach to the patient under treatment with bisphosphonates. Oncologist, dentist, oral surgeon and the maxillofacial surgeon must cooperate and control the patient who is under treatment with bisphosphonates before therapy start, as well as during and after it.

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CASE REPORT

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Hydroxyurea and nonmelanoma skin cancers: report on three cases and review of the literature

Hidroksiurea i nemelanomski karcinomi kože: prikaz tri bolesnika i pregled litrature

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Abstract

Introduction. Hydroxyurea (HU) is a cytostatic agent, frequently used for the treatment of myeloproliferative disorders, sickle cell anemia and severe forms of psoriasis. Cutaneous side effects occur in up to one third of patients taking hydroxyurea, with the most serious side effect being susceptibility to develop non-melanoma skin cancers. **Case report.** We report 3 patients using HU that have developed multiple skin malignancies on the head and neck region and dorsa of the hands, arranged according to the level of the overall squamous dysplasia expressed. **Conclusion.** A cumulative dose of hydroxyurea affects skin cancer promotion in concordance with other risk factors determining cumulative ultraviolet exposure (age of the patients, skin phototype, sun habits), but the exact influence of each of them and enrollment of other possible cofactors remains to be elucidated. We point out the importance of adequate skin cancer preventive and therapeutic approach to the patients treated with hydroxyurea.

Key words:
skin neoplasms; ultraviolet rays; hydroxyurea; treatment outcome.

Apstrakt

Uvod. Hidroksiurea je citostatik koji se često koristi u terapiji mijeloproliferativnih oboljenja, anemije srpastih ćelija i težih oblika psorijaze. Neželjena dejstva na koži manifestuju se u oko trećine bolesnika lečenih hidroksiureom, među kojima je najozbiljnije neželjeno dejstvo sklonost pojavi nemelanomskih karcinoma kože. **Prikaz bolesnika.** U radu su prikazana tri bolesnika lečena hidroksiureom kod kojih je došlo do razvoja brojnih maligniteta kože u predelu glave, vrata i dorzuma šaka, redosledom prema nivou ukupne prisutne skvamozne displazije. **Zaključak.** Kumulativna doza hidroksiuree utiče na nastanak karcinoma kože u sadejstvu sa drugima faktorima rizika koji određuju kumulativnu izloženost ultraljubičastom zračenju (životno doba, fototip kože, obrazac izlaganja suncu), ali precizniji uticaj svakog od navedenih, kao i uticaj mogućih drugih kofaktora tek treba razjasniti. Ističemo važnost adekvatne prevencije i terapije karcinoma kože kod bolesnika lečenih hidroksiureom.

Ključne reči:
koža, neoplazme; ultravioletni zraci; hidroksiurea; lečenje, ishod.

Introduction

Hydroxyurea (HU) is a cytostatic agent that inhibits cellular DNA synthesis. Due to its high therapeutic efficiency and manageable dose-related toxicity, HU is frequently used for the treatment of myeloproliferative disorders, sickle cell anemia and severe forms of psoriasis [1, 2]. In up to one third of patients taking HU a wide variety of cutaneous side effects occur: dryness and scaling, hyperpigmentation of skin and nails, partial non-scarring alopecia, skin atrophy, skin and mucosal ulcerations, facial and acral erythema, palmoplantar keratoderma, lichenoid and dermatomyositis-like eruption (DMLE) [3, 4]. It has been observed that HU contributes to photo damage of sun exposed skin areas, primarily the head and neck region and dorsa of the hands, inducing skin changes described as "HU-associated squamous dysplasia" (HUSD) [5] and promoting development of non-melanoma skin cancers (NMSC), namely actinic keratoses (AK), keratoacanthomas (KA), squamous cell carcinomas (SCC) and basal cell carcinomas (BCC).

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Case report

We reported three patients using HU as a single specific therapy that developed multiple skin malignancies on the head and neck region and dorsa of the hands, arranged according to the level of the overall squamous dysplasia expressed (Figures 1, 2 and 3). Two patients suffered from *polycythemia rubra* vera (PRV) where HU is usually prescribed in lower doses, and one patient from chronic granulocytic leukemia (CGL) periodically taking up to 3 g of HU daily. All three patients were Caucasian, males, non-smokers, denied alcohol abuse, with family history negative for skin cancers. All were long-term occupationally sun exposed and had no sun protection habits. Other significant data are presented in Table 1. For flat lesions with AK features dignosis was made by dermoscopy and they were treated mainly by conservative options, while all nodular and/or hyperkeratotic lesions were biopsized or excised. Apart from anticancer treatment performed, presented in Table 1, all three patients were advised of meticulous sun protection.

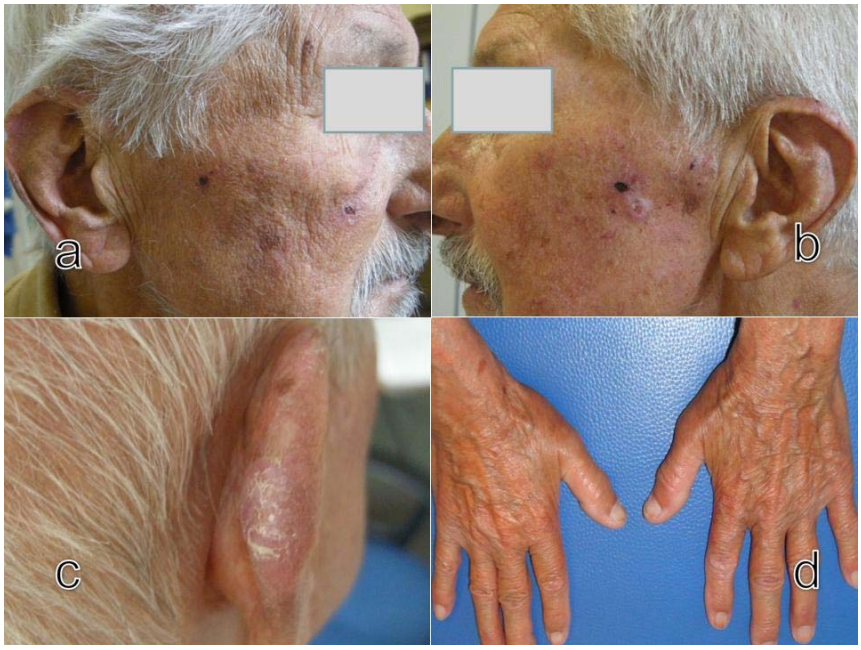

Fig. 1 – Fotoexposed skin areas in the patient 1: a) basal cell carcinoma (BCC) and multiple acinic keratoses (AK) on the right cheek; b) squamous cell carcinoma (SCC) and multiple AK on the left cheek; c) SCC on the right helix; d) clinically intact skin of the dorsa of the hands.

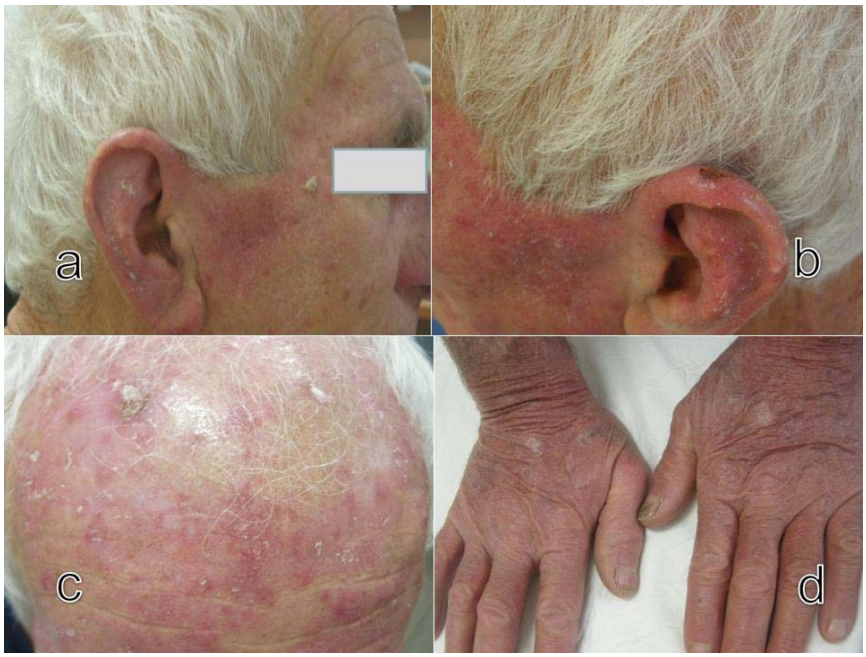

Fig. 2 – Fotoexposed skin areas in the patient 2 – multiple actinic keratoses (AK): a) on the right cheek; b) on the left cheek; c) multiple squamous cell carcinoma (SCC) *in situ* on the frontoparietal region; d) on the dorsa of the hands.

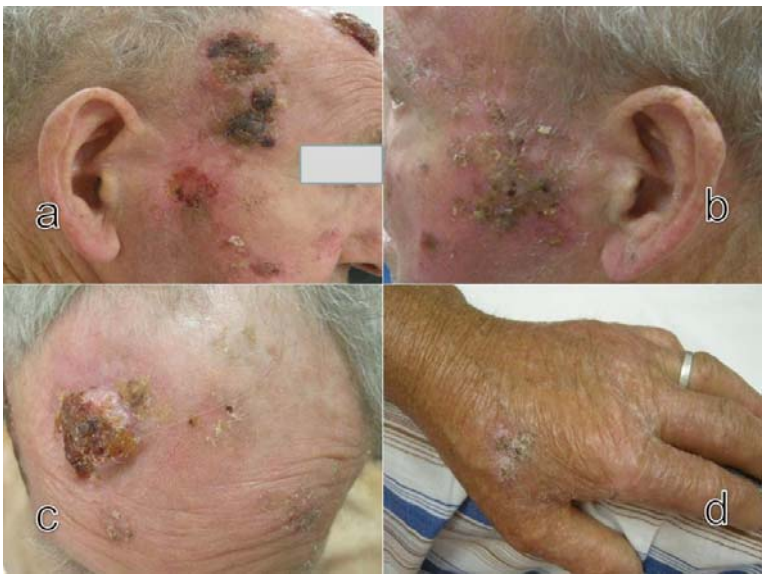

Fig. 3 – Fotoexposed skin areas in the patient 3 – multiple actinic keratoses (AK) and multiple invasive squamous cell carcinoma (SCC): a) on the right temporal and preauricular region; b) on the left preauricular region; c) on the frontoparietal region; d) on the dorsa of the hands.

Table 1

Additional relevant features of reported patients

Features	Case 1 (Figure 1)	Case 2 (Figure 2)	Case 3 (Figure 3)
Age (years)	82	72	73
Skin phototype*	III	II	II
Sunburns in childhood or adulthood	No	Yes, both	Unreliable
Hematological disorders	PRV	PRV	CGL
HU daily doses (g)	1–1.5	1–1.5	2–3
HU cumulative dose and length of therapy at the onset of the first skin malignancy	1.8 kg in 5 years	1.4 kg in 4 years	2.9 kg in 4 years
HU total dose and total length of therapy	> 2.9 kg during 8 years	> 4 kg during 11 years	> 4.3 kg during 6 years
Skin tumors during follow-up	Multiple AK 1 BCC 2 invasive SCC	Multiple AK 1 KA 1BCC 3 *in situ* SCC 1 invasive SCC	Multiple AK Multiple invasive SCC
Treatment applied	Cryosurgery, 5-FU cream, Surgical excisions	Imiquimod cream Surgical excisions Acitretin chemo-prevention	Following multiple biopsies patient refused radical surgery and was lost to follow-up

***Skin phototype according to Fitzpatrick [6]**
HU – hydroxyurea; PRV – *polycytemia rubra vera*; CGL – chronic granulocytic leukemia; AK – actinic keratose; KA – keratocanthomas; BCC – basal cell carcinoma; SCC – squamous cell carcinoma; FU – fluorouracil.

Discussion

Hydroxyurea became an antiproliferative treatment option in the 1960s [7], and is widely used ever since in coping with numerous hematological and other conditions.

HU-associated skin eruption was initially reported in 1975, described as lichen planus like eruption with histology of an interface dermatitis. In 1995 a form of HU-induced eruption with a tendency to mimic true dermatomiositis was recognized and named DMLE [3]. The first report of a potential HU influence on promoting skin cancers was given in 1991 [8], followed by multiple similar case reports [9–12], drawing attention to the most serious HU adverse effect and changing perspective on HU-induced skin lesions. The term "hydroxyurea dermopathy" was introduced in 1997, describing "focal to more extensive squamous changes of basal cell keratinocytes" [13], while in 2004 a more precise terminology proposition of HUSD was made [5]. Further research of the number and distribution of p53 mutated keratinocytes along the lower layers of the epidermis suggested that HU-associated DMLE is a premalignant state, but to a lesser degree compared to HUSD, since DMLE showed focal p53 expression in a confluent nuclear pattern, while HUSD showed diffuse p53 expression [3, 5]. Mutant p53 keratinocyte clones represent hallmark of AK and SCC [14]. The observation that DMLE lesions have a latency period of 3 to 5 years, compared to longer latency for NMSC [3], concurs with

theory that DMLE is a predecessor of HUSD, which precedes HU-NMSC, all being just different evolutionary phases.

The exact interdependence between the HU mechanism of action and neoplastic skin alterations is still not completely understood. In general, HU acts as an inhibitor of cellular DNA synthesis, through inactivation of the enzyme ribonucleotide reductase, thus leading to cell apoptosis [3].

It has been proposed that a direct cumulative damage, as well as a cytotoxic effect of HU on basal keratinocytes results in promoting skin cancers. In experimental models HU produces carcinogenic agents such as N-methyl-N-nitrosourea, induces direct chromosomal damage and inhibits DNA repair in ultraviolet (UV)-irradiated cells [15–17]. The cytotoxic effect of HU promotes atrophy of the skin that enables a higher degree of UV penetration and UV damage [3].

The results of the study [18] that examined chromosomal HU effect in the presence of metal ions show that HU causes DNA damage in the presence of copper ion Cu(II), and inhibition of DNA damage in the presence of bathocuproine, a copper ion specific chelator. No HU induced DNA damage was recorded in the presence of cobalt, nickel, manganese or iron ions. Researchers observed that characteristic oxidative DNA lesions increased with increasing concentration of hydroxyurea in the presence of Cu(II). Copper is an essential component of chromatin, but obviously has the ability to catalyze the production of reactive oxygen species to mediate oxidative DNA damage [19]. Whatever the cause (excess intake or constitutional excess), the elevated copper metal ion concentration could affect pharmacological properties of HU.

The suspected role of human papillomavirus infection as a cofactor in the development of HU-NMSC has not been proved yet [20].

An interesting evidence of HU influence reports female monozygotic twins, with only difference that lies in the fact that of 1 of them who was taking HU developed severely sun damaged skin, multiple Bowenoid AK and SCC *in situ* [21].

The true incidence of NMSC in patients taking HU is unknown. In the case series of 26 patients taking HU, 8 patients had AK and 2 of them developed SCC [4]. There are also no established criteria that can help us recognize the group of patients taking HU with higher risk of developing NMSC. Concomitant excessive UV exposure, duration of treatment and the cumulative dose of HU are recognized as important risk factors.

Like the majority of the reported cases, our patients were older subjects with mostly fair skin types, all had positive history of excessive UV exposure and no sun protection habits. NMSC are not recorded in patients taking HU for the treatment of sickle cell anemia, which is a hereditary disease affecting young black individuals [22]. On the other hand, a report of a patient developing exclusively mucosal SCC at a cumulative dose of more than 6 kg HU, with no associated risk factors for oral SCC [23], as well as a case of a 59-year-old male with multiple SCCs taking 1 g of HU per day during 6 years, with skin phototype IV and a lack of over photo exposure [1], indicate importance of other factors apart from UV exposure.

The duration of HU intake as a cofactor is well illustrated in a study reporting 158 patients receiving HU for chronic myeloid leukemia (CML), where only 5 of them developed NMSC. Patients with NMSC received HU for an average of 76 months, compared to the average of 38 months in all the other patients from the cohort [7]. NMSC are not recorded in patients taking HU for the treatment of psoriasis, and the explanation may be significantly shorter treatment courses. Our patients developed first NMSC lesions after 4 to 5 years of continuing HU therapy.

Cumulative HU dose is another important cofactor. A case series of 5 patients with NMSC reports cumulative doses varying from 0.65 to 3.6 kg HU, in average taken during 6.5 years [12]. Our patients developed first NMSC lesions at the individual cumulative dose of 1.8 kg; 1.4 kg and 2.9 kg, respectively. It is unclear whether HU intake played any role in promoting NMSC in a case report of a 74-year old female with PRV who developed just one SCC after a cumulative dose of only 0.6 kg, and no other NMSC appeared during next 6 years of follow up despite ongoing HU therapy [24].

Besides abovementioned risks, patients suffering from CML have additional burden of disease-related immunosuppression [17] which modifies tumor behavior and prognosis. The latency period is usually shorter and tumors are more aggressive [8, 16]. Some of the reported CML patients developed NMSC even years after the discontinuation of the HU therapy [9, 15, 25].

There are reports of Merkel cell carcinoma in 2 patients taking HU [26, 27], but until larger number of cases are registered, these should be considered as a coincidental association.

Marked predominance of AK-SCC spectrum compared to BCC lesions in a total body of reported cases, including our case series, could be due to a combined UV and HU influence on specific molecular pathway [28], but further investigations are needed.

The management of the patients developing NMSC while taking HU is complex and multidisciplinary approach is mandatory. Most of the HU associated NMSCs were treated by surgery, but repeated topical 5% imiquimod treatment is a good therapeutic option for well preselected lesions [9, 29] and chemoprevention with retinoids is a possible viable tool. Close collaboration between general practitioner, hematologist, dermatologist, oncologist and reconstructive and/or maxillofacial surgeon is mandatory.

Conclusion

Our findings indicate that a cumulative dose of HU affects skin cancer promotion in concordance with other risk factors determining cumulative UV exposure (age of the patients, skin phototype, sun habits). The level of HU and possible cofactor influence remains to be elucidated, since the case-control studies are still lacking.

Nevertheless, it is important to recognize the higher risk for development of NMSC in patients taking HU, to take preventive measures through educating patients in photo protection and self-examination of the skin, to organize periodic preventive thorough skin examinations by professionals and to discontinue or replace HU therapy at the onset of the first NMSC, if possible.

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CASE REPORT

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Magnets ingestion as a rare cause of ileus in adults: a case report

Progutani magneti kao redak uzrok ileusa kod odraslih

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Abstract

Introduction. Magnetic foreign bodies are harmless when ingested as a single object. However, if numerous of individual magnets are ingested at certain intervals, one after the other, they may attract each other through the bowel wall and cause severe bowel damage. **Case report**. We reported a case of a man, age 21, who swallowed 2 very small magnets, presented with clinical and radiographic signs of acute bowel obstruction and intestinal fistula. The cause of obstruction was detected during laparotomy. At laparotomy, one magnet was found in the proximal jejunum and the other in the distal ileum, strongly attracting each other followed by small bowel twisting around this point of rotation, causing a complete small bowel obstruction with strangulation and jejuno-ileal fistula. The intestinal segments were devolvulated and both intestine perforations were primarily sutured. The patient recovered successfully after the surgery and was discharged 5 days after the laparotomy. **Conclusion**. Patients who ingested magnetic objects must be seriously considered and emergency laparotomy should be performed to prevent serious gastrointestinal complications.

Key words:
foreign bodies; magnetics; intestinal obstruction; digestive system surgical procedures.

Apstrakt

Uvod. Progutano magnetno strano telo je bezazleno ukoliko je pojedinačno. Kod gutanja većeg broja pojedinačnih magneta u vremenskim razmacima oni se mogu međusobno snažno privući kroz zid creva i izazvati ozbiljna oštećenja. **Prikaz bolesnika**. Prikazali smo slučaj 21-godišnjeg muškarca koji je, nakon što je progutao dva mala magneta, razvio kliničke i radiografske znake akutne crevne okluzije i intestinalne fistule. Uzrok ileusa detektovan je tek tokom laparotomije. Pri laparotomiji jedan magnet pronađen je u vijuzi proksimalnog jejunuma, a drugi u distalnom ileumu. Bili su snažno međusobno privučeni, sa uvrtanjem preostalih vijuga tankog creva oko formirane tačke rotacije, uzrokujući kompletnu crevnu opstrukciju sa strangulacijom tankog creva i jejuno-ilealnom fistulom. Crevne vijuge su oslobođene i mesta perforacije su zbrinuta primarnom suturom creva. Oporavak je protekao bez komplikacija. Bolesnik je otpušten iz bolnice petog postoperativnog dana. **Zaključak**. Ukoliko je pacijent progutao više pojedinačnih magneta i razviju se znaci akutne intestinalne okluzije, potrebna je brza hirurška intervencija da bi se sprečile ozbiljne komplikacije.

Ključne reči:
strana tela; magnetika; creva, opstrukcija; hirurgija digestivnog sistema, procedure.

Introduction

In adult population swallowing foreign bodies (FB) is less common than in children, but it is possible as accidental ingestion or ingestion in mentally ill patients. Reaching pylorus it usually can pass through the intestine and if it reaches the colon, the patient can expel them spontaneously by defecation. Nevertheless swallowed FB can lead to severe abdominal complications in < 1% [1]. A danger in FB ingestion exists if a clinician does not think about swallowing FB or when the FB is difficult to detect by commonly used diagnostic methods. Adults swallow, usually accidentally, fish bones, chicken bones, parts of tools being held in the mouth, toothpicks or needles [2]. Among swallowed FB the most dangerous are sharp objects, large objects, toxic FB, button batteries and magnets [3, 4].

Magnetic FB are seemingly harmless but they are very dangerous if patient swallows several individual magnets at certain intervals, one after the other. In this case, magnets attract each other through the walls of hollow abdominal organs and their contact leads to intestinal wall ischemia and necrosis [3–6]. Magnets can be seen on radiographs, but the di-

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agnosis is usually delayed if patient does not give information that he/she has swallowed magnets. Significant sign of the magnet presence is the FB shadow that does not change the position of the repeated follow-up radiography in patients with worsening abdominal symptoms [6, 7]. When multiple swallowing of magnets at certain sequences is suspected, patient requires emergency surgical treatment [8].

The goal of this report was to point out a rare cause of acute intestinal obstruction in previously healthy young man, who has never had prior abdominal surgery which occured when he swallowed two or more magnets at certain intervals.

Case report

A 21 years-old man with severe abdominal pain, nausea and vomiting was admitted to the Clinic for Emergency Surgery Clinical Center of Serbia, Belgrade. His symptoms started suddenly about 12 hours before arriving to the hospital. He had no previous abdominal surgery, no history of malignancy or hereditary diseases. On admission, the patients vital signs were stable. Physical examination showed distended and tender abdomen without peritoneal signs. Auscultation detected weakening peristaltic rushes. Rectal examination was regular. Laboratory analyses showed leucocytosis (13×10^9/L). Ultrasound (US) of the abdomen showed a moderate amount of free intraperitoneal liquid. First abdominal radiograph demonstrated a several air-fluid levels of small bowel, without pneumoperitoneum (Figure 1a). Unexpectedly, abdominal radiography revealed a shadow of the foreign body presented in the middle abdomen region (Figure 1a). However, the patient denied having swallowed a foreign body. As there was no evidence of serious complications, the initial treatment was consisted of fluid resuscitation and placement of a nasogastric tube. After a few hours, physical examination showed distended abdomen with guarding and rebound tenderness. A follow-up abdominal radiograph showed the object in an unchanged position, but demonstrated increased bowel distension with air-fluid levels and signs of ileus (Figure 1b).

The patients condition was rapidly deteriorated and clinical as well as radiological signs pointed to an acute intestinal obstruction, so the laparotomy was indicated. The laparotomy performed through a middle incision, revealed intestinal segments volvulated 30 cm distal to the Treitz ligament and distal ileum. The cause of obstruction was discovered only after the release of intestinal segments. Surprisingly, two small magnetic balls were found: one was set in the proximal jejunal loop 20 cm distal to the ligament of Treitz and the other in the distal ileum 20 cm proximal from Bauchini valve, with small bowel twisting around this point of rotation, causing a complete small bowel obstruction and strangulation (Figure 2). The strangulated bowel between these areas was not necrotic, but bowel loops proximal to occlusion were edematouse and succulent. In addition, the magnetic balls connected through the bowel wall were leading to necrosis and the formation of a jejunoileal fistula (Figure 2a). The intestinal segments were devolvulated and both perforations were primarily sutured. After surgical removal magnets were measured and each magnetic ball was 3 mm in size (Figure 3). The patient successfully recovered after the surgery, without complications during postoperative course. He was discharged from hospital 5 days after laparotomy. The patient did not know that he had swallowed magnets; he only remembered that he had kept pieces of jewelry in his mouth, finding out after reexamination that two small parts were missing.

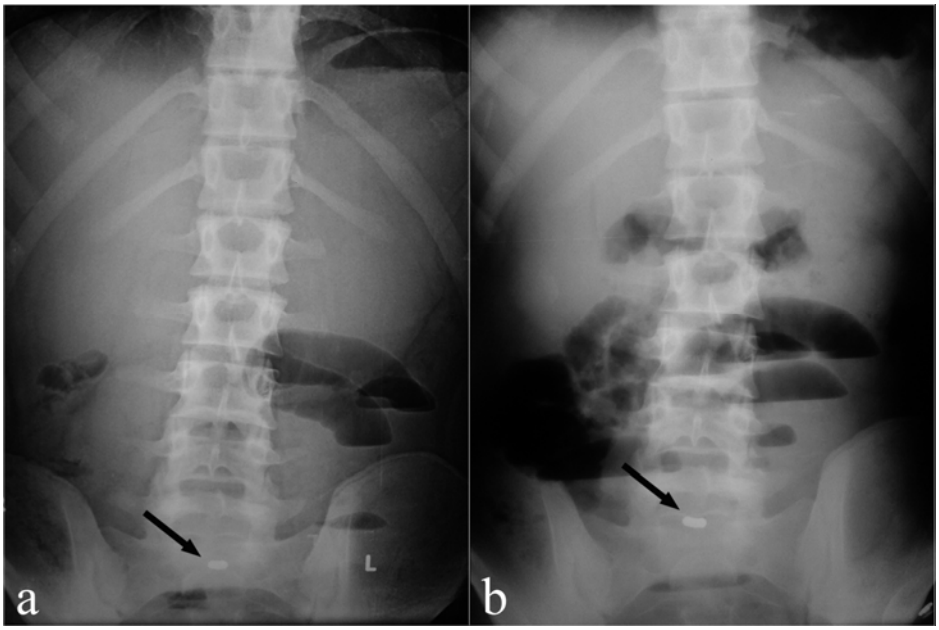

Fig. 1 – Abdominal radiography performed: a) on admittance showing a several air-fluid levels and foreign body (black arrow); b) after several hours showing a radiographic signs of small bowel obstruction and foreign body in an unchanged position (black arrow).

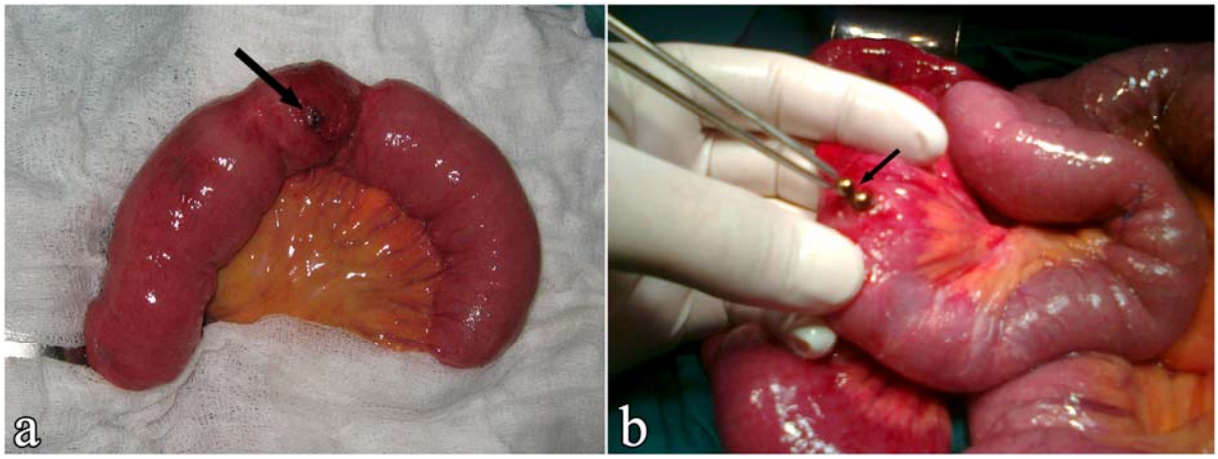

Fig. 2 – Intraoperative photograph showing: a) jejunal loop containing foreign body and local necrosis of the bowel wall (black arrow); b) the cause of obstruction after the release of intestinal segments [we found two magnetic balls (black arrow)].

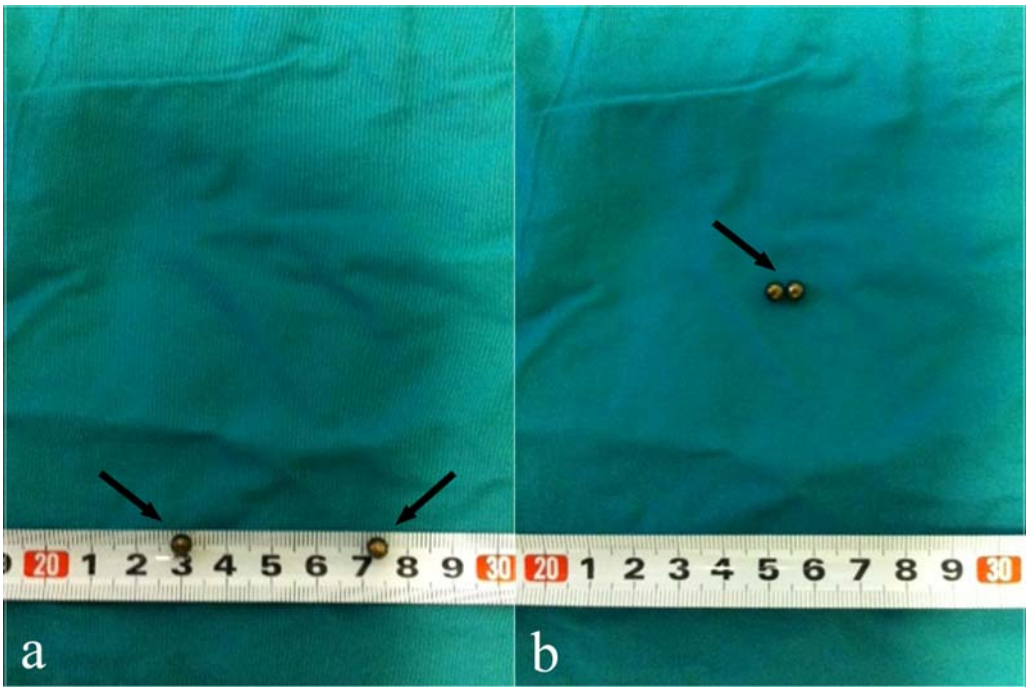

Fig. 3 – Photo of a foreign body: a) after it was removed from the intestinal loops [two small magnetic balls, 3 mm size (black arrow)]; b) two small magnetic balls are capable of attracting each other (black arrow).

Discussion

Only about 20% of ingested FB were observed in adults, while the remaining 80% of cases were reported in children [8]. Reviewing the available literature data from June 1987 to October 2010, out of 149 cases identified as magnet ingestion injuries, only 6 (4.02%) patients were ≥ 18 years and the oldest patient was 48 years [3, 5, 9–11]. In 80% of cases, swallowed FB passed stomach, small intestine and colon without no complications [12]. Swallowed FB that cause severe abdominal complications are usually sharp objects such as toothpicks, needles, chicken bones and fish bones [13, 14]. Swallowed magnetic FB are especially interesting. If a patient swallows a single magnet it is usually harmless. Nevertheless over 50% of patients swallow two or more magnets [6]. The problem arises when a patient swallows two or more magnets, one after the other, at certain intervals, which results in a strong mutual attraction of magnets through the wall of the hollow organs of the abdomen, and can cause intestinal wall ischemia, necrosis and perforation with peritonitis, or intestinal fistula formation [1, 15–18].

We reported a case of a young patient who swallowed 2 very small magnets, presented as acute bowel obstruction that required emergency surgery.

The presence of ingested magnets usually can be seen on radiography or abdominal ultrasound, but clinicians rarely think of swallowing magnets in adults [4, 6]. On abdominal radiography with air-fluid levels, sometimes the metallic shadow of two magnets can be seen with a small free space between them, just in the place where they attached through the wall of the hollow organs [6]. These FB shadows do not change position on repeated radiography. In unclear clinical

cases who require additional diagnostic methods, swallowed magnetic FB may be disclosed on the abdominal ultrasound or abdominal CT [16]. If magnetic FB are highly suspected as a cause of abdominal complications the magnetic resonance imaging should be avoided [6, 16]. In our case, the patient was not aware that he had swallowed magnets; on abdominal we did not recognize that FB are magnets; so, finally, 2 small magnetic balls were discovered at surgery. Our patient probably swallowed two magnetic balls one by one at a certain time interval, therefore, there has been a magnet attracted by one in the jejunal loop and the other in the distal ileum loop causing small bowel obstruction due to the small bowel twisting around this point of rotation and intestinal fistula, which required urgent surgical treatment. If the magnets still remain in the esophagus or stomach, they must be urgently removed endoscopically [15, 17]. In other cases the emergency explorative laparotomy should be performed because of the potential severe abdominal complication following magnets ingestion [15, 17].

Conclusion

Swallowing magnets one by one at a certain time interval can lead to severe acute complications that require emergency surgery. Multiple magnets ingestion is expected rarely in adults, but if unrecognized , they could carry a significant risk of morbidity. Clinicians should always keep in mind a possibility of magnetic FB ingestion, especially when the etiology of the acute abdominal symptoms is unknown.

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vsp@vma.mod.gov.rs

Časopis „Vojnosanitetski pregled“ izlazi godišnje u 12 brojeva.

Godišnja pretplata za 2016. godinu iznosi: 5 000 dinara za građane Srbije, 10 000 dinara za ustanove iz Srbije i 150 € za strane državljane i ustanove. Pretplate: Žiro račun br. 840-314849-70 MO – Sredstva objedinjene naplate – VMA (za Vojnosanitetski pregled), poziv na broj 12274231295521415. Uplatnicu (dokaz o uplati) dostaviti lično ili poštom (pismom, faksom, *e-mail*-om). Za zaposlene u MO i Vojsci Srbije moguća je i pretplata u 12 mesečnih rata putem trajnog naloga, tj. „odbijanjem od plate“. Popunjen obrazac poslati na adresu VSP-a.

PRIJAVA ZA PRETPLATU NA ČASOPIS „VOJNOSANITETSKI PREGLED“

Ime i prezime ili naziv ustanove	
Jedinstveni matični broj građana	
Poreski identifikacioni broj (PIB) za ustanove	
Mesto	
Ulica i broj	
Telefon / telefaks	

Pretplata na časopis „Vojnosanitetski pregled“ (zaokružiti):

1. Lično. Dokaz o pretplati dostavljam uz ovu prijavu.
2. Za pripadnike MO i Vojske Srbije: Dajem saglasnost da se prilikom isplate plata u Računovodstvenom centru MO iz mojih prinadležnosti obustavlja iznos mesečne rate (pretplate).
3. Virmanom po prijemu profakture.

Datum _____	Potpis _____

VOJNOSANITETSKI PREGLED
VOJNOMEDICINSKA AKADEMIJA
Crnotravska 17, 11040 **Beograd**, **Srbija**
Tel/Fax: +381 11 2669689
vsp@vma.mod.gov.rs

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Godišnja pretplata za 2016. godinu iznosi: 5 000 dinara za građane Srbije, 10 000 dinara za ustanove iz Srbije i 150 € za strane državljane i ustanove. Pretplate: žiro račun br. 840-314849-70 MO – Sredstva objedinjene naplate – VMA (za Vojnosanitetski pregled), poziv na broj 12274231295521415. Uplatnicu (dokaz o uplati) dostaviti lično ili poštom (pismom, faksom, *e-mail*-om). Za zaposlene u MO i Vojsci Srbije moguća je i pretplata u 12 mesečnih rata putem trajnog naloga, tj. „odbijanjem od plate“. Popunjen obrazac poslati na adresu VSP-a.

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