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"DA LI STE PAŽLJIVO ČITALI RADOVE"

TEST JE OBJAVLJEN U ČASOPISU SKS INFORMATOR BR.6, JUN 2018. GODINE

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Ako nemate drugih dokaza o sebi,
govorite loše o onima koji su
bolji od vas. To stvara iluziju o
sopstvenoj vrednosti.
Dušan Radović

Možda će se u ovom citatu mnogi prepoznati, ali moja namera je pre svega bila da ukažem na hrabrost, ali i „svežinu" i aktuelnost velikana pisane i izgovorene reči. Njegova hrabrost i vera je, uprkos strahovima, bila nadahnuta mudrošću koja je oblikovala kulturu jednog vremena, a njegova nadarenost da kritički misli i odgovorno stvara pružala je priliku za avanturu i ispunjen život u svakodnevnom bitisanju.

Aktuelna svakodnevica je potpuno drugačija jer je svaka, pa i intelektualna hrabrost svedena na nivo „incidenta". Svekolika estradizacija isključuje svaku želju za promenom u društvu, a nauku, kulturu, obrazovanje i umetnost postavlja na pijedestal „srama". Jedini oblik kulture je „kultura straha" koja kontroliše svaku odvažnost i svaku želju za promenom, gušeći ih u svom začetku.

Umesto borbe ideja, otkrivanja novih vidika, traženja alternativnih prečica za razumevanje osnovne istine, u ponudi je ogoljena borba za vlast u svakom segmentu života. A kada se umesto racionalnih objašnjenja nudi zapaljiva retorika, onda je „požar" mnogo izvesniji od mirne luke. Činjenica je takođe da tamo gde ne postoje konsekvence za nasilje, to postaje opšti i prihvatljiv standard ponašanja. U takvom sistemu vrednosti odgovorni ljudi teško dopiru do realnosti i često svoje bitisanje samo usmeravaju ka „rijalitijima" sopstvenog života.

Realnost je u stvari „vulgarna" tekovina političkih naloga i neuspešan pokušaj šminkanja sramote. A tamo gde žrtve olako postaju okrivljeni, tamo gde je potrebno samo malo da se neistina preoblikuje u istinu, malo je šansi za svetlu i perspektivnu budućnost.

Civilizacijska inverzija je i činjenica da uprkos svekolikom „napretku" u svim sferama života naše bitisanje u brojnim segmentima tavori na najnižim lestvicama. Poznato je takođe da samo neodgovorni ljudi mogu i znaju sve. Oni kojima je odgovornost na vrhu svih lestvica ništa ne prihvataju olako, jer znaju da se uspeh i ambicija baziraju isključivo na znanju i upornom radu, a ne na podobnosti i poslušnosti. Osim toga, podanički odnos gazi osnovne etičke i naučne principe, ali i elementarno dostojanstvo.

Treba biti slobodan i hrabar u svakom smislu, jer je hrabrost vrlina učenih i slobodoumnih, a ne poslušnih. Hrabre ljude samo znanje, istina i poštenje čine slobodnim. Neko je davno rekao da jedino onaj ko je savijen nad knjigom uspravno hoda.

Urednički komentar ću ovog puta završiti citatom našeg Nobelovca Ive Andrića, jer jasno oslikava sadašnji trenutak i njegove aktuelne aktere na daskama naših života: „Slabe i plašljive ljude strah nagoni da rade upravo ono čega se najviše boje".

Prof. dr Slavoljub Živković

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ORIGINAL ARTICLE
ORIGINALNI RAD

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Compressive strength of calcium silicate-based cement

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SUMMARY

Introduction The aim of this study was to compare compressive strength (Cs) of new nanostructural calcium silicate based cement (nCS) with commercial calcium silicate cement and conventional GIC.

Methods Four nanostructural materials were tested: nanostructural calcium silicate based cement (nCS) (Jokanović et al.), MTA Plus (Cerkamed, Poland), Fuji IX (GC Corporation, Japan) and Ketac Universal Aplicap (3M ESPE, USA). Five samples of each material were mixed in accordance with manifecturer's guidelines and positioned in metal moulds (φ4mm and 6mm). Compressive strength (Cs) expressed in MPa was determined after 24 hours, 7 days and 28 days respectively. Measurements were performed on universal testing equipment (Tinius Olsen, USA) at a crosshead speed of 1mm/min. For processing the results one-way ANOVA and post-hoc test were used.

Results The highest values of compressive strength after 24h was found in conventional GIC Fuji IX (mean 38.56±13.31) and Ketac Universal (mean 40.77±7.96). Calcium silicate cements after 24h showed low values of compressive strength (MTA Plus 5.91±0.28 MPa, nCS 1.35±0.36 MPa). After 7 days, FUJI IX 47.42±9.33 MPa and Ketac Universal 35.25±10.60 MPa showed higher value of compressive strength than MTA Plus (15.09±2.77 MPa) and nCS (11.06±0.88 MPa). After 28 days the Cs value for conventional GIC Fuji IX was 48.03±7.82 MPa and Ketac Universal 36.65±11.13 MPa while for calcium silicate cements it was 16.47±1.89 MPa and nCS 14.39±1.63 MPa. There was statistically significant difference ($p<0.05$) in Cs between conventional GIC and CS cements after 24h, 7 and 28 days.

Conclusions Calcium silicate cements initially showed lower values of compressive strength than conventional GIC that increased over time.

Keywords: calcium silicate cement; nanoparticle; glass ionomer cement; compressive strength

INTRODUCTION

Ideal material for root reparation should be able to close communication between the root canal and surrounding tissue, is biocompatible, dimensionally stable and insoluble when in contact with tissue fluids. The material is often placed in the root with an acidic environment, frequently with bacterial inflammation; therefore low pH level is an important factor that adds to the hardness and other properties of the cement [1]. In the past, materials such as calcium hydroxide, zinc oxide eugenol cements, resin composites, glass ionomer cements have been used for root canal perforation treatment but not all of them meet criteria of an ideal material [2].

GIC are developed by combining two different cements: silicate and zinc polycarboxylate cements [3]. Conventional GIC are made by an acid-base reaction of glass ions with a water solution of polyacrylic acid. They are considered potential biomaterials for orthopedic application because of their ability to adhere to bone and metals and good stability in wet environment. However, lack of bioactive potential and poor mechanical characteristics are some of the issues of this cement.

In the past few years, biocompatible calcium silicate hydraulic cements have been introduced in endodontic therapy. Mineral trioxide aggregate (MTA) is usually used as biomaterial for root and functional perforation reparation, as well as in other indications [4]. MTA is a bioactive material that forms an apatite layer on its surface when in contact with phosphates from tissue liquids but it also forms hybrid layer between dentin and calcium silicate materials [5]. It also releases some of its components in phosphate saliva puffers that encourage biomineralization processes [6]. There are number of calcium silicate cements on the market with the goal to surpass the deficiencies of the original formulation. MTA Plus is a nanostructural MTA released in 2012, with shorter binding time and lower concentration of heavy metals (up to 90%) in its formulation.

Nanoparticles allow uniform and homogenous structure, as well as lower temperature release while hydrating the cement (source: manufacturer). The use of nanoparticles has become an important research aspect in dentistry, with the focus on improving mechanical characteristics and antibacterial effect of the particles. The size of nanomaterial particles (<100nm) that is similar to the size of

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Table 1. Force (N) needed to break samples after 24 h, 7 and 28 days
Tabela 1. Vrednosti sile (N) koje dovode do lomljenja uzorka posle 24 sata, 7 i 28 dana

	24 hrs 24 sata				7 days 7 dana				28 days 28 dana			
	MAX	MIN	MEAN	SD	MAX	MIN	MEAN	SD	MAX	MIN	MEAN	SD
nCS	24.05	12.15	16.91	4.54	149.10	121.50	138.94	11.08	197.15	145.20	188.90	18.38
MTA	79.40	69.70	74.25	3.55	220.00	134.35	189.53	34.86	230.33	170.66	213.17	21.24
FUJI IX	675.17	289.33	484.37	167.13	688.00	423.67	595.00	116.70	543.30	321.83	512.67	87.90
KETAC 3M	593.17	399.00	512.07	99.96	569.17	234.00	442.77	133.09	569.00	224.00	381.17	125.00

biological molecules and structures (proteins, DNA, water) indicates possible uses in biomedical researches.

Newly synthesized nanostructural material used in our study uses tri-calcium and di-calcium silicates as a base. This calcium silicate system is produced with hydrothermal sol-gel method and self-expanding burning reaction [7], which secures its high activity and short bonding time. The smallest parts of this system are about 19.9 nm and show notable system activity [8, 9].

Compressive strength tests are used in dentistry for simulations of masticatory forces that clinically affect restoration or materials for covering or replacing tissue. The majority of masticatory forces are of compressive nature and their exact value is hard to determine.

The aim of this study was to test the compressive strength of a newly synthesized nanostructural CS cement and compare it to the commercial MTA Plus and conventional GIC that are used in functional or crown perforation reparations. The null hypothesis was that there was no difference in compressive strength between conventional and calcium silicate cements.

Table 2. Compressive strength (Cs) values (MPa) of the tested materials in the function of time
Tabela 2. Vrednosti kompresivne čvrstoće (MPa) testiranih materijala u funkciji vremena

	24 hrs 24 sata	7 days 7 dana	28 days 28 dana
nCS	1.35 ± 0.36	11.06 ± 0.88	14.39 ± 1.63
MTA	5.91 ± 0.28	15.09 ± 2.77	16.47 ± 1.89
FUJI IX	38.56 ± 13.31	47.42 ± 9.33	48.03 ± 7.82
KETAC 3M	40.77 ± 7.96	35.25 ± 10.60	36.65 ± 11.13

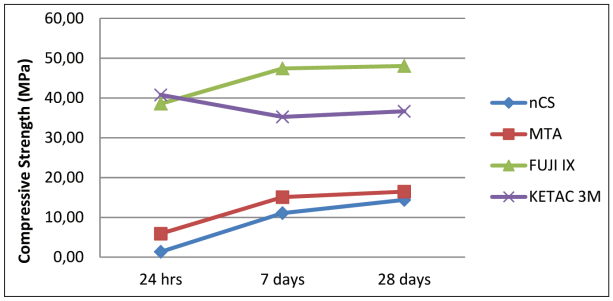

Figure 1. Compressive strength values (MPa) in the function of time
Slika 1. Vrednosti kompresivne čvrstoće (MPa) u funkciji vremena

nCS – nanostructural calcium silicate cement
nCS – nanostrukturni kalcijum-silikatni cement

MATERIAL AND METHODS

Four cements were used in the research: nanostructural calcium silicate system (nCS) (Jokanovic et al.) where 60% of the total mass were β-C_2S i C_3S phases, 20% calcium carbonate and 20% $BaSO_4$ (Merck, Germany) as X-ray contrast. Water/powder mixing ratio was 1:2; MTA Plus (Cerkamed, Poland) hand mixed in the ratio 0,34g distilled water and 1gMTA powder; conventional GIC Fuji IX (GC Corporation, Japan), by the product instructions mechanically mixed in capsules for 10 seconds in an amalgamator at 4500 rpm; and self-adhesive and self-bonding GIC Ketak Universal Aplicap (3M ESP, USA), with glass oxides and liquid component that consisted of copolymer of acrylic and maleic acid and tartaric acid. As per manufacturer's instruction the capsules were mechanically mixed for 10 seconds in an amalgamator at 4500 rpm.

Sample preparation

After mixing all materials were placed in two parted metal moulds 4mm in diameter and 6mm tall and they were condensed with a hand plunger, 5 samples for each material. The samples were kept on 37°C in a steam bath for 24h, 7 and 28 days. All cylindrical samples were polished with the finest abrasive paper and minimal pressure and visually checked. Samples with visible structural damage were eliminated from the study.

Compressive strength testing was done according to international standard ISO 9917-1:2007 (*Dentistry-water-based cements- Part 1: powder/liquid acid-base cements)* using a universal test machine (Tinius Olsen, USA; 5KN) at the speed of 1 mm/min along the longer axis of cylindrical samples [10]. The force needed to break the sample was noted and compressive strength in MPa was calculated with the formula $Cs= 4P/\pi d$ where P was the maximum force needed to break the sample measured in N, and d was the diameter in mm.

For processing the results one-way ANOVA and post-hoc test was used. The level of significance was set at $p<0.05$.

RESULTS

The results are showed in the Tables 1-2 and Figure 1. The highest values of compressive strength after 24h were shown by conventional GIC Fuji IX (mean 38.56±13.31) and Ketac Universal (mean 40.77±7.96), but without sta-

tistical difference between them. Calcium silicate cements showed low values of compressive strength (MTA Plus 5.91±0.28 MPa and nCS 1.35±0.36 MPa), without statistical difference. A statistically significant difference was noticed between GIC and CS cements ($p<0.05$).

After 7 days, the highest compressive strength value was shown by FUJI IX 47.42±9.33 MPa and Ketak Universal 35.25±10.60 MPa, but without a statistically significant difference between them. The compressive strength of MTA Plus was 15.09±2.77 MPa and nCS 11.06±0.88 MPa, without statistically significant difference. There was a statistically significant difference between conventional GIC and CS cements ($p<0.05$).

After 28 days Cs value for conventional GIC Fuji IX was 48.03±7.82 MPa and Ketak Universal 36.65±11.13 MPa. There was no statistically significant difference between them. After four weeks, an increase in Cs value was noticed in calcium silicate cements, MTA Plus 16.47±1.89 MPa, and nCS 14.39±1.63 MPa but without statistically significant difference between them. Between the conventional GIC and CS cements there was a statistically significant difference ($p<0.05$).

DISCUSSION

Compressive strength is an indirect measure of bonding and strength of the material [11, 12]. It is an important property that may affect clinical performance [13]. This factor plays an important role in the treatment of functional perforations where cements are directly exposed to occlusal forces [14].

In the literature, significant variations in measured comprehensive strength have been reported as numerous factors can affect it. The cylindrical shape of the samples is convenient but sample surface perfection and intimate contact between samples and testing machine is hard to achieve [8]. Also, the size and shape of the samples, the preparation of the samples and hydration time, water/powder ratio, mixing technique, pressure while compacting, as well as the moisture and temperature of the room affect results [15, 16].

Conventional GIC are in wide use in clinical practice as cements or restorative materials. Many researches have been done with the goal to enhance mechanical and biological properties of GIC with incorporation of bioactive ceramic particles, glass powder and similar. Adding Zn has shown to have a stimulating effect on bone formation in *in vitro* and *in vivo* conditions, as well as antibacterial activity, similar to silver. Adding MgO increased cell proliferation [3]. Titanium oxide is added because it is chemically stable, biocompatible and has antibacterial properties, and has shown significant activity against *Streotococcus mutans* in nano-formulation [8].

It has been confirmed in our research, as well as by other researchers, that cement reaction continues after one day, because crosswise bonds are established in the cement matrix [3]. Shiowaza [17] pointed out that cement maturation (acid-base reaction) is continued in the first week that can be seen as an increase in compressive strength and it is then stable for the next 12 months. These results are consistent with the results of our research, where there was no further compressive strength value increase between 7 and 28 days.

Compressive strength is considered as one of the most important physical properties of hydraulic cements and it is in correlation with the degree of hydration [2], where hydration reaction is the key for hardening of hydraulic silicate cements.

Compressive strength of calcium silicate cements is initially, after 24h low. Bonding and hardening of the hydraulic cements depends on the formation of the CSH gel and ettringate (hydrated calcium sulfoaluminate) on the nucleation points of calcium hydroxyl crystals [18]. The presence or absence of these crystal formations (*ettringate crystals*) in different formulations of calcium silicate cements is probable reason for different values of compressive strengths between them [2]. In the ISO standard compressive strengths are still not defined for pulp covering or perforation materials, there is only a suggestion that materials are to be compared to the value of stress that occurs during amalgam condensation [19]. After seven days, the value of compressive strength of calcium silicate cements increases, where Cs of nCS further increases even after 28 days due to cement hydration.

The difference in compressive strength values between materials with similar or even the same composition can be explained by the size of the particles [11, 19], as well as experimental conditions. That is how Akbari et al. [6] found that the Cs of White MTA (Angelus, Brazil) was 1.16 MPa after 24h and 2.19 MPa after 7 days, while Natale et al. [20] found Cs to be 18 MPa after 7 days. Noh et al. [21] found that WMTA (ProRoot MTA) after 24h had an average value of 19.41 and after 7 days 46.18 MPa, while Basturk et al. [16] showed results as high as 84.17 MPa after 4 days for ProRoot MTA. The microstructure and homogenicity of the cement affect its strength because finer particles have greater ability to absorb moisture.

Hand mixing of materials can result in inadequate hydration due to the limited formation of micropores inside the material that compromise water penetration in the material. Mitchell and Douglas pointed out that hand mixed cements have lower comprehensive strength due to trapped air, while capsulated cements mixed in a centrifuge have higher Cs [22, 16].

Nanostructural materials have particles that are not over 100 nm in size (most often between 5 and 50 nm), but therefore have up to ten times bigger surface area, which stimulates greater ettringate crystal formation [23]. Nanostructures strive to solve one of the key problems of endodontic cements like bonding time. Experiments indicate that in almost all nano-powders kinetic absorption and desorption can be improved simply by reducing the particle size [14].

Perfecting materials that can be used as biological bone "substitutes" is currently one of the most valuable and most active fields of biomaterial research. Biocompatibility and bioactivity of these materials secure the interaction with biological systems. Bioactive materials like calcium silicate cements, especially with nanostruc-

ture, stimulate regeneration of damaged tissue, therefore renewing the function of damaged tissue or organs [7].

CONCLUSION

The null hypothesis that there is no difference in Cs between conventional and calcium silicate cements is rejected. The compressive strength of conventional glass ionomer cements was significantly higher after 24 h, increased after 7 days and remained the same after 28 days. MTA Plus showed higher compressive strength after 24 h and 7 days than newly synthesized nanostructural calcium silicate cement (nCS) but the values were similar after 28 days. Compressive strength of calcium silicate cement grows with time and cement hydration.

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Ispitivanje kompresivne čvrstoće kalcijum-silikatnih cementa

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KRATAK SADRŽAJ

Uvod Cilj ovog rada je bio da se proveri kompresivna čvrstoća (KČ) novog nanostrukturnog kalcijum-silikatnog cementa (nCS) i uporedi sa komercijalnim kalcijum-silikatnim cementom i konvencionalnim GJC u funkciji vremena.

Materijal i metod Testirana su četiri materijala – nanostrukturni CS (Jokanović i sar.), MTA Plus (Cerkamed, Poland), Fuji IX (GC Corporation, Japan) and Ketac Universal Aplicap (3M ESPE, USA). Po pet uzoraka za svaki materijal je zamešano po proizvođačkom uputstvu i postavljano u metalne kalupe (φ 4 mm i 6 mm visoke). KČ, izražena u megapaskalima, merena je posle 24 sata i posle 7 i 28 dana na univerzalnoj test mašini (Tinius Olsen, USA) sa brzinom utiskivača od 1 mm/min. Dobijeni rezultati su statistički obrađeni one-way ANOVA i post hoc Tukeys testovima.

Resultati Posle 24 sata najveću KČ imao je FUJI IX (38,56 ± 13,31 MPa), zatim Ketac Univerzal (40,77 ± 7,96 MPa). Kalcijum-silikatni cementi su pokazali niže vrednosti KČ 24 sata posle mešanja i to MTA 5,91 ± 0,28, a nCS 1,35 ± 0,36 MPa. Posle sedam dana KČ za FUJI IX je bila 47,72 ± 9,33 MPa, a za Ketac Universal 35,25 ± 10,60 MPa, dok je vrednost za MTA bila 15,09 ± 2,77 MPa, a nCS 11,06 ± 0,88. Posle 28 dana KČ za FUJI IX je bila 48,03 ± 7,82 MPa, a za Ketac 36,65 ± 11,13 MPa. KČ kalcijum-silikatnih cemenata posle 28 dana je bila 16,47 ± 1,89 za MTA, a za nCS 14,39 ± 1,63 MPa, bez statističi značajne razlike između njih. Između konvencionalnih GJC i CS cemenata postoji statistički značajna razlika ($p < 0,05$) posle 24 h, kao i posle 7 i 28 dana.

Zaključak Kalcijum-silikatni cementi su inicijalno pokazali niže vrednosti KČ u odnosu na konvencionalne GJC, ali su se one povećavale u funkciji vremena.

Ključne reči: kalcijum-silikatni cement; nanočestice; glasjonomer cement; kompresivna čvrstoća

UVOD

Idealan materijal za reparaciju oštećenja korena zuba treba da omogući zatvaranje komunikacije između kanala korena i okolnog tkiva, da je biokompatibilan, dimenziono stabilan i nerastvorljiv u kontaktu sa tkivnim fluidima. Materijal se često plasira u koren, gde je kiselo okruženje, a često i sa bakterijskom inflamacijom, pa je nizak pH važan faktor koji utiče na tvrdoću i druga svojstva cemenata [1]. Dugi niz godina u terapiji perforacija kanala korena se koriste različiti materijali kao što su kalcijum-hidroksid, cink-oksid eugenol cementi, kompozitne smole, glasjonomer cementi, ali nijedan u potpunosti ne ispunjava zahteve idealnog materijala [2].

GJC su razvijeni iz kombinacije dva različita cementa: silikatnih i cink-polikarboksilatnih [3]. Konvencionalni GJC nastaju acido-baznom reakcijom jona stakla sa vodenim rastvorom poliakrilne kiseline i u širokoj su upotrebi u kliničkoj praksi. Smatraju se potencijalnim biomaterijalima za ortopedske aplikacije zbog njihove sposobnosti da adheriraju na kost i metale i stabilnosti u vlažnoj sredini. Ipak, nedostatak bioaktivnog potencijala i loše mehaničke osobine još su uvek nerešivi.

Poslednjih godina se u endodontsku terapiju uvode izrazito biokompatibilni kalcijum-silikatni hidraulični cementi. Mineral trioksid agregat (MTA) uobičajeno se koristi kao biomaterijal za reparaciju korenskih i furkacionih perforacija, ali i u drugim indikacijama [4]. MTA je bioaktivan materijal koji na svojoj površini formira apatitni sloj u kontaktu sa fosfatima iz tkivnih tečnosti, ali i hibridni sloj između dentina i kalcijum-silikatnih materijala [5]. On takođe otpušta neke od svojih komponenti u fosfatne pufere pljuvačke, čime podstiče procese biomineralizacije [6]. Na tržištu postoji veći broj kalcijum-silikatnih cemenata sa ciljem da se prevaziđu neki nedostaci izvorne formulacije. MTA Plus je nanostrukturni MTA predstavljen 2012. godine, sa kraćim vremenom vezivanja i smanjenjem sadržaja teških metala (i do 90%) u svojoj formulaciji. Nanočestice omogućavaju uniformnu i homogenu strukturu, kao i oslobađanje niže toplote prilikom hidratacije cementa (izvor: proizvođač).

Upotreba nanopartikula je postala značajan aspekt istraživanja u stomatologiji, sa fokusom na poboljšanje mehaničkih osobina i antibakterijski efekat tih čestica. Sama veličina čestica nanomaterijala (<100 nm), koja je slična veličini bioloških molekula i struktura (proteini, organele, molekul DNK i vode), upućuje na moguću primenu nanomaterijala u biomedicinskim istraživanjima.

Novosintetisani nanostrukturni materijal korišćen u ovom radu je na bazi trikalcijum i dikalcijum silikata. Ovaj kalcijum-silikatni sistem je dobijen hidrotermalnom sol-gel metodom i samoširećom reakcijom sagorevanja [7], što mu je obezbedilo visoku aktivnost i kratko vreme vezivanja. Najmanji deo ovog sistema su kristaliti veličine oko 19,9 nm, koji pokazuju značajnu aktivnost sistema.

Testovi kompresivne čvrstoće (KČ) koriste se u stomatologiji za laboratorijsku simulaciju stresa koji je posledica sila koje klinički deluju na restaurativne materijale, ili materijale za prekrivanje i nadoknadu tkiva. Većina mastikatornih sila su kompresivne prirode i njihovu tačnu vrednost je teško utvrditi [8, 9].

Cilj ovog rada je bio da se proveri KČ novosintetisanog nanostrukturnog CS cementa i uporedi sa komercijalnim MTA Plus i najčešće korišćenim konvencionalnim GJC koji se koriste u reparaciji furkacionih ili kruničnih perforacija. Nulta hipoteza je da nema razlike u KČ između konvencionalnih i kalcijum-silikatnih cemenata.

MATERIJAL I METOD

U istraživanje su uključena četiri cementa:

Nanostrukturni kalcijum-silikatni sistem (nCS) (Jokanović i sar.) sadrži 60% ukupne količine β-C_2S i C_3S faza, 20% kalcijum-karbonata i 20% $BaSO_4$ (Merck, Nemačka) kao rendgen

kontrastno sredstvo. Prah je mešan sa destilovanom vodom u odnosu 1 : 2 voda : prah.

MTA Plus (Cerkamed, Poljska) ručno je mešan u odnosu 0,34 g destilovane vode sa 1 g praha MTA.

Konvencionalni GJC Fuji IX (GC Corporation, Japan) – prema uputstvu proizvođača mehanički su mešane kapsule 10 sek. u amalgamatoru na 4500 rpm.

Samoadhezivni i samovezujući GJC Ketak Universal Aplicap (3M ESP, USA), sa oksidima stakla, a tečna komponenta je kopolimer akrilne i maleične kiseline i vinska kiselina. Prema proizvođačkom uputstvu kapsule su mehanički mešane 10 sek. u amalgamatoru na 4500 rpm.

Priprema uzoraka

Materijali su nakon mešanja plasirani u dvodelne metalne kalupe promera 4 mm i visoke 6 mm, gde su kondenzovani ručnim nabijačem, po pet uzoraka za svaki materijal. Uzorci su držani na 37 C u parnom kupatilu 24 sata, 7 i 28 dana. Svi cilindrični uzorci su polirani najfinijim abrazivnim papirom sa minimalnim pritiskom i vizuelno proveravani, a uzorci sa oštećenjima u strukturi su odbačeni.

Testiranje KČ testiranih materijala je sprovedeno u skladu sa međunarodnim standardom ISO 9917-1:2007 (Dentistry-water-based cements- Part 1: powder/liquid acid-base cements) [10]. KČ je testirana na univerzalnoj test mašini (Tinius Olsen, USA 5KN) sa brzinom utiskivača od 1 mm/min. po dužoj osi cilindričnog uzorka. Sila potrebna da polomi uzorak je beležena, a KČ izražena u MPa je izračunavana po formuli $Cs = 4P/\pi d^2$, gde je P maksimalna sila potrebna da polomi uzorak izražena u N, a d je prečnik uzorka u mm.

Za obradu dobijenih rezultata korišćeni su one-way ANOVA i post-hoc testovi. Nivo značajnosti je određen kao $p < 0,05$.

REZULTATI

Dobijeni rezultati su prikazani u Tabeli 1 i 2 i Grafikonu 1.

Najveće vrednosti KČ posle 24 dana pokazali su konvencionalni GJC FUJI IX (38,56 ± 13,31) i Ketak Universal (40,77 ± 7,96), ali bez statističke razlike. Kalcijum-silikatni cementi su posle 24 sata pokazali niske vrednosti KČ, MTA Plus 5,91 ± 0,28 MPa, a nCS 1,35 ± 0,36 MPa, bez statističke značajnosti. Statistički značajna razlika je uočena između konvencionalnih GJC i CS cemenata ($p < 0,05$).

I posle sedam dana najveće vrednosti KČ su pokazali FUJI IX 47,42 ± 9,33 MPa i Ketak Universal 35,25 ± 10,60 MPa, ali bez statistički značajne razlike među njima. KČ MTA Plus je 15,09 ± 2,77 MPa, a nCS 11,06 ± 0,88 MPa, bez statistički značajne razlike između njih. Između konvencionalnih GJC i CS cemenata postoji statistički značajna razlika ($p < 0,05$).

Posle 28 dana uočena je stagnacija vrednosti KČ za konvencionalne GJC FUJI IX 48,03 ± 7,82 MPa i Ketak Universal 36,65 ± 11,13 MPa. Nije bilo statistički značajne razlike između GJC. Posle četiri nedelje uočava se porast vrednosti KČ za kalcijum-silikatne cemente, MTA Plus 16,47 ± 1,89 MPa, a nCS 14,39 ± 1,63 MPa, ali bez statistički značajne razlike između njih. Između konvencionalnih GJC i CS cemenata postoji statistički značajna razlika ($p < 0,05$) i posle 28 dana.

DISKUSIJA

KČ je indirektna mera vezivanja i čvrstoće materijala [11, 12] i važno je svojstvo koje može uticati na kliničke performanse materijala [13]. Ovaj faktor igra važnu ulogu u tretmanu furkacionih perforacija, gde su cementi direktno izloženi delovanju okluzalnih sila [14].

U literaturi se uočavaju velike varijacije u rezultatima, jer na njih utiču brojni faktori. Cilindričan oblik uzoraka je pogodan, ali savršenost površine uzoraka i intimni kontakt između uzorka i uređaja za testiranje je teško postići [8]. Osim toga, na reprezentativnost rezultata utiču veličina i oblik uzorka, priprema uzorka i vreme hidratacije, odnos voda/prah, tehnika mešanja, pritisak prilikom kompakcije, kao i vlažnost i temperatura prostorije [15, 16]. Ovi faktori utiču na fizička svojstva materijala, pa i na KČ.

Konvencionalni GJC su u širokoj upotrebi u kliničkoj praksi kao cementi ili kao restaurativni materijali. Mnoga istraživanja su rađena u cilju poboljšanja mehaničkih i bioloških osobina GJC inkorporacijom bioaktivnih keramičkih čestica, staklenog praha i sl. Dodavanje Zn je pokazalo da ima stimulativni efekat na formiranje kosti u *in vitro* i *in vivo* uslovima, kao i na antibakterijsku aktivnost, slično srebru. Dodavanje MgO je poboljšalo ćelijsku proliferaciju [3]. Titanijum-dioksid se dodaje jer je hemijski stabilan, biokompatibilan i ima antibakterijska svojstva, a u nanoformulaciji je pokazao i značajnu aktivnost protiv *Streptococcus mutansa* [8].

U ovom radu je potvrđeno, kao i kod drugih istraživača, da se cementna reakcija nastavlja i posle jednog dana, jer se u cementnom matriksu odvijaju poprečne veze [3]. Shiozawa [17] ističe da se maturacija cementa, odnosno acido-bazna reakcija nastavlja u prvoj nedelji, što se ogleda u porastu KČ, a onda ostaje na tom nivou sledećih 12 meseci. Ovi nalazi su u saglasnosti sa rezultatima ovog istraživanja, gde između 7 i 28 dana nije došlo do daljeg porasta vrednosti KČ konvencionalnih glasjonomer cemenata.

KČ se smatra jednom od glavnih fizičkih karakteristika hidrauličnih cemenata i u korelaciji je sa stepenom hidratacije [2], a reakcija hidratacije je ključna za očvršćavanje hidrauličnih silikatnih cemenata.

KČ kalcijum-silikatnih cemenata je inicijalno, posle 24 sata, niska. Vezivanje i jačina hidrauličnih cemenata zavisi od formiranja CSH gela i etringita (hidrirani kalcijum-sulfoaluminat) na mestima nukleacije kalcijum-hidroksilnih kristala [18] (Oloomi i sar. 2013). Prisustvo ili manjak ovih kristalnih formacija (*ettringite crystals*) u različitim formulacijama kalcijum-silikatnih cemenata su verovatno razlog nižih/različitih vrednosti KČ među njima [2]. U ISO standardu još uvek nisu definisane vrednosti KČ za materijale za prekrivanje pulpe ili perforacija, pa se sve svodi na preporuku da se materijali porede sa vrednostima stresa koji nastaje usled kondenzacije amalgama [19]. Posle sedam dana vrednosti KČ kalcijum-silikatnih cemenata izrazito se povećava, a kao posledica dalje hidratacije cementa, KČ nCS i dalje raste, posle 28 dana.

Razlike u vrednostima KČ između materijala koji imaju sličan ili pak isti sastav može se objasniti razlikama u veličini čestica [11, 19], kao i uslovima eksperimenta. Tako Akbari i sar. [6] nalaze da je KČ za White MTA (Angelus, Brazil) 1,16 MPa posle 24 sata, a 2,19 MPa posle sedam dana, dok Natale i sar. [20] nalaze da mu je KČ posle sedam dana 18 Mpa. Noh i sar.

[21] nalaze da je WMTA (ProRoot MTA, USA) posle 24 sata imao prosečne vrednosti 19,41, a posle sedam dana 46,18 MPa, dok Basturk i sar. [16] iznose vrednosti i do 84,17 MPa posle četiri dana za ProRoot MTA. Mikrostruktura i homogenost cementa utiču na njegovu čvrstoću, jer sitnije čestice imaju veću sposobnost apsorpcije vlage.

Ručno mešanje i unošenje materijala takođe može uticati na neadekvatnu hidrataciju zbog ograničenog formiranja mikropora unutar materijala, što kompromituje prodor vode u hidrat materijala. Mitchell i Douglas ističu da ručno mešani cementi imaju slabiju čvrstoću, zbog zarobljenog vazduha, dok inkapsulirani cementi koji se mešaju i centrifugiraju imaju veću KČ [22, 16].

Nanostrukturni materijali imaju čestice koje ne prevazilaze veličinu od 100 nm (najčešće između 5 i 50 nm), ali zato imaju i do deset puta veću interaktivnu površinu, što utiče na povećano formiranje etringit kristala [23]. Nanostrukture pokušavaju da reše jedan od ključnih problema endodontskih cemenata kao što je vreme vezivanja. Eksperimenti ukazuju na to da kod praktično svih nanoprahova kinetika apsorpcije i desorpcije može biti unapređena jednostavno smanjenjem veličine zrna [14].

Usavršavanje materijala koji bi mogli da se koriste kao biološke „zamene“ kosti danas je jedna od najznačajnijih i najaktivnijih oblasti istraživanja biomaterijala. Biokompatibilnost i bioaktivnost ovih materijala obezbeđuje interakciju sa biološkim sistemima. Bioaktivni materijali kakvi su kalcijum-silikatni cementi, posebno sa nanostrukturom, stimulišu regeneraciju oštećenih tkiva, a time i obnavljanje funkcije oštećenih tkiva ili organa [7].

ZAKLJUČAK

Nulta hipoteza da nema razlike u KČ između konvencionalnih i kalcijum-silikatnih cemenata je odbačena. KČ za konvencionalne glasjonomer cemente je bila značajno viša posle 24 sata, a rasla je posle sedam dana i ostala ista 28 dana. MTA Plus je pokazao veću KČ posle 24 sata i sedam dana u odnosu na novosintetisani nanostrukturni kalcijum-silikatni cement (nCS), ali ove vrednosti su se izjednačile posle 28 dana. KČ kalcijum-silikatnih cemenata značajno raste u funkciji vremena i sa hidratacijom cemenata.

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Lead concentration in hard dental tissues – SEM/EDS analysis

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SUMMARY

Introduction Currently, one of the most important ecological issues is exposure to lead in environment, since it is a metal with evident toxic effects on human organism. Hard dental tissues are suitable structures for assessing long-term effects of exposure to toxic metals.
The aim of this paper was to determine the concentration of lead in hard dental tissues of a rat with experimentally induced DM using SEM/EDS analysis, after 14 and 30 days of exposing animals to lead.

Material and methods The study was conducted in rats of Wistar strains divided into the three groups. The first group consisted of 8 rats (128 molars and premolars of the upper and lower jaws) with experimentally induced DM, taking lead in the course of 14 days at the concentration of 1500 ppm; the second group included 8 rats (128 molars and premolars of the upper and lower jaws) taking lead in the course of 30 days at the concentration of 1500 ppm, while the third control group consisted of 5 healthy rats (80 molars and premolars of the upper and lower jaws). Experimental animals received lead-acetate every day at the concentration of 1500 ppm via water ad libitum. In these animals, diabetes mellitus was induced by Alloxan. The teeth samples were analysed using scanning electron microscopy (SEM). EDS analysis determined the mass fraction of lead and other elements in hard dental tissues.

Results No lead was detected in a single tooth layer in the teeth of rats that received lead in drinking water in the course of 14 days. The average values of the mass fraction of lead, calcium, and phosphorus in enamel of teeth of rats receiving lead in the course of 30 days amounted to: lead 0.36%, calcium 15.48%, and phosphorus 10.62%. Lead was registered only in enamel.

Conclusion Lead was detected in enamel only in rats receiving lead in the course of 30 days while it was not detected in teeth after the course of 14 days.

Keywords: lead; enamel; dentin; SEM/EDS analysis

INTRODUCTION

Hard dental tissues consist of several different minerals that together with calcium represent major macromineral and therefore represent ideal tissues for assesing the long-term effects of exposure to toxic metals [1, 2, 3]. Exposure to lead is significant health problem in many countries because it is associated with the impact on general health (anemia, hypertension), or the pathology of bones and teeth, including dental caries [4, 5, 6].

Although lead levels in hard dental tissues are useful indicators of lead exposure, information on its time effects and lead compounds in dental tissues is very limited. Some studies have shown that the influence of lead on developing teeth does not have to be related only to its cytotoxic effects but also to interaction with proteins and enzymes of extracellular matrix. It has also been confirmed that the pulp metabolism is significantly delayed in some metabolic disorders such as diabetes [7, 8].

There are reports in the literature indicating that the presence of lead in chemical composition of enamel can alter its ultrastructure and lead to its damage. Thus, Gomes et al. in their study found in pre-school children's teeth, who lived in the industrial city area, higher lead concentration in enamel than in children who lived outside this area [9]. Data from ancient populations revealed high prevalence of hypoplastic enamel with high levels of lead in bones and teeth. Correlation between the presence of lead in dental tissues and clinical alteration of enamel was noticed in the form of discoloration. An increase of enamel hypoplasia has been confirmed in children exposed to high lead concentrations [10, 11, 12].

In vitro studies have shown that the presence of lead during amelogenesis can lead to ultrastructural alterations of enamel associated with modifications in physicochemical relationship and making enamel more sensitive to demineralization [13]. It has been found that the content of metal in teeth (in the population in areas polluted with lead) was associated with an increased incidence of caries lesions. However, the correlation between the influence of lead and development of dental caries in hard dental tissues is still subject of numerous studies [14].

Moss et al. confirmed correlation between lead exposure during dentin formation and increased caries prevalence [15], and Martin et al. found that lead affects formation of caries but only in deciduous teeth [16]. Gomes et

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al. investigated association between lead concentrations in deciduous teeth enamel and found no connection between lead and dental caries in children's teeth living in industrial zone [9].

Cenić-Milošević et al. examined correlation between the concentration of lead in extracted teeth of the inhabitants of Pančevo and Belgrade (among members of different age groups) and concluded that one of the possible causes of tooth loss and damages caused by caries was long-term exposure to lead [17]. Barmes and Ludmgh found correlation of lead concentration in the teeth and tooth decay, and concluded that subjects with high lead concentration had more teeth with dental caries. It has also been confirmed that lead, as a cytotoxic agent, can have an effect on ameloblasts, i.e. an alteration in the amount of proteins and delay in amelogenesis [18]. Gerlach et al. concluded that lead increases the concentration of proteins and slows down enamel mineralization of incisors of rats that consumed water contaminated with lead [19]. Tvinnereim et al. found association between lead exposure during dentin formation and increased sensitivity to caries in rats' teeth [20].

The aim of this paper was to determine using SEM/EDS analysis the concentration of lead in hard dental tissues of rats with experimentally induced DM after 14 and 30 days of animal exposure to lead.

MATERIAL AND METHODS

Wistar strain rats (21) and 336 teeth were selected for the experiment due to similarities in the physiology of dental pulp of rats and pulp physiology of human teeth. The study was approved by the Ethics Committee of the Institute of Dentistry of the Faculty of Medicine in Banja Luka. All rats were divided into the two groups: the first group consisted of 8 rats (128 molars and premolars of the upper and lower jaws) with experimentally induced DM taking lead in the course of 14 days at a concentration of 1500 ppm. The second experimental group consisted of 8 rats (128 molars and premolars of the upper and lower jaws) taking lead in the course of 30 days at a concentration of 1500 ppm. The control group consisted of 5 healthy rats (80 molars and premolars of the upper and lower jaws). The protocol of experimenatly induced diabetes mellitus in rats included the use of Alloxan solution which was applied intraperitoneally and the protocol for lead intoxication included the intoxication of adult rats using lead – acetate at the concentration of 1500 ppm via water ad libitum. All animal procedures, nurturing, experimental treatments, sacrificing without pain and stress were conducted in accordance with the guidelines for the care of animals used for experimental research („Guide for the Care and Use of Laboratory Animals", 1996 National Academy Press, Washington, DC). After decapitation, upper jawbones of rats were separated from soft tissues and stored in fixative (10% neutral buffered Formalin) and then prepared for the SEM-EDS analisys. The tooth samples were cut and polished with a diamond disc through the middle of the tooth in the medio-distal direction in order to expose the cross-section of the enamel zone and dentine mass. Recording and analyses were performed using the Scanning electron microscope (JEOL JSM 6460LV) and connected OXFORD INCAx-sight spectrum analyzer. For the purposes of this analysis, images were obtained using Back-scatter or Primary emissions of reflected electrons in Compo mode (BEIc), as it proved to be the most useful emphasizer of the enamel zone and dentine mass. The samples were observed at an acceleration of 20kV at a working distance (WD) of 10 mm, at the angle of incidence that was suitable for the inclination of polished surface of premolars and molars. The general image was given in clear magnification of 35x, and for the purposes of more precise EDS analysis, the magnification of 100x was used. The obtained results were analysed and processed statistically.

RESULTS

Table 1 shows the average values of the mass fraction of phosphorus, calcium, and lead in the teeth parts of all examined groups. The average values of the mass fraction of phosphorus in the teeth of the rats receiving lead in the course of 14 days was highest in the area of enamel - dentine junction (15.61%), dentin (13.96%) and the lowest values were in enamel (13.92%). In the rats receiving lead in the course of 30 days, the highest mass fraction was found in dentin (13.96%), then in enamel – dentin junction (21.91%), and the lowest in enamel (10.62%).

The highest average values of calcium, in teeth of the rats receiving lead through drinking water in the course of 14 days, were in enamel – dentin junction (25.66%), then in enamel (23.28%), and the lowest in dentin (22.35%). In the rats receiving lead in the course of 30 days, the mass fraction was 20.13% in the area of enamel – dentin junction, 15.48% in enamel, and 21.74% in dentin.

No lead was detected in any of the tooth layers in the teeth of rats receiving lead through drinking water in the course of 14 days. It was detected only in enamel of rats receiving lead in the course of 30 days (0.36).

Analysis of the obtained values of the mass fraction of certain elements in enamel indicated statistically significant difference only in the values of calcium ($p<0.05$). Also, lower values were recorded in the group of rats receiving lead through drinking water in the course of 14 days compared to the group of rats receiving lead in the course of 30 days. There was no statistically significant difference in the mass fraction of other elements (Table 1, Figure 1).

The relation between obtained values of the mass fraction of examined elements in all tooth parts was estimated by the Spearman's correlation coefficient. Statistically significant negative correlation between the mass fraction of calcium and mass fraction of lead was found, as well as statistically significant positive correlation with the mass fraction of phosphorus. The existence of statistically significant negative correlation between the mass fraction of lead and mass fraction of calcium was also found (Table 2).

Table 1. The average values of the mass fraction of certain elements in hard dental tissues in the examined groups
Tabela 1. Prosečne vrednosti masenog udela pojedinih elemenata u delovima zuba kod ispitivanih grupa

	Hard dental tissue Deo zuba	Group Grupa											
		Pb 14 days Pb 14 dana						Pb 30 days Pb 30 dana					
		N	X̄	SD	Med	Min	Max	N	X̄	SD	Med	Min	Max
Wt% P	Enamel Gleđ	12	13.92	3.39	14.66	8.14	19.22	7	10.62	4.49	10.90	1.36	14.51
	EDJ GDG	11	15.61	2.40	15.87	10.24	18.77	8	12.91	4.54	13.43	3.50	18.23
	Dentin	12	13.95	2.13	13.86	10.55	17.40	6	13.95	3.06	14.50	9.08	17.10
	Pulp Pulpa	0	.	.	.	.	.	1	16.55	.	16.55	16.55	16.55
	Total Ukupno	35	14.46	2.74	14.68	8.14	19.22	22	12.63	4.18	13.43	1.36	18.23
Wt% Ca	Enamel Gleđ	12	23.28	5.68	23.02	14.37	31.76	7	15.48	8.26	17.10	.67	25.88
	EDJ GDG	11	25.66	4.35	25.28	15.98	31.73	8	20.13	10.82	20.72	1.92	37.40
	Dentin	12	22.35	4.52	23.11	14.69	27.79	6	21.74	8.49	21.65	9.70	33.57
	Pulp Pulpa	0	.	.	.	.	.	1	29.10	.	29.10	29.10	29.10
	Total Ukupno	35	23.71	4.96	24.86	14.37	31.76	22	19.50	9.33	19.38	.67	37.40
Wt% Pb	Enamel Gleđ	12	.00	.00	.00	.00	.00	7	.36	.62	.00	.00	1.30
	EDJ GDG	11	.00	.00	.00	.00	.00	8	.00	.00	.00	.00	.00
	Dentin	12	.00	.00	.00	.00	.00	6	.00	.00	.00	.00	.00
	Pulp Pulpa	0	.	.	.	.	.	1	.00	.	.00	.00	.00
	Total Ukupno	35	.00	.00	.00	.00	.00	22	.11	.37	.00	.00	1.30

EDJ – enamel-dentin junction
GDG – gleđno-dentinska granica

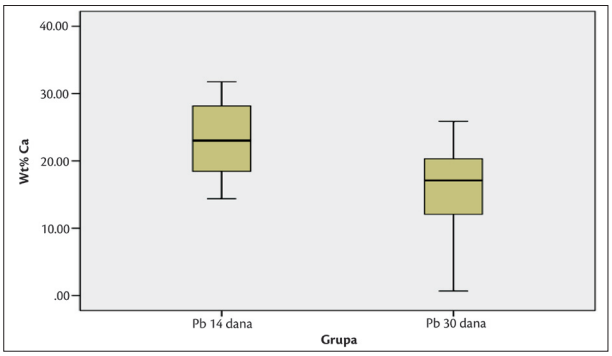

Figure 1. Mass fraction of calcium in enamel in the examined groups
Slika 1. Maseni udeo kalcijuma u gleđi u ispitivanim grupama

Table 2. Spearman's correlation coefficient of the mass fraction of certain elements in hard dental tissues
Tabela 2. Spirmanov koeficijent korelacije masenih udela pojedinih elemenata u tvrdim zubnim tkivima

Spearman's Rho Spirmanov koeficijent		Wt% P	Wt% Ca	Wt% Pb
Wt% P	Ro	1.000	.927**	-.249
	P	.	.000	.062
	N	57	57	57
Wt% Ca	Ro	.927**	1.000	-.272*
	P	.000	.	.040
	N	57	57	57
Wt% Pb	Ro	-.249	-.272*	1.000
	P	.062	.040	.
	N	57	57	57

** Correlation is statistically significant at the probability level of 0.01
** Korelacija je statistički značajna na nivou verovatnoće od 0,01
* Correlation is statistically significant at the probability level of 0.05
* Korelacija je statistički značajna na nivou verovatnoće od 0,05

DISCUSSION

Although the level of lead in hard dental tissues is useful indicator of lead exposure, information on its effects is very limited. Some heavy metals can replace calcium in hydroxyapatite crystals, and therefore the assessment of lead levels in the tooth should not be based solely on the absolute content of this metal, but also its relation to calcium. The aim of one of the more recent studies was to examine the presence of cadmium and lead in deciduous teeth in children suffering from celiac disease and food allergy in the industrial areas of Polland. Using flame atomic absorption spectrophotometry, it was confirmed that these metals were mainly accumulated in deciduous teeth. It has also been observed that toxic heavy metals in teeth remained in a dynamic balance with normal tooth formation, i.e. that they were replaced by calcium in hydroxyapatite crystals [1–4, 15]. Orzechowska-Wylęgała et al. came upon similar results. They found significantly

higher percentage of lead and cadmium compared to calcium in deciduous teeth of children with celiac disease and food allergies compared to the teeth of healthy children [21].

In their study, Malara et al. examined the content of lead and cadmium from tobacco smoke in deciduous teeth of children, whose parents were smokers, in relation to calcium using the method of atomic absorption spectrophotometry. The study found that toxic heavy metals accumulated in the teeth remained in a dynamic balance with normal tooth content (heavy metals replaced calcium in hydroxyapatite crystals) [22].

The results of these studies indicate that lead can replace calcium in hydroxyapatite crystals, which is consistent with the findings of our study. Analysing the obtained values of the mass fraction of certain elements it was noticed that the highest average value of calcium in the rats's teeth receiving lead through drinking water in the course of 14 days was in the area of enamel–dentin junction, then in enamel, and the lowest was in dentin. The lowest average value of calcium in the teeth of rats receiving lead in the course of 30 days was in the area of enamel-dentin junction. Lead in the teeth of rats who received it in the course of 30 days was observed in enamel only, indicating possibility of replacing calcium by lead in the area of enamel-dentin junction and enamel.

Liu et al. who analyzed the mass fraction of elements in enamel and dentin using induced mass spectrometry reported similar results. The samples were human teeth (third molars) collected in ambulances in Taiwan. The obtained results showed detected P in enamel with the mass fraction of 2.19%, Ca (27.91%), and Pb (0.72), and the concentrations of P, Ca and Pb were higher in dentin than enamel. Also, Ca/P ratio was constant [23].

The results of our study and the values of the mass fractions of P, Ca and Pb are consistent with the study conducted by Arora et al. where decreased mass fraction of Ca (24.35%) and P (12.41%) were found in the teeth of patients exposed to lead. They concluded that the concentration of lead in enamel and dentin has been increasing over the years, indicating that teeth can be realiable biomarker of lead contamination [24].

In our study, the SEM analysis protocol was performed on the same segments of all teeth (enamel, enamel-dentin junction and dentin) and lead was not detected in significant concentration. In the experimental groups, such alterations can be explained by the presence of carious lesions or the enamel defects with accumulated lead, detected in the surface parts of enamel.

The study of Bercovitz et al. included the analysis of the presence of lead in the teeth of children and adults using atomic absorption spectrophotometry, indicating higher lead concentration in children's teeth [25]. Youravong et al. examined enamel and dentin in children with high lead concentration in blood using the secondary ion mass spectrometry (SIMS) and x-ray microanalysis. These methods confirmed higher lead concentration in the area between dentin and pulp. X-ray microanalysis could not detect lead while the secondary ion mass spectrometry detected it in dentin near the border with pulp. SEM analysis with multi-element detectors is also a reliable method that can be effective in conventional chemical testing since it does not require standard protocols for sample preparation and it is faster [10].

Anttila examined lead concentration in enamel of deciduous incisors of children in Finland, Askola, a rural area, rich in radon. The study utilized Proton Induced X-rays, and the results showed that lead concentration (8.8+/-6.6 ppm) in enamel was similar to those found in other areas of Finland, indicating that radon does not cause significant increase in lead levels in enamel [26].

The results of these studies are consistent with the results obtained by Appleton, who examined lead concentration in rats's teeth using the SEM analysis. X-ray analysis showed the localization of lead in the form of, so-called, lead line as well as rapid drop of intracellular calcium that was replaced by lead ions [27]. Some studies reported similar results when administering an intravenous lead-acetate injection to mice caused response in dentin by forming so-called lead lines. This was associated with rapid but temporary increase in serum calcium and phosphorus due to the fact that lead replaced calcium and phosphorus in hydroxyapatite crystals [25, 10].

In our study, phosphorus was detected by SEM analysis and the analysis of the obtained results showed that phosphorus could have been replaced by lead, in addition to calcium, in hydroxyapatite crystals.

Issa's results showed that lead was detected in all molars from the experimental group. Higher lead concentration was found close to gingival part of the tooth than occlusal part, with a significant decrease in calcium concentration. These results are consistent with other studies that also have shown lower lead concentration in enamel. It should be emphasized that experimental animal species (rats) in the study by Issa et al. were given lead in the course of 60 days (30 mg / L) through drinking water and the rats in our study were given lead-acetate at the concentration of 1500 ppm (in water ad libitum) in a much shorter period. Much lower values of the mass fraction of lead and solely in the enamel also explain this [28].

Results that were not consistent with ours are the findings of Grobler et al. who examined pregnant female rats who received lead through drinking water during pregnancy and during lactation. Lead concentration in molars was tested by the atomic absorption spectrophotometry. It was found that the highest embedded concentration of lead in dental tissues was in females who drank water with the highest lead concentration. However, this research was conducted by applying high concentrations of lead in water that does not normally happen in the nature [29].

CONCLUSION

Based on the obtained results of this study, it can be concluded that the mass fraction of lead in the teeth of rats who received lead in the course of 30 days, was detected only in enamel, while lead in the teeth of rats who received lead in the course of 14 days was not detected in

any tooth structure. The mass fraction of calcium and phosphorous in enamel of rats who received lead in the course of 30 days was lower than in rats receiving lead in the course of 14 days.

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Koncentracija olova u tvrdim zubnim tkivima – SEM/EDS analiza

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KRATAK SADRŽAJ

Uvod Izloženost olovu u životnoj sredini je danas jedna od važnijih ekoloških tema, s obzirom na to da se radi o metalu sa izrazitim toskičnim efektima na ljudski organizam. Čvrsta tkiva zuba predstavljaju dobre strukture za procenu dugoročnih efekata izlaganja toksičnim metalima.

Cilj ovog rada je bio da se SEM/EDS analizom odredi koncentracija olova u tvrdim zubnim tkivima pacova sa eksperimentalno izazvanim DM-om, nakon 14 i 30 dana izlaganja životinja olovu.

Materijal i metode Istraživanje je sprovedeno kod pacova soja Vistar podeljenih u tri grupe. Prvu grupu je činilo osam pacova (128 molara i premolara gornje i donje vilice) sa eksperimentalno izazvanim DM-om koji su uzimali olovo tokom 14 dana u koncentraciji od 1500 ppm, drugu grupu osam pacova (128 molara i premolara gornje i donje vilice) koji su uzimali olovo tokom 30 dana u koncentraciji od 1500 ppm, dok je treću kontrolnu grupu činilo pet zdravih pacova (80 molara i premolara gornje i donje vilice). Eksperimentalne životinje su svakog dana dobijale olovo-acetat u koncentraciji od 1500 ppm putem vode *ad libitum*. Dijabetes melitus kod ovih životinja je indukovan aloksanom. Uzorci zuba su analizirani skening elektronskom mikroskopijom (SEM). EDS analizom je određen maseni udeo olova i ostalih elemenata u tvrdim zubnim tkivima.

Rezultati U zubima pacova koji su dobijali olovo u vodi za piće tokom 14 dana nije detektovano olovo ni u jednom sloju zuba. Prosečne vrednosti masenih udela olova, kalcijuma i fosfora u gleđi zuba pacova koji su dobijali olovo 30 dana iznosile su: za olovo 0,36%, za kalcijum 15,48% i za fosfor 10,62%. Olovo je registrovano samo u predelu gleđi.

Zaključak Olovo je detektovano u zubima pacova koji su dobijali olovo toko 30 dana i to samo u gleđi, dok olovo u zubima pacova koji su ga dobijali u vodi za piće tokom 14 dana nije detektovano ni u jednom sloju zuba.

Ključne reči: olovo; gleđ; dentin; SEM/EDS analiza

UVOD

Čvrsta zubna tkiva se sastoje od nekoliko različitih minerala koji sa kalcijumom predstavljaju glavni makromineral pa zato predstavljaju idealna tkiva za procenu dugoročnih efekata izlaganja organizma toksičnim metalima [1, 2, 3].

Izloženost olovu je danas značajan zdravstveni problem u mnogim zemljama jer je ono povezano sa uticajem na opšte zdravlje (anemija, hipertenzija), odnosno na patologiju kostiju i zuba, uključujući pre svega karijes zuba [4, 5, 6].

Iako su nivoi olova u tvrdim zubnim tkivima korisni pokazatelji izloženosti olovu, informacije o njegovom vremenskom delovanju i jedinjenjima olova u tkivima zuba su vrlo ograničene. U nekim studijama se pokazalo da uticaj olova na zube u razvoju ne mora biti povezan samo sa njegovim citotoksičnim efektima već i sa međusobnom interakcijom sa proteinima i enzimima ekstraćelijskog matriksa. Potvrđeno je takođe da je metabolizam pulpe značajno usporen u nekim metaboličkim oboljenjima, kao što je dijabetes [7, 8].

Postoje izveštaji u literaturi koji ukazuju na to da prisustvo olova u hemijskom sastavu gleđi može promeniti njegovu dentalnu ultrastrukturu i dovesti do oštećenja gleđi. Tako je i Gomes sa saradnicima u svojoj studiji utvrdio u zubima predškolske dece koja su živela u industrijskoj oblasti grada veće koncentracije olova u gleđi nego kod dece koja su živela van ove oblasti [9].

Podaci drevnih populacija otkrili su visoku rasprostranjenost defekata hipoplastične gleđi kod populacije koja je imala visoke nivoe olova u kostima i zubima. Uočena je povezanost između prisustva olova u dentalnom tkivu i kliničkih promena u gleđi u vidu diskoloracije i potvrđeno da je hipoplazija gleđi povećana kod dece izložene visokim koncentracijama olova [10, 11, 12].

In vitro studije su pokazale da prisustvo olova u toku amelogeneze može dovesti do ultrastrukturnih promena gleđi, koje mogu biti povezane sa modifikacijama u fizičko-hemijskom odnosu i time gleđ učiniti osetljivijom na demineralizaciju [13].

Jedno od istraživanja je pokazalo da je sadržaj metala u zubima (kod stanovništva u oblastima zagađenim olovom) povezan sa povećanom incidencom karijesa. Međutim, povezanost uticaja olova i nastanka karijesa u tvrdim zubnim tkivima još uvek je predmet brojnih istraživanja [14].

Moss i saradnici su potvrdili povezanost između izloženosti olova u vreme obrazovanja dentina i povećane zastupljenosti karijesa [15], a Martin i saradnici su zaključili da olovo utiče na nastanak karijesa samo kod mlečnih zuba [16]. Gomes i saradnici su procenjivali povezanost između koncentracije olova u gleđi mlečnih zuba i nisu pronašli povezanost između olova i dentalnog karijesa kod dece u industrijskoj zoni [9].

Cenić-Milošević i saradnici su pokušali da utvrde korelaciju između koncentracije olova u izvađenim zubima stanovnika Pančeva i Beograda (kod pripadnika različitih starosnih grupa) i zaključili da je jedan od mogućih uzroka gubitka zuba i karijesnih oštećenja upravo dugotrajna izloženost olovu [17].

Barmes i Ludmgh su ustanovili korelaciju koncentracije olova u zubima i zubnog kvara, i zaključili da su ispitanici sa visokim koncentracijama olova imali veći broj karijesnih zuba.

Potvrđeno je takođe da olovo kao citotoksični agens može dovesti do uticaja na ameloblaste, to jest promena u količini proteina i kašnjenja u amelogenezi [18].

Gerlach i saradnici su zaključili da olovo povećava koncentraciju proteina, a usporava mineralizaciju gleđi kod sekutića pacova koji su pili vodu sa prisutnim olovom [19].

Tvinnereim i saradnici su uočili vezu između izlaganja olovu u trenutku formiranja dentina i povećanja osetljivosti na karijes kod zuba pacova [20].

Osnovni cilj ovog rada je bio da se SEM/EDS analizom odredi koncentracija olova u tvrdim zubnim tkivima pacova sa eksperimentalno izazvanim DM-om, nakon 14 i 30 dana izlaganja životinja olovu.

MATERIJAL I METOD RADA

Za uzorak su odabrani pacovi soja Vistar, zbog velike sličnosti u fiziologiji pulpe zuba pacova sa fiziologijom pulpe humanih zuba. U eksperiment je uključen 21 laboratorijski pacov soja Vistar, odnosno 336 zuba. Studija je odobrena od strane Etičkog komiteta Zavoda za stomatologiju Medicinskog fakulteta u Banjaluci. Eksperimentalne grupe pacova su podeljene u dve grupe: prvu grupu je činilo osam pacova (128 molara i premolara gornje i donje vilice) sa eksperimentalno izazvanim DM-om koji su uzimali olovo tokom 14 dana u koncentraciji od 1500 ppm. Drugu eksperimentalnu grupu je činilo osam pacova (128 molara i premolara gornje i donje vilice) koji su uzimali olovo tokom 30 dana u koncentraciji od 1500 ppm. Kontrolnu grupu je činilo pet zdravih pacova (80 molara i premolara gornje i donje vilice). Protokol eksperimentalno indukovanog dijabetes melitusa kod pacova je uključivao primenu rastvora aloksan, koji je aplikovan intraperitonealno, a protokol za intoksikaciju olovom je obuhvatao intoksikaciju adultnih pacova olovnim acetatom u koncentraciji od 1500 ppm putem vode *ad libitum*. Sve procedure na životinjama, negovanje, eksperimentalni tretman, žrtvovanje bez bola i stresa izvedeni su u skladu sa Smernicama za brigu o životinjama u eksperimentalnim istraživanjima (Guide for the Care and Use Laboratory Animals, 1996 National Academy Press, Washington, DC). Nakon dekapitacije, gornjovilične kosti pacova su odvajane od mekih tkiva, pohranjene u fiksativ (10% neutralni puferovani formalin) i potom su u uzorku zuba pripremljeni za SEM/EDS analizu. Uzorci zuba su sečeni i polirani dijamantskim diskom kroz sredinu zuba u mediodistalnom smeru kako bi se eksponirao poprečni presek zone gleđi i dentinske mase. Snimanje i analize su urađeni na Skening elektronskom mikroskopu (JEOL JSM 6460LV) i priključenom OXFORD INCAx-sight spektralnom analizatoru. Za potrebe ove analize slike su dobijene Back-scatterovanom ili Primarnom emisijom odbijenih elektrona u Compo modu (BEIc) jer se pokazalo da najkorisnije ističe zone gleđi i dentinske mase. Uzorci su posmatrani pri ubrzanju od 20kV na radnoj distanci (WD) od 10 mm i pod upadnim uglom koji je bio primeren nagibu polirane površine premolara i molara. Opšti snimak dat je u preglednom uvećanju 35×, a za potrebe preciznije EDS analize upotrebljeno je uvećanje 100×. Dobijeni rezultati su analizirani i statistički obrađeni.

REZULTATI

U Tabeli 1 prikazane su prosečne vrednosti masenih udela fosfora, kalcijuma i olova u delovima zuba kod svih ispitivanih grupa. Prosečne vrednosti masenih udela fosfora u zubu pacova koji su dobijali olovo 14 dana bila je najveća u predelu gleđno-dentinske granice (15,61%), potom u predelu dentina (13,96%) a najmanja u predelu gleđi (13,92%). Kod pacova koji su dobijali olovo 30 dana najveći maseni udeo je uočen u dentinu (13,96%), zatim u oblasti gleđno-dentinske granice (21,91%), a najmanja u gleđi (10,62%).

Prosečne vrednosti udela kalcijuma u zubima pacova koji su dobijali olovo u vodi za piće tokom 14 dana bila je najveća u predelu gleđno-dentinske granice (25,66%), potom u gleđi (23,28%) i najmanja u dentinu (22,35%). Kod pacova koji su dobijali olovo 30 dana maseni udeo u oblasti gleđno-dentinske granice je iznosio 20,13%, u gleđi 15,48%, dok je u dentinu iznosio 21,74%.

U zubima pacova koji su dobijali olovo u vodi za piće tokom 14 dana nije detektovano olovo ni u jednom sloju zuba. Kod pacova koji su dobijali olovo 30 dana, ono je bilo detektovano samo u gleđi (0,36).

Analiza dobijenih vrednosti masenog udela pojedinih elemenata u gleđi je ukazala da postoji statistički značajna razlika jedino u vrednostima kalcijuma ($p < 0,06$). I ovde su manje vrednosti zabežene u grupi pacova koji su dobijali olovo u vodi za piće tokom 14 dana u odnosu na grupu pacova koji su dobijali olovo tokom 30 dana. U masenim udelima ostalih elemenata nije dobijena statistički značajna razlika (Tabela 1, Slika 1).

Povezanost dobijenih vrednosti masenog udela pojedinih elemenata u određenim delovima zuba procenjivana je Spiromanovim koeficijentom korelacije. Uočeno je da postoji statistički značajna negativna korelacija između masenog udela kalcijuma i masenih udela olova i statistički značajna pozitivna korelacija sa masenim udeom fosfora. Takođe je uočeno da postoji statistički značajna negativna korelacija između masenog udela olova i masenog udela kalcijuma (Tabela 2).

DISKUSIJA

Iako je nivo olova u tvrdim zubnim tkivima koristan pokazatelj izloženosti olovu, informacije o njegovim efektima u tkivima zuba su vrlo ograničene. Pojedini teški metali mogu zameniti kalcijum u kristalima hidroksiapatita pa se zato i procena nivoa olova u zubu ne bi smela bazirati samo na apsolutnom sadržaju ovog metala već i na njegovom odnosu prema kalcijumu. Jedna od savremenijih studija imala je za cilj da ispita zastupljenost kadmijuma i olova u mlečnim zubima kod dece koja pate od celijaklije i alergije na hranu u industrijskim oblastima Poljske. Upotrebom plamene atomske apsorpcione spektrofotometrije uvrđeno je da su se ovi metali najviše akumulirali u mlečnim zubima. Takođe je uočeno da su toksični teški metali u zubima ostali u dinamičnom balansu sa normalnom građom zuba, tj. da su zamenjeni kalcijumom u hidroksiapatitnim kristalima [1–4,15].

Do sličnih rezultata su došli Orzechowska-Wylęgała i saradnici, koji su utvrdili da u mlečnim zubima dece sa celijaklijom i alergijama na hranu postoji znatno veći maseni udeo olova i kadmijuma u odnosu na kalcijum nego u zubima zdrave dece [21].

U svojoj studiji Malara i saradnici su ispitivali sadržaj olova i kadmijuma iz duvanskog dima u mlečnim zubima dece čiji su roditelji pušači, odnosno njihov odnos sa kalcijumom, metodom atomske apsorpcione sektrofotometrije. Studija je utvrdila da su toksični teški metali koji se talože u zubu ostali u dinamičnom

balansu sa normalnim sadržajem zuba (teški metali su zamenili kalcijum u hidroksiapatičnim kristalima) [22].

Rezultati navedenih studija ukazuju da olovo može zameniti kalcijum u kristalima hidroksiapatita, što je u skladu i sa nalazima ovog istraživanja. Analizom dobijenih vrednosti masenog udela pojedinih elemenata uočeno je da je prosečna vrednost udela kalcijuma u zubu pacova koji su dobijali olovo u vodi za piće tokom 14 dana bila najveća u predelu gleđno-dentinske granice, potom u gleđi i najmanja u dentinu, a da je prosečna vrednost masenog udela kalcijuma u zubu pacova koji su dobijali olovo 30 dana bila najniža u oblasti gleđno-dentinske granice. Prosečna vrednost masenog udela olova u zubu pacova koji su dobijali olovo 30 dana uočena je samo u gleđi, što ukazuje na mogućnost zamene kalcijuma olovom u oblasti gleđno-dentinske granice i gleđi kod testiranih pacova.

Do sličnih rezultata su došli i Liu i saradnici, koji su sproveli studiju u kojoj su određivali maseni udeo elemenata u gleđi i dentinu pomoću indukovane masene spektrometrije. Uzorke su činili humani zubi (treći molari) sakupljeni u ambulantama u Tajvanu. Dobijeni rezultati su pokazali da je u gleđi detektovan P čiji je maseni udeo iznosio 2,19%, zatim Ca (27,91%) i Pb (0,72), a koncentracija P, Ca i Pb je bila veća u dentinu nego u gleđi. Takođe je i odnos Ca i P bio konstantan [23].

Rezultati ovog istraživanja i vrednosti masenih udela P, Ca i Pb su saglasni sa istraživanjem Arore i saradnika, gde je takođe uočen smanjen maseni udeo Ca (24,35%) i P (12,41%) u zubima pacijenata izloženih uticaju olova. Arora i saradnici su zaključili da koncentracija olova u gleđi i dentinu raste sa godinama i ukazuju da zub može biti pouzdan biomarker za olovo [24].

U ovom istraživanju je protokol SEM analize bio sproveden na istim segmentima svih zuba (gleđi, gleđno-dentinskoj granici i u dentinu), a olovo nije detektovano u značajnoj koncentraciji. U eksperimentalnim grupama ove studije ovakve promene se mogu objasniti prisustvom karijesnih šupljina ili defekata gleđi u koje se nataložilo olovo, a koje je SEM analizom detektovano u površinskim delovima gleđi.

Studija Bercovitza i saradnika je obuhvatala analizu prisustva olova u zubima dece i odraslih pomoću atomske apsorpcione spektrofotometrije i ukazala na veću koncentraciju olova u dečjim zubima [25].

Youravonga i saradnici su ispitivali gleđ i dentin kod dece sa visokom koncentracijom olova u krvi metodom sekundarne jonske masene spektrometrije (secondary ion mass spectrometry-SIMS) i mikroanalizom x-zracima. Ove metode su ukazale na vidljiv nivo olova u dentinu na granici sa pulpom. Mikroanaliza x-zracima nije mogla detektovati olovo, dok ga je sekundarna jonska masena spektrometrija detektovala u dentinu blizu granice sa pulpom. SEM analiza sa multielementnim detektorima je takođe pouzdana metoda koja može biti efikasna kod konvencionalnog hemijskog ispitivanja jer ne zahteva standardne protokole pripreme uzoraka i kraće traje [10].

Anttila je ispitivala koncentraciju olova u gleđi mlečnih sekutića dece iz Askole, ruralnog područja, najbogatijeg radonom, u Finskoj. Ispitivanje je rađeno pomoću protonske indukcije x-zracima, a rezultati su pokazali da je koncentracija olova (8,8 ± 6,6 ppm) u gleđi zuba bila slična nalazima iz drugih oblasti Finske, ukazujući na to da radon ne utiče na značajan porast nivoa olova u gleđi zuba [26].

Rezultati ovih istraživanja su saglasni sa rezultatima Appletona, koji se bavio ispitivanjem olovne linije u zubima pacova pomoću SEM-a. Analizom uz pomoć x-zraka je uočena lokalizacija olova u vidu tzv. olovne linije i brzi pad intracelularnog kalcijuma, koji je bio zamenjen jonima olova [27]. Do sličnih rezultata su došle neke studije koje su pokazale da se davanjem intravenske injekcije olovo-acetata miševima javlja odgovor u dentinu zuba, formiranjem tzv. olovne linije. Ovo je bilo povezano sa brzim, ali privremenim porastom serumskog kalcijuma i fosfora, tj. činjenice da olovo zamenjuje kalcijum i fosfor u kristalima hidroksiapatita [25, 10].

U ovoj studiji je takođe SEM analizom detektovan fosfor, a analiza dobijenih rezultata je pokazala da je olovo pored kalcijuma moglo zameniti i fosfor u kristalima hidroksiapatita.

Rezultati Issa su pokazali da je olovo detektovano u svim molarima iz eksperimentalne grupe, a veća koncentracija olova je uočena uz gingivalnu nego uz bukookluzalnu ivicu, uz značajno smanjenje koncentracije kalcijuma. Ovi rezultati su u skladu sa ovim istraživanjima jer je i ovde uočena manja koncentracija olova u gleđi zuba. Treba naglasiti da su eksprimentale životinje (pacovi) u studiji Issa i saradnika dobijali olovo 60 dana (30 mg / L) u vodi za piće, a pacovi u našoj studiji dobijali olovo-acetat u koncentraciji 1500 ppm (u vodi *ad libitum*) u mnogo kraćem periodu. To objašnjavaju i mnogo manje vrednosti masenog udela olova i to samo u gleđi [28].

Rezultati koji nisu bili u skladu sa našim su nalazi Groblera i saradnika, koji su ispitivali trudne ženke pacova koje su dobijale olovo u vodi za piće tokom trudnoće i tokom laktacije. Koncetracija olova u molarima je ispitivana atomskom apsorpcionom spektrofotometrijom i utvrđeno je da je olovo najviše ugrađeno u zubno tkivo kod ženki koje su pile vodu sa najvećom koncentracijom olova. Međutim, ovo istraživanje je urađeno primenom visokih koncentracija olova u vodi koja se inače ne nailazi u životnoj sredini [29].

ZAKLJUČAK

Na osnovu dobijenih rezultata ovog istraživanja može se zaključiti da je maseni udeo olova u zubima pacova koji su dobijali olovo 30 dana bio detektovan samo u gleđi, dok olovo u zubima pacova koji su dobijali olovo tokom 14 dana nije detektovano ni u jednom sloju zuba.

Maseni udeo kalcijuma u zubima pacova koji su dobijali olovo 30 dana u oblasti gleđi je bio niži u odnosu na vrednosti udela kalcijuma u gleđi zuba pacova koji su dobijali olovo tokom 14 dana, a maseni udeo fosfora u zubima pacova koji su dobijali olovo tokom 30 dana bio je najmanji u gleđi, te je bio niži nego u gleđi zuba pacova koji su dobijali olovo tokom 14 dana.

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Evaluation of Adhesive Remnant Index after metal brackets removal using AutoCAD software

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SUMMARY

Introduction After the completion of treatment with fixed orthodontic appliances, it is necessary to remove the brackets and bands from teeth using an appropriate method. The aim of this study was to determine the most common way of bond failure between teeth and metal brackets, as well as to compare bond failure between the brackets and upper and lower premolars.

Material and Method Metal brackets were bonded with Aspire composite material on 154 human premolars, extracted for orthodontic purposes. After debonding, the surface of remaining adhesive on the teeth and brackets was measured. Adhesive Remnant Index (ARI) was used to estimate bond failure between teeth and metal brackets.

Results The average size of remaining adhesive surface after removing brackets from the upper premolars was 12.06 mm^2, while it was 9.32 mm^2 on the lower premolars. The average size of the remaining adhesive surface area on the brackets removed from the upper premolars was 0.37 mm^2, while it was 2.08 mm^2 on the brackets removed from lower premolars. A statistically significant difference was found between these values. The most common score of ARI_{teeth} was 3 (85.71%) and the most frequent score of $ARI_{brackets}$ was 0 (85.71%).

Conclusion The most common way of bond failure between teeth and metal brackets was between the bracket base and adhesive surface. A statistically significant difference was found between the values of the size of residual adhesive surface on the upper and lower premolars as well as on the brackets debonded from them.

Keywords: bracket removal; surface of residual adhesive; Adhesive Remnant Index (ARI)

INTRODUCTION

Treatment with fixed orthodontic appliances is one of the most frequently used procedures for correcting orthodontic irregularities and achieving proper occlusal relationships. After completion of orthodontic treatment, it is necessary to remove brackets and bands from the teeth as well as the remaining adhesive. Debonding of the brackets depends on the type of brackets used (metal, ceramic or plastic). Metal brackets are usually removed using the appropriate orthodontic pliers and force of traction, torsion or shearing [1, 2].

Several factors may influence enamel-bracket shear bond strength: the type of adhesive systems used for bonding brackets, the type and duration of enamel etching, the size and type of brackets, the design of bracket base and oral environmental factors. These factors and the type of debonding procedure affect the type of bond failure between teeth and brackets as well as the amount of remaining adhesive on both [3–6].

Optimal bond strength between teeth and metal brackets ranges from 5.9 MPa to 8 MPa. If bond strength is greater than these values, it is possible to cause enamel damage during debonding procedure. The highest risk of enamel fracture is present at the moment of bracket debonding. Plaque accumulation and tooth pigmentation often occurs in the area of these fractures. During bracket debonding, bond failure can occur: on the bracket, between the bracket and adhesive, on adhesive, between enamel and adhesive, on enamel or mixed type of bond failure may occur. It is desirable that, after removing brackets, adhesive completely remains on the surface of a tooth to avoid possibility of enamel microcracks and fractures [2, 7, 8, 9].

As per literature, bond failure between adhesive and a bracket base is the most common [10–14]. Some authors correlated morphological characteristics of teeth and mode of bond failure between teeth and brackets, due to larger amounts of adhesive found to remain on buccal surfaces of upper teeth in comparison to lower teeth, after bracket removal. Studies by Hobson at al. and Ozturk at al. concluded that differences in morphological characteristics among groups of teeth affect shear bond strength between brackets and teeth, regardless of the type of adhesive system used

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[15, 16]. Zannarini at al. also noted difference between the values of remaining adhesive surface on bracket bases on the upper and lower teeth, namely between upper and lower incisors and upper and lower canines. They found higher amount of adhesive remained on the brackets removed from the upper teeth in relation to the lower teeth [17].

One of the most commonly used methods for determining the mode of bond failure between a tooth surface and a bracket base is the use of Adhesive Remnant Index (ARI), which was introduced by Artun and Bergland in 1984 [18]. According to this index, it is possible to precisely determine location of bond failure using different types of brackets and adhesive systems. To analyze the surface of residual adhesive on teeth and brackets, stereomicroscopy is often used, as well as the appropriate computer software [17, 19, 20].

The aim of this study was to determine the most frequent mode of bond failure between teeth and metal brackets using the ARI index and AutoCAD program, and to compare bond failure between upper and lower premolars and metal brackets.

MATERIAL AND METHOD

For the study, 154 human premolars extracted for orthodontic purposes were collected (77 upper premolars and 77 lower premolars). The tooth selection criteria implied that teeth had an intact oral and buccal surface without visible damage, white spots and carious lesions, and they had not been previously exposed to chemicals such as hydrogen peroxide or an acid for the purpose of etching. The teeth were kept until the beginning of the study in saline with 0.1% thymol to prevent dehydration. Prior to the experiment, the teeth were mechanically cleaned with a brush and non-abrasive non-fluoride paste. The middle third of the buccal surface of each tooth was treated with 38% orthophosphoric acid for 20 seconds and then rinsed with water for 30 seconds and dried for another 30 seconds. Buccal surface of 154 teeth was primed with Aspire orthodontic adhesive 7GM (OC Orthodontics, USA), according to the manufacturer's instructions and then polymerized for 10 seconds. Metal brackets (Ortho Organizer Elite OptiMIM, Henry Schein Orthodontics, USA) were bonded to the prepared surface of tooth with Aspire orthodontic adhesive 5GM (OC Orthodontics, USA) and light cured for 40 seconds according to the manufacturer's instructions (Figure 1 and 2).

The teeth were left in artificial saliva at the room temperature for 48 hours to allow the adhesive system to reach its maximum strength. *Biotene gel* (*GlaxoSmith-Kline, Belgium*) was used as a source of artificial saliva. The brackets were debonded with Ixion bracket removing pliers (DB Orthodontics, West Yorkshire, UK). After a sample preparation process, digital photographs of the buccal surfaces and the bracket bases were taken with a digital camera Nikon D5100. The procedure was carried out using a macroscopic lens at an appropriate distance of 20 cm. The sample was placed on a millimeter scale foil that served as a reference measure for the calibration for AutoCAD software (*Autodesk inc. San Rafael, CA, USA*) [21]. AutoCAD software is the most commonly used Autodesk program for designing engineering project documentation in 2D and 3D design. In this study, it was used to determine the surface of the remaining adhesive on a tooth and bracket as well as to determine the surface of a bracket base. Since the measurement was performed in millimeters, the images were calibrated so that the lengths in the program are set in millimeters. After calibrating the images in AutoCAD, the polyline lines in the form of "closed polygons" overlaid the surface of the remaining adhesive on the tooth and the base of the bracket, as well as the bracket base surface, and the values of marked parameters were automatically obtained (Figure 3).

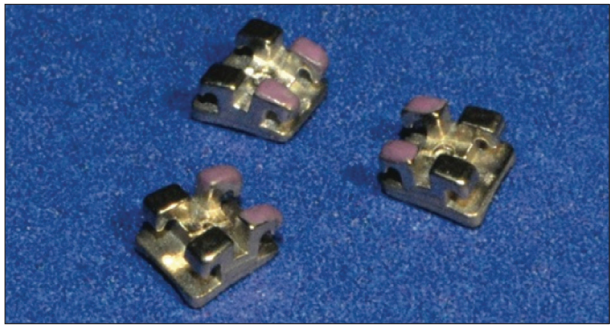

Figure 1. Brackets Ortho Organizers Elite OptiMIM
Slika 1. Bravice Ortho Organizers Elite OptiMIM

Figure 2. Aspire orthodontic adhesive system
Slika 2. Ortodontski adhezivni sistem Aspire

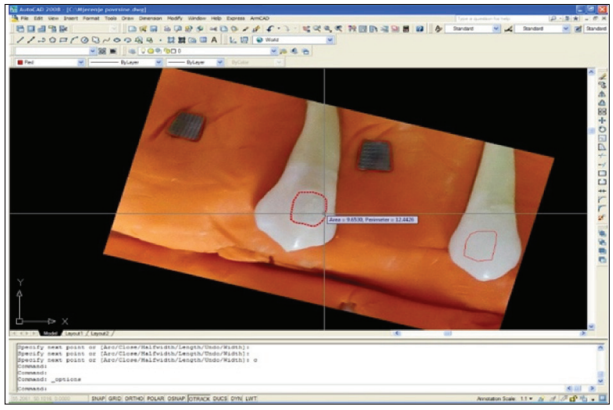

Figure 3. Analysis of the remaining adhesive on the teeth and brackets and measurement of the brackets surface size using AutoCAD software
Slika 3. Određivanje površine preostalog adheziva na zubu i na bravici, kao i površine baze bravice korišćenjem programa AutoCAD

The value of the remaining adhesive surface on teeth was evaluated according to the ARI_{teeth} scores from 0 to 3 as follows:

score 0 - 0% of the remaining adhesive on enamel surface,

score 1 - less than 50% of the remaining adhesive on the surface of a tooth,

score 2 - more than 50% of the remaining adhesive on the tooth surface and

score 3 - 100% of the remaining adhesive on the tooth surface.

The residual adhesive surface on bracket bases was evaluated by the $ARI_{brackets}$ as follows:

score 0 - less than 10% of remaining adhesive on the surface of the bracket,

score 1 - more than 10% but less than 50% of remaining adhesive on the surface of the bracket,

score 2 - more than 50% but less than 90% of adhesive remaining on the surface of the bracket and

score 3 - more than 90% of adhesive on the surface of the bracket [18].

Qualitative data (ARI scores) were presented by the number of occurrences and percentages. Descriptive statistic indicators (arithmetic mean, standard deviation, extreme values) were used to show the surface of the remaining adhesive on teeth and brackets after debonding. Parametric Student t-test for independent samples was used to compare the mean values of the characteristics. A statistically significant difference was defined as $p<0.05$, with values in which $p<0.01$ were stated as statistically very significant. Pearson's parametric correlation was used to determine the degree of correlation of the observed characteristics.

RESULTS

The average value of metal bracket base surfaces Ortho Organizer Elite OptiMIM for premolars, which were bonded to 154 premolars, was 11.07 mm^2. After removing the brackets, the AutoCAD program determined the surface of the remaining adhesive on the teeth and brackets. The average value of the remaining adhesive after removing the brackets from the upper premolars was 12.06 mm^2, while this value at the lower premolars was 9.32 mm^2. Student t-test showed that there was a statistically significant difference between the values of the remaining adhesive surfaces on the upper and lower premolars after removing the brackets (Table 1).

The average value of the remaining adhesive surface on the brackets removed from the upper premolars was 0.37 mm^2, while this value at the lower premolars was 2.08 mm^2. Student t-test showed a statistically significant difference between the values of the remaining adhesives on the brackets removed from the upper and lower premolars (Table 2).

Tables 3 and 4 show the distribution of ARI_{teeth} and $ARI_{brackets}$ for the upper and lower premolars. The most common score of ARI_{teeth} was score 3. Even for 132 teeth (85.71%), score 3 was determined after removing the brackets, for 13 teeth (8.44%) score was 0, 7 teeth had score 2 (1.3%), while only 2 teeth (1.3%) had score 1. The most common score of $ARI_{brackets}$ was score 0 (132, 85.71%). 13 brackets (8.44%) had score 3, 7 brackets (4.54%) had score 1, while only 2 brackets (1.3%) had score 2.

Table 1. The surface of remaining adhesive on the upper and lower premolars
Tabela 1. Površina preostalog adheziva na gornjim i donjim premolarima

Surface of residual adhesive (mm^2) Površina preostalog adheziva (mm^2)	n	M ± SD	Min	Max	t	df	p
Upper premolars Gornji pretkutnjaci	77	12.06 ± 2.14	0	15.48			
Lower premolars Donji pretkutnjaci	77	9.32 ± 4.45	0	14.91	4.86	109.5	**<0.01**
Total Ukupno	154	10.69 ± 3.74	0	15.48			

Table 2. The surface of remaining adhesive on brackets removed from the upper and lower premolars
Tabela 2. Osnovni pokazatelji deskriptivne statistike za površinu preostaloga adheziva na bravicama koje su uklonjene sa gornjih i donjih premolara

Surface of residual adhesive on the bracket (mm^2) Površina preostalog adheziva na bravici (mm^2)	n	M±SD	Min	Max	t	df	p
Upper premolars Gornji pretkutnjaci	77	0.37 ± 1.74	0	12.21			
Lower premolars Donji pretkutnjaci	77	2.08 ± 4.27	0	13.3	3.26	100.5	**<0.01**
Total Ukupno	154	1.23 ± 3.36	0	13.3			

Table 3. Distribution of ARI_{teeth} scores for upper and lower premolars
Tabela 3. Raspodela ocena ARI_{zuba} kod gornjih i donjih pretkutnjaka

Premolars Pretkutnjaci	ARI_{teeth} score ARI_{zuba} ocena				Total Ukupno
	0	1	2	3	
Upper Gornji	1 (1.3%)	1 (1.3%)	3 (3.89%)	72 (93.51%)	77 (100%)
Lower Donji	12 (15.58%)	1 (1.3%)	4 (5.2%)	60 (77.92%)	77 (100%)
Total Ukupno	13 (8.44%)	2 (1.3%)	7 (4.54%)	132 (85.71%)	154 (100%)

Table 4. Distribution of $ARI_{brackets}$ scores
Tabela 4. Raspodela ocena $ARI_{bravica}$

Brackets Bravice	$ARI_{brackets}$ $ARI_{bravica}$				Total Ukupno
	0	1	2	3	
For upper premolars Za gornje pretkutnjake	72 (93.51%)	3 (3.89%)	1 (1.3%)	1 (1.3%)	77 (100%)
For lower premolars Za donje pretkutnjake	60 (77.92%)	4 (5.2%)	1 (1.3%)	12 (15.58%)	77 (100%)
Total Ukupno	132 (85.71%)	7 (4.54%)	2 (1.3%)	13 (8.44%)	154 (100%)

Table 5. Pearson correlation
Tabela 5. Pirsonova korelacija

Pearson correlation Pirsonova korelacija		$ARI_{brackets}$ for upper premolars $ARI_{bravica}$ za gornje pretkutnjake	Pearson correlation Pirsonova korelacija		ARI_{teeth} for upper premolars ARI_{zuba} za gornje pretkutnjake
$ARI_{brackets}$ for lower premolars $ARI_{bravica}$ za donje pretkutnjake	r	.523**	ARI_{teeth} for lower premolars ARI_{zuba} za donje pretkutnjake	r	.523**
	p	.001		p	.001

** correlation significant at 0.01
** korelacija je značajna na nivou 0,01

Correlation between the average values of ARI_{teeth} for the upper and lower premolars was determined by the Pearson coefficient of linear correlation. The statistically significant, positive and very strong correlation between the average values of ARI_{teeth} for the upper and lower premolar ($r = .53$, $p < .01$) was found. Also, a statistically significant, positive and very strong correlation was found between the average values of $ARI_{brackets}$ for brackets bonded to the upper and lower premolar ($r = .53$, $p < .01$) (Table 5).

DISCUSSION

Since fixed appliances with metal brackets often represent a method of choice for achieving successful orthodontic treatment, metal brackets have been used in our research. After removing brackets from examined teeth, the difference between the amount of remaining adhesive surface on the brackets on the upper and lower premolars was determined, as well as the difference between the values of $ARI_{brackets}$.

Some authors reported difference between the size of remaining adhesive on the upper and lower teeth. Therefore, Zanarini at al. conducted their research on 100 metal brackets that were removed after completed treatment with fixed orthodontic appliances in 60 patients. Using the Image J program on photomicrographs obtained by scanning electron microscopy, they measured the size of remaining adhesive on brackets. They noted statistically significant difference in the size of remaining adhesive on the base of brackets between the teeth from upper and lower dental arch, which is in agreement with results of the current study [17].

The most commonly reported ARI_{teeth} score in this study was 3, followed by 0, 2 and 1. This result indicates that the most common mode of bond failure was between the adhesive and the base of the metal bracket that decreased the possibility of enamel microcracks. *In vitro* studies of Ryf at al., as well as Sing and Kumari, evaluated the mode of bond failure between the teeth and brackets, according to the ARI index. They also reported greater amount of remaining adhesive on teeth after metal brackets removal. The most frequent ARI score was 3 and it indicated that bond failure was most commonly found between the base of the bracket and resin [22, 23].

ARI index can also be used in clinical studies where precise impressions of the teeth from which brackets have been removed are required. These studies may point significant influence of certain factors on the mode of bond failure between teeth and brackets that cannot fully be mimicked in *in vitro* studies. Some of them are: longer exposure time of brackets and adhesive to saliva and pH changes caused by certain type of drink and food, exposure of a fixed appliance to mastication force etc. Using teeth impressions study models can be obtained and observed with stereomicroscope. Photomicrographs can be taken as well. Scanning electron microscopy (SEM) can also be used for impressions analysis, and it can even more accurately determine the size of remaining adhesive on teeth. Some clinical studies did not show any difference in the ARI index values from the same parameters determined by experimental studies [24].

Bonetti at al. conducted their research on 12 dental students with metal brackets Victory Series; 3 Munitek, Monrovia; California, which were bonded using Transbond XT composite material, and metal brackets APC II Victory Series; 3M Unitek, with already fabricated adhesive. After removing brackets, they evaluated the surface of remaining adhesive on teeth and brackets using ARI index. They found no statistically significant difference in the distribution of ARI score between the two premolar groups, and the most common score was 3, which is in accordance with the results of our study [25].

One of the factors affecting the mode of bond failure between teeth and brackets is also the type of brackets used. Thus, Mirzakouchaki at al. in their study on 120 intact human premolars with metal and ceramic brackets concluded that more adhesive remained on teeth that had ceramic brackets compared to metal one [7].

However, beside the bracket material, the retention mode (chemical or mechanical) and bracket base design can affect shear bond strength and the mode of bond failure during bracket removal. Henkin at al. investigated mode of bond failure for 7 different types of metal brackets (MorelliTM, American OrthodonticsTM, TP OrthodonticsTM, OrthometricTM, TecnidentTM, UnidentTM, Abzil-3MTM) attached to 105 bovine teeth. After their removal, the ARI index was determined by stereomicroscopy. They noted different distribution of ARI scores for teeth with different types of brackets attached. The most common score for the teeth with the UnidentTM brackets was 3, which is in accordance with the results of our research. On the teeth with other types of metal brackets attached, the most common score was 1, which can be explained by different shape and structure of the metal bracket base. UnidentTM brackets have similar shape and mesh base structure as the OrthoOrganizer brackets used in our research, which could be the reason for obtaining similar results of the ARI index [10].

For the purpose of the current study, light-curing composite material Aspire (Ortho Classic Orthodontics, USA)

was used, characterized by extended working hours. This resin is not transparent; therefore it is easier to notice it on the tooth surface. Some studies also emphasized the importance of the type of adhesive system used [26].

There has been noticed premature polymerization of the composite material used for bonding brackets under the influence of dental chair reflector in the process of bonding fixed orthodontic appliances. This applies to situations when material is bonded before bracket is placed in the correct position on a tooth surface. Tiwari et al. examined the influence of light of dental chair reflector on shear bond strength, as well as mode of bond failure between teeth and brackets when Transbond XT (3M Unitec, Monrovia, Callif, USA) light-cured composite material was used. Their study was performed on 60 human premolars, extracted for orthodontic purposes. The most common ARI score after bracket debonding was 3, indicating that the most frequent mode of bond failure was between a bracket base and adhesive. The authors concluded that dental chair reflector light did not have any significant effect on the shear bond strength and mode of bond failure between teeth and brackets [14].

In our study, difference in size of remaining residual adhesive after bracket debonding was found both on the brackets and examined teeth. However, our *in vitro* study included only premolars, while further studies could investigate the same parameters on incisors and molars due to different morphological characteristics of these teeth.

CONCLUSION

After removing metal brackets, the most frequent mode of bond failure was between adhesive and the base of bracket. A significant difference was found in the size of remaining adhesive both on the teeth and brackets between upper and lower premolars.

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Procena indeksa zaostalog adheziva posle uklanjanja metalnih bravica primenom programa AutoCAD

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KRATAK SADRŽAJ

Uvod Po završetku terapije fiksnim ortodontskim aparatima neophodno je ukloniti bravice i prstenove sa zuba, odgovarajućim postupkom. Cilj ovog rada je bio da se utvrdi najčešći način prekida veze između zuba i bravica prilikom uklanjanja metalnih bravica, kao i da se uporedi način prekida veze između metalnih bravica i gornjih i donjih premolara.

Materijal i metod rada Na 154 humana premolara, ekstrahovana u ortodontske svrhe, lepljene su metalne bravice Aspire kompozitnim materijalom. Posle njihovog odlepljivanja izmerena je površina preostalog adheziva na zubima i na bravicama. Primenom Indeksa zaostalog adheziva (Adehsive remnant index – ARI) izvršena je procena načina prekida veze između zuba i metalnih bravica.

Rezultati Prosečna vrednost površine preostalog adheziva nakon uklanjanja bravica sa gornjih premolara je iznosila 12,06 mm^2, dok je ova vrednost kod donjih premolara iznosila 9,32 mm^2. Prosečna vrednost površine preostalog adheziva na bravicama koje su uklonjene sa gornjih premolara je iznosila 0,37 mm^2, dok je ova vrednost kod bravica uklonjenih sa donjih premolara iznosila 2,08 mm^2, te je između ovih vrednosti utvrđena statistički značajna razlika. Najčešće zastupljena ocena ARI_{zuba} na ukupnom nivou je bila ocena 3 (85,71%), dok je najčešće zastupljena ocena $ARI_{bravica}$ na ukupnom nivou bila ocena 0 (85,71%).

Zaključak Najčešći način prekida veze između zuba i bravice prilikom uklanjanja metalnih bravica je bio između baze bravice i površine lepka. Između vrednosti površine preostalog adheziva na gornjim i donjim premolarima kao i na bravicama utvrđena je statistički značajna razlika.

Ključne reči: uklanjanje bravica; površina adheziva; indeks zaostalog adheziva (ARI)

UVOD

Terapija fiksnim ortodontskim aparatima predstavlja jedan od najčešće korišćenih postupaka za ispravljanje ortodontskih nepravilnosti i postizanje pravilnih okluzalnih odnosa. Po završetku ortodontske terapije neophodno je ukloniti bravice i prstenove sa zuba, kao i ostatke adheziva. Način odlepljivanja bravica sa površine zuba zavisi od vrste bravica koje se koriste tokom terapije (metalne, keramičke ili plastične). Metalne bravice se uklanjaju odgovarajućim kleštima primenom vuče, torzije ili smicanja [1, 2].

Nekoliko faktora utiču na jačinu veze između zuba i bravica: vrsta adhezivnih sistema koji se koriste za lepljenje bravica, vrsta kiseline i dužina perioda kondicioniranja gleđi tokom pripreme zuba, veličina i vrsta bravica, dizajn baze bravice i prisustvo pljuvačke, odnosno mogućnost obezbeđivanja suvog radnog polja tokom sprovođenja postupka postavke fiksnog ortodontskog aparata. Navedeni faktori, zajedno sa vrstom i načinom primene instrumenata za uklanjanje bravica, utiču na način prekida veze između zuba i bravica, a samim tim i na količinu preostalog adheziva na zubima i na bravicama [3–6].

Smatra se da optimalna jačina veze između zuba i metalnih bravica iznosi od 5,9 MPa do 8 MPa. Ukoliko je jačina veze veća od navedenih vrednosti, pojavljuje se mogućnost oštećenja gleđi zuba prilikom samog postupka uklanjanja fiksnog ortodontskog aparata. Upravo u momentu uklanjanja bravica kleštima postoji najveći rizik od nastanka frakture gleđi. Na mestu frakture dolazi do pojačane akumulacije plaka i pojave pigmentacija. Prilikom postupka uklanjanja fiksnog ortodontskog aparata, do prekida veze između bravice i površine zuba može doći na nekoliko mesta: u bravici, između bravice i adheziva, u adhezivu, između gleđi i adheziva, u gleđi i mešoviti prelom (kombinacija navedenih). Poželjno je da nakon uklanjanja bravica lepak ostane na površini zuba, kako bi se izbegla mogućnost pojave gleđnih pukotina [2, 7, 8].

Prilikom uklanjanja metalnih bravica, po završenoj terapiji fiksnim ortodontskim aparatima, najčešće dolazi do prekida veze između adheziva i baze metalne bravice, prilikom čega se smanjuje mogućnost pojave mikrooštećenja gleđi tokom izvođenja ovog postupka [10–14].

Pojedini autori su ukazali na međuzavisnost morfoloških karakteristika zuba i načina prekida veze između zuba i bravica, jer su uočili da veće količine lepka ostaju na bukalnim površinama zuba gornje vilice nakon uklanjanja metalnih bravica. Studije Hobsona i saradnika i Ozturka i saradnika su istakle da razlike u morfološkim karakteristikama između zuba utiču na jačinu ostvarene veze između bravice i zuba, bez obzira na vrstu korišćenog adhezivnog sistema [15, 16].

Zanarini i saradnici su određujući površinu preostalog lepka samo na bravicama, uočili razliku između vrednosti površine preostalog adheziva na bazama bravica uklonjenih sa zuba gornjeg i donjeg zubnog luka. Utvrdili su da veća količina adheziva ostaje na bravicama koje su uklonjene sa zuba gornjeg zubnog luka [17].

Jedna od najčešće korišćenih metoda za utvrđivanje načina i mesta prekida veze između površine zuba i baze bravice jeste upotreba indeksa zaostalog adheziva (Adhesive Remnant Index – ARI), kojeg su u istraživanje uveli Artun i Bergland 1984. godine [18]. Primenom ovog indeksa omogućeno je određivanje mesta prekida veze prilikom korišćenja različitih vrsta bravica i adhezivnih sistema, na jednostavan način. Procena preostalog adheziva na zubima i bravicama sprovodi se primenom ARI indeksa zuba (ARI_{zuba}) i ARI indeksa bravica ($ARI_{bravica}$). Za određivanje površine zaostalog adheziva na zubima i bravicama često se koristi stereomikroskopija, ali i određeni kompjuterski programi [17, 19, 20].

Cilj ovog istraživanja je bio da se utvrdi najčešći način prekida veze između zuba i metalnih bravica primenom ARI indeksa i programa AutoCAD, te da se ispita postojanje razlike u prekidu veze između gornjih i donjih premolara i metalnih bravica.

MATERIJAL I METOD RADA

U toku ovog istraživanja su prikupljena 154 humana premolara ekstrahovana u ortodontske svrhe (77 gornjih premolara i 77 donjih premolara). Kriterijum za izbor zuba je podrazumevao da zubi imaju intaktnu oralnu i bukalnu površinu, bez vidljivih oštećenja, belih mrlja i karioznih lezija i da nisu prethodno bili izlagani hemijskim agensima poput vodonik-peroksida ili nekoj od kiselina u svrhu nagrizanja. Stereomikroskopijom (uvećanje 10×) procenjeno je da li prikupljeni uzorak ispunjava kriterijum. Zubi su se čuvali do početka istraživanja u fiziološkom rastvoru sa 0,1% timolom, kako bi se sprečila pojava dehidracije gleđi zuba. Pre samog eksperimenta zubi su mehanički očišćeni četkicom i neabrazivnom pastom bez fluora. Srednja trećina bukalne površine svakog zuba je tretirana 38% ortofosfornom kiselinom u trajanju od 20 sekundi, a potom isprana mlazom voda-vazduh 30 sekundi i posušena još 30 sekundi. Na bukalnu površinu 154 zuba je nanošen *Aspire orthodontic adhesive 7GM (OC Orthodontics, USA)* prema uputstvu proizvođača, a potom je izvršena njegova polimerizacija u trajanju od 10 sekundi. Nakon toga, metalne bravice za premolare (*Ortho Organizers Elite OptiMIM, Henry Schein Orthodontics, USA)* postavljane su instrumentom za pozicioniranje (*Ixion positioner, DB Orthodontics, Zapadni Jorkšir, UK*) na pripremljenu površinu zuba i lepljene su pomoću *Aspire orthodontic adhesive 5GM* (*OC Orthodontics, USA)* (slika 1 i 2). Polimerizacija je izvršena LED lampom u trajanju od 40 sekundi, po uputstvu proizvođača.

Zubi su ostavljeni u veštačkoj pljuvački na sobnoj temperaturi, tokom 48 sati, kako bi adhezivni sistem dostigao svoju maksimalnu snagu. Kao izvor veštačke pljuvačke korišćen je *Biotene* gel *(GlaxoSmithKline, Belgija).* Nakon 48 sati bravice su odlepljene kleštima za skidanje bravica *(Ixion bracket removing plier, DB Orthodontics, West Yorkshire, UK)*. Nakon sprovođenja postupka pripreme uzorka koji se sastojao u fiksiranju zuba i bravica, omogućeno je fotografisanje bukalnih površina zuba i baza bravica digitalnim fotoaparatom Nikon D5100. Fotografisanje je sprovedeno uz upotrebu makroskopskog objektiva, pri adekvatnom svetlu sa udaljenosti od 20 cm. Fiksiran uzorak je bio postavljen na foliju sa milimetarskom podelom koja je služila kao referentna mera u okviru fotografisanog kadra i osnova za kalibraciju fotografije u programu AutoCAD *(Autodesk inc. San Rafael, CA, USA)* [21]. Za određivanje površine preostalog adheziva na zubu i bazi bravice, kao i za određivanje površine baze bravice, korišćen je program AutoCAD, najčešće korišćen program firme Autodesk za izradu inženjerske projektne dokumentacije u 2D i 3D projektovanju. Nakon kalibracije fotografije u AutoCAD-u, polilinijama u vidu „'zatvorenih poligona" preko fotografija označene su površine preostalog adheziva na zubu i bazi bravice, kao i površina bravice, te su automatski dobijene vrednosti označenih parametara (Slika 3). Procena preostalog adheziva na zubima i bravicama urađena je primenom ARI indeksa zuba i ARI indeksa bravica. ARI_{zuba} se koristio za procenu površine preostalog adheziva na gleđi i određen je po sledećoj formuli: površina zuba sa preostalim adhezivom/površina baze bravice ×100. $ARI_{bravica}$ se koristio za procenu površine preostalog adheziva na bravicama, a dobijen je po formuli: površina bravice sa preostalim adhezivom/površina baze bravice ×100.

Vrednost površine preostalog adheziva na zubima se ocenjivala pomoću ARI_{zuba}, tako da je za određene vrednosti preostalog adheziva dodeljena ocena od 0 do 3 na sledeći način:

ocena 0 – 0% adheziva na površini zuba,

ocena 1 – manje od 50% adheziva preostalog na površini zuba,

ocena 2 – više od 50% adheziva preostalog na površini zuba i

ocena 3 – 100% adheziva na površini zuba.

Površina preostalog adheziva na bravicama je bila ocenjena primenom $ARI_{bravica}$, na sledeći način:

ocena 0 – manje od 10% adheziva preostalog na površini bravice,

ocena 1 – više od 10% ali manje od 50% adheziva preostalog na površini bravice,

ocena 2 – više od 50% ali manje od od 90% adheziva preostalog na površini bravice i

ocena 3 – više od 90% adheziva na površini bravice [18].

Statistička obrada podataka

Kvalitativni podaci (ocene ARI indeksa) prikazani su kroz broj pojava i procentualnu zastupljenost. Za prikaz površine preostalog adheziva na zubu i na bravici nakon uklanjanja bravica korišćeni su pokazatelji deskriptivne statistike (aritmetička sredina, standardna devijacija, ekstremne vrednosti). Za upoređivanje srednjih vrednosti obeležja korišćen je parametarski Student t test za nezavisne uzorke. Kao statistički značajne uzimane su vrednosti u kojima je $p < 0,05$, s tim da su vrednosti u kojima je $p < 0,01$ isticane kao statistički veoma značajne. Za utvrđivanje stepena povezanosti posmatranih obeležja korišćena je Pirsonova parametarska korelacija.

REZULTATI

Prosečna vrednost površine bravica *Ortho Organizers Elite OptiMIM* za premolare, koje su lepljene na površinu 154 premolara, iznosila je 11,07 mm^2. Nakon uklanjanja bravica, programom AutoCAD su određene vrednosti površine preostalog adheziva na zubima i na bravicama. Prosečna vrednost preostalog adheziva nakon uklanjanja bravica sa gornjih premolara je iznosila 12,06 mm^2, dok je ova vrednost kod donjih premolara iznosila 9,32 mm^2. Primenom parametarskog Student t testa, za upoređivanje srednjih vrednosti obeležja utvrđeno je da je bilo statistički značajne razlike u vrednostima preostalog adheziva na gornjim i donjim premolarima nakon uklanjanja bravica. Ovi rezultati su prikazani u Tabeli 1.

Prosečna vrednost površine preostalog adheziva na bravicama koje su uklonjene sa gornjih premolara je iznosila 0,37 mm^2, dok je ova vrednost kod donjih premolara iznosila 2,08 mm^2. Primenom parametarskog Student t testa, za upoređivanje srednjih vrednosti obeležja je utvrđeno da je bilo statistički veoma značajne razlike u vrednostima preostalog adheziva na bravicama uklonjenim sa gornjih i donjih premolara. Ovi rezultati su prikazani u Tabeli 2.

U tabelama 3 i 4 prikazana je distribucija ARI_{zuba} i $ARI_{bravica}$ za gornje i donje premolare i na ukupnom nivou. Najčešće za-

stupljena ocena ARI_{zuba} na ukupnom nivou je bila ocena 3. Čak kod 132 zuba (85,71%) utvrđena je ocena 3 nakon uklanjanja bravica, 13 zuba (8,44%) je dobilo ocenu 0, ocenu 2 je imalo sedam zuba (1,3%), dok su ocenu 1 imala svega dva zuba (1,3%). Najčešće zastupljena ocena $ARI_{bravica}$ na ukupnom nivou je bila ocena 0 (132, 85,71%), 13 bravica (8,44%) je dobilo ocenu 3, ocenu 1 je imalo sedam bravica (4,54%), dok su ocenu 2 imale svega dve bravice (1,3%).

Povezanost između prosečnih vrednosti ARI_{zuba} za gornje i donje premolare izrežena je pomoću koeficijenta Pirsonove linearne korelacije. Utvrđena je statistički značajna, pozitivna i veoma jaka povezanost između prosečnih vrednosti ARI_{zuba} za gornje i donje premolare ($r = 0,53$, $p < 0,01$). Takođe, utvrđena je statistički značajna, pozitivna i veoma jaka korelacija između prosečnih vrednosti $ARI_{bravica}$ za bravice lepljene na gornje i donje premolare ($r = 0,53$, $p < 0,01$) (Tabela 5).

DISKUSIJA

U ovom istraživanju korišćene su metalne bravice, s obzirom na to da fiksni ortodontski aparati sa metalnim bravicama često predstavljaju metod izbora za postizanje uspešnog terapijskog rezultata. Nakon uklanjanja bravica sa zuba utvrđena je statistički značajna razlika u vrednostima površine preostalog adheziva na gornjim i donjim premolarima,

kao i na bravicama koje su uklonjene sa navedenih zuba, ali i razlika u vrednostima ARI indeksa. Postojanjem jasnih razlika između morfoloških karakteristika krune gornjih i donjih premolara, a samim tim i konveksiteta bukalne površine različitog stepena izraženosti, mogla bi se objasniti utvrđena statistički značajna razlika u vrednostima površine preostalog adheziva na njima. Pojedini autori su ukazali na postojanje razlike u vrednostima površine preostalog adheziva na gornjim i donjim zubima koristeći bravice u svrhu ispitivanja. Tako su Zanarini i saradnici svoje istraživanje sproveli na 100 metalnih bravica, koje su uklonjene po završetku terapije fiksnim ortodontskim aparatima kod 60 pacijenata. Primenom programa *Image J* na fotomikrografijama dobijenim skenirajućom elektronskom mikroskopijom, merili su površinu preostalog adheziva na bazama bravica. Uočili su statistički značajnu razliku između vrednosti površina preostalog adheziva na bazama bravica, između zuba gornjeg i donjeg zubnog luka, što je u skladu i sa rezultatima ovog istraživanja [17].

Najčešće zastupljena ocena ARI_{zuba} u ovom istraživanju bila je ocena 3, koja je utvrđena kod čak 132 zuba. Kod 13 zuba je dodeljena ocena 0, sedam zuba je dobilo ocenu 2, a samo dva zuba su imala ARI ocenu 1. Ovaj rezultat ukazuje na to da je najčešće mesto prekida veze zub–bravica bilo upravo između adheziva i baze metalnih bravica, čime je onemogućeno nastajanje mikropukotina prilikom uklanjanja adheziva. *In vitro* studijama Ryfa i saradnika, kao i Singa i Kumaria, koji su primenom ARI_{zuba} ispitivali prekid veze između zuba i bravice prilikom uklanjanja metalnih bravica kleštima sa humanih premolara, takođe je uočena veća količina preostalog lepka na zubu. Najviše zastupljena ARI ocena je bila ocena 3, koja je ukazala na to da je mesto prekida veze najčešće nastajalo između baze bravice i lepka [22, 23].

ARI indeks takođe se koristi i u *in vivo* studijama, prilikom čijeg izvođenja je neophodno uzeti precizne otiske zuba sa kojih su uklonjene bravice. Ovakve studije još mogu da ukažu na značaj pojedinih faktora na mesto nastanka prekida veze između zuba i bravice, koje u *in vitro* studijama nije moguće u potpunosti ostvariti, kao što su: duži period izlaganja bravica i lepka dejstvu pljuvačke te promenama pH vrednosti koje se dešavaju u usnoj duplji prilikom unosa određene vrste pića i hrane, kao i izloženost postavljenih bravica silama tokom mastikacije. Na osnovu otisaka zuba mogu se napraviti studijski modeli čija se površina može posmatrati stereomikroskopijom, što dalje pruža mogućnost pravljenja i fotomikrografija. Otisci takođe mogu da budu korišćeni kao uzorci sa SEM analizu, čime se još preciznije može utvrditi vrednost površine preostalog adheziva na zubu. Rezultati većine *in vivo* studija, koje su ispitivale mesto prekida veze između metalnih bravica i zuba, nisu pokazale veća odstupanja u dobijenim vrednostima ARI indeksa u odnosu na *in vitro* studije [24].

Bonetti i saradnici su svoje istraživanje sproveli na 12 studenata dentalne medicine, kojima su na druge premolare lepili metalne bravice i to na 12 premolara *Victory Series*; na tri *Munitek*, *Monrovia*; *California*, pomoću kompozitnog materijala *Transbond XT*, a na preostalih 12 premolara APC II *Victory Series*; *3M Unitek*, sa već fabrički nanesenim adhezivom. Nakon uklanjanja bravica ispitivali su površinu preostalog adheziva na zubima i bravicama primenom ARI indeksa. Utvrdili su da nije bilo statistički značajne razlike u distribuciji ARI ocena između dve grupe premolara, a najčešća ocena je bila ocena 3, što je u skladu i sa rezultatima ovog istraživanja [25].

Jedan od faktora koji utiče na način prekida veze između zuba i bravice prilikom uklanjanja bravica predstavlja i vrsta bravice koja se koristi tokom terapije. Tako Mirzakouchaki i saradnici u svojoj studiji koju su sproveli na 120 intaktnih humanih premolara, na koje su postavljali metalne i keramičke bravice, zaključuju da je kod zuba na koje su bile postavljene metalne bravice veći deo adheziva ostao na zubu, za razliku od zuba na koje su bile postavljene keramičke bravice [7].

Osim materijala koji se koristi za izradu bravice, način retencije (hemijska ili mehanička) i dizajn baze bravice utiču kako na jačinu ostvarene veze sa zubom, tako i na način njenog prekida prilikom uklanjanja bravica. Henkin i saradnici su u svom istraživanju koje je sprovedeno na 105 goveđih zuba, ispitivali način prekida veze između zuba i sedam različitih tipova metalnih bravica (*Morelli*^{TM}, *American Orthodontics*^{TM}, *TP Orthodontics*^{TM}, *Orthometric*^{TM}, *Tecnident*^{TM}, *Unident*^{TM}, *Abzil-3M*^{TM}). Nakon njihovog odlepljivanja određivali su ARI indeks stereomikroskopijom. Uočili su različitu distribuciju ARI ocena kod zuba sa različitim tipom bravica. Najčešća ocena kod zuba na koje su lepljene bravice *Unident*^{TM} bila je ocena 3, što je u skladu sa rezultatima ovog istraživanja. Na zubima na kojima su lepljeni drugi tipovi metalnih bravica najčešća ocena je bila ocena 1, što se može objasniti različitim oblikom i strukturom baze metalnih bravica. Bravice *Unident*^{TM} imaju sličan oblik i mrežastu strukturu baze kao bravice *OrthoOrganizer* koje su korišćene u ovom istraživanju, što je moglo uticati na sličan rezultat za ARI indeks [10].

Prilikom sprovođenja ovog istraživanja korišćen je svetlosno-polimerizujući kompozitni materijal *Aspire* (*Ortho Classic Orthodontics*, *USA*), kojeg karakteriše produženo radno vreme. Korišćeni materijal ne poseduje transparentnost, što omogućava njegovo lakše uočavanje na površini zuba. Veliki broj istraživanja u kojima je određivan ARI indeks ističe značaj vrste adhezivnog sistema koji se koristi za postavku fiksnog ortodontskog aparata [26].

Prilikom postavke fiksnog ortodontskog aparata primećeno je da ponekad može doći do prevremene polimerizacije kompozitnog materijala koji se koristi za lepljenje bravica pod uticajem reflektora na stomatološkoj stolici. Ovo se odnosi na situacije kada dođe do vezivanja materijala pre nego što se bravica postavi u pravilan položaj na površinu zuba. Pojedini autori, kao što su Tiwari i saradnici, ispitivali su i uticaj svetla, odnosno jačine reflektora stomatološke stolice na jačinu veze, kao i na mesto nastanka prekida veze između zuba i bravica lepljenih *Transbond XT* (*3M Unitec*, Monrovia, Callif, USA) svetlosno-polimerizujućim kompozitnim materijalom. Svoju studiju su sproveli na 60 humanih premolara, ekstrahovanih u ortodontske svrhe. Najčešća ocena ARI indeksa nakon odlepljivanja metalnih bravica je bila 3, što je ukazalo da je najčešće mesto prekida veze između zuba i bravica bilo na granici lepka i baze bravice, a to je u skladu sa rezultatima ovog istraživanja. Autori su uočili da svetlo reflektora stomatološke stolice ipak nije imalo značajnijeg uticaja na jačinu i način prekida veze između zuba i bravice [14].

U ovom istraživanju je pronađena razlika u vrednostima površine preostalog adheziva između gornjih i donjih premolara nakon odlepljivanja bravica. Razlika je pronađena i u vrednostima površine preostalog lepka na bravicama koje su odlepljene sa ispitivanih premolara. S obzirom na to da je studija obavljena isključivo na premolarima, u daljim istraživanjima mogla bi se ispitati razlika u površini preostalog adheziva i na sekutićima i molarima gornjeg i donjeg zubnog luka nakon uklanjanja bravica, zbog postojanja morfoloških razlika u obliku ovih zuba.

ZAKLJUČAK

Prilikom uklanjanja metalnih bravica najčešći način prekida veze je bio između adheziva i baze bravice. Prilikom uklanjanja bravica utvrđena je statistički značajna razlika između vrednosti površine preostalog adheziva na gornjim i donjim premolarima, kao i između vrednosti preostalog adheziva na bazama bravica uklonjenih sa gornjih i donjih premolara.

CASE REPORT
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Hypodontia and WNT10A mutation: a case report

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SUMMARY

Tooth agenesis is common dentofacial malformation in humans. Its etiology is still not clear. Hypodontia has been regarded as a multifactorial condition influenced by gene function, environmental interaction and developmental timing. More than 300 genes have been related with patterning, morphogenesis and cell differentiation in teeth. According to data WNT10A gene is considered to have an important role in odontogonesis.

The aim of this study was to show mutation status in WNT10A gene in a family with two members with diagnosis of hypodontia/oligodontia. In the reported family (father, mother, son, daughter) children were diagnosed with congenital tooth agenesis (son-2 teeth, daughter-11 teeth), while parents negated congenital absence of teeth. We identified a heterozygous missense mutation, c.682T>A (p.Phe228Ile) within the exon 3 of WNT10A in mother and father and the same homozygous mutation was detected in the same region of WNT10A gene in daughter and son. Observed differences in our study, from no symptoms to mild/severe hypodontia, could be the consequence of genetic influence of c.682T>A(p.Phe228Ile) mutation, but also the contribution of many environmental factors during odontogenesis.

Keywords: hypodontia/ oligodontia; homozygous; heterozygous; mutation; WNT10A gene

INTRODUCTION

Tooth agenesis is common dentofacial malformation in humans [1]. It can occur either as an isolated characteristic (non-syndromic form) or as a part of recognized clinical syndrome [2]. Different terms are used to describe this anomaly, depending on the number of congenitally missing teeth. Hypodontia is used when one to six teeth (excluding third molars) are congenitally missing, while oligodontia means that more than six teeth are missing (excluding third molars). Term anodontia is used for extreme case of complete absence of teeth [3].

The etiology of tooth agenesis is still not clear. Hypodontia has been regarded as a multifactorial condition influenced by gene function, environmental interaction and developmental timing [4, 5].

More than 300 genes have been related with patterning, morphogenesis and cell differentiation in teeth so far [6]. Tooth agenesis can be the result of different nucleotide changes in genes that are involved in the process of tooth formation. Their products are signal molecules and transcription factors that control gene expression in different phases of tooth morphogenesis. According to data, WNT10A is one of the most important candidate genes expressed in epithelial cells and through Wnt/ß-catenin signal pathway its signal protein may activate mesenchymal cells during early phase of odontogenesis. Moreover, WNT10A mutations can be possible cause of tooth agenesis in affected individual [6–9].

The aim of this study was to present detected mutations in the WNT10A gene in a family where two members were affected by congenital tooth agenesis.

CASE REPORT

A Serbian family (mother, father, son and daughter) presented two members (son and daughter) with diagnosis of congenital tooth agenesis. Panoramic radiographs and clinical examination confirmed hypo/oligodontia. All family members were examined for other symptoms of ectodermal dysplasia but none was detected. No panoramic radiographs were available for the parents, however they denied congenital absence of any teeth in a detailed interview. The daughter was diagnosed with oligodontia – 11 teeth congenitally missing (upper lateral incisors, upper second molars, lower central and lateral incisors, lower canines, and lower left second molar), while the son was diagnosed with hypodontia of lower central incisors. Panoramic radiographs and odontograms of the daughter and the son are shown in the Figure 1.

Mutational analysis

Informed consent was obtained from the patients and the Human Research Ethics Committee of the Faculty of Dental Medicine, University of Belgrade, approved the study. Buccal swabs from family members were used to obtain DNA, and WNT10A mutational analysis of "hot spot" regions, exon 2 and 3, was performed by method of direct sequencing. Heterozygous missense mutation, c.682T>A, within the exon 3 of WNT10A in mother and father (I-1 and I-2) and homozygous mutation in the same region of WNT10A gene in daughter and son (II-1 and II-2) were detected. Reported mutation lead to amino-acid exchange, p.Phe228Ile, that had pathological effect (Table 1, Figure 2).

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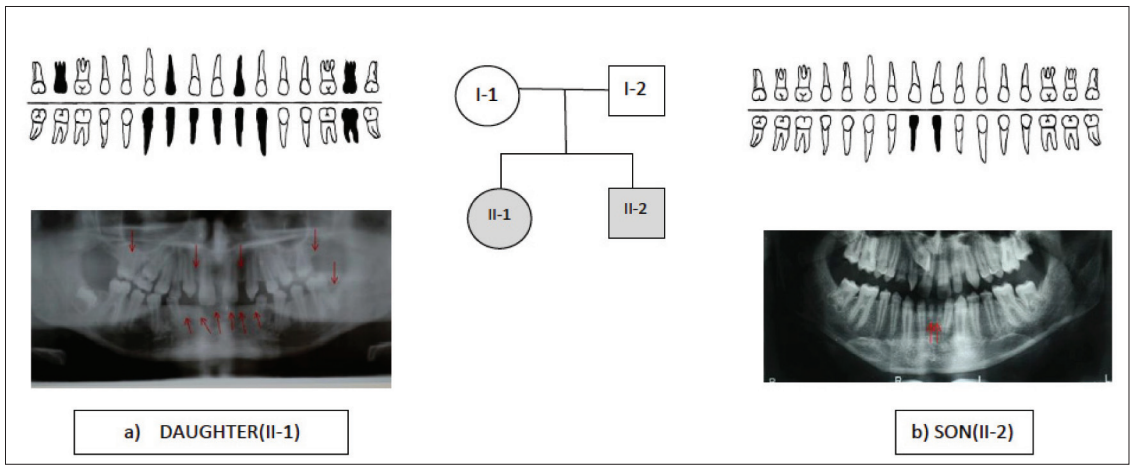

Figure 1. Heredity of the family
Panoramic radiographs and odontograms of daughter and son
a) Oligodontia (daughter) – 11 congenitally missing teeth, b) Hypodontia (son) – 2 congenitally missing teeth
Slika 1. Rodoslovno stablo
Ortopantomografski snimci ćerke i sina i odontogrami
a) Oligodoncija (ćerka) – urođeni nedostatak 11 stalnih zuba, b) Hipodoncija (sin) – urođeni nedostatak dva zuba

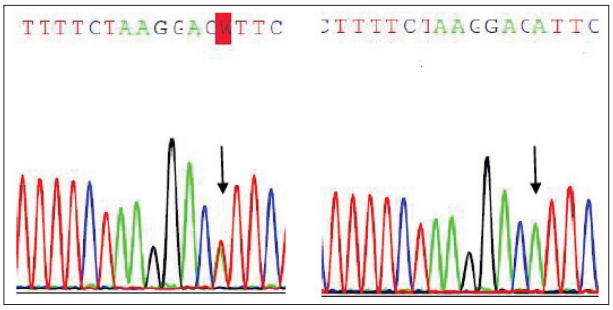

Figure 2. Missense WNT10A mutation (rs121908120) c.682T>A[TTT→ATT] p.Phe228Ile a) TA* (heterozygous mutation), b) A*A* (homozygous mutation)
Slika 2. „Missense" mutacija gena WNT10A (rs121908120) c.682T>A[TTT→ATT] p.Phe228Ile
a) TA* (heterozigotna mutacija), b) A*A* (homozigotna mutacija)

Table 1. Family members with p.Phe228Ile mutation in WNT10A gene
Parents with heterozygous mutation and not affected
Affected daughter and son have homozygous mutation
Tabela 1. Članovi porodice sa p.Phe228Ile mutacijom gena WNT10A
Roditelji sa heterozigotnom mutacijom bez promena u broju zuba
Sin i ćerka sa homozigotnom mutacijom i urođenim nedostatkom zuba

Family member Član porodice	Mother Majka	Father Otac	Daughter Ćerka	Son Sin
Number Broj	I-1	I-2	II-1	II-2
Gender Pol	F	M	F	M
Missing teeth Nedostajući zubi	0	0	11	2
Mutation WNT10A (p.Phe228Ile)	T/A* heterozygous heterozigotni	T/A* heterozygous heterozigotni	A*/A* homozygous homozigotni	A*/A* homozygous homozigotni

DISCUSSION

The reported Serbian family had no manifestations of ectodermal dysplasia. Missense mutation c.682T>A (p.Phe228Ile) within the exon 3 of WNT10A gene was detected in all members of the family, in heterozygous or homozygous form. In our case report family, the pattern of inheritance probably could be autosomal recessive or autosomal dominant with different gene penetrance, since there was no data of parents' congenitally missing teeth. Kantaputra and Sripathomsawat found the same mutation in a family with non-syndromic hypodontia and without other changes in ectodermal tissues [9]. They also detected a c.682T>A mutation for the father (missing maxillary first premolars) and two sons (one had missing upper lateral incisors and lower second premolars, and the other one presented with microdontia of the lower left second premolar). The mother did not have mentioned mutation, and she was not affected with hypodontia. Interestingly, the mother had p.Asp217Asn mutation that was also detected in the WNT10A genes of the two sons.

According to these clinical observations and obtained data in our analyzed family, the mode of c.682T>A inheritance of WNT10A gene could probably be autosomal dominant. Moreover, in a study of Bohring at al. pathogenic mutation c.682T>A was detected in patients with ectodermal dysplasia and the same mutation, considered as disease causing, was found in healthy individuals (0.5%) [10]. Analyzing phenotype manifestations in patients with heterozygous mutation p.Phe228Ile, Bohring et al. also reported that heterozygotes showed minor phenotype manifestations associated with teeth (small, conical, sharp, or missing upper lateral permanent incisors; agenesis of lower right central incisor or agenesis of 2 to 6 permanent teeth except third molars), or no manifestations at al. [10].

Similarly, in our reported family, diverse clinical manifestations were reported in carriers of c.682T>A WNT10A mutation (heterozygote parents and homozygote son and daughter). Observed differences in our study, from no symptoms to mild/severe hypodontia, could be the con-

sequence of genetic influence of the suspected gene, but also the impact of many environmental factors during odontogenesis.

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Hipodoncija i mutacija WNT10A gena: prikaz slučaja

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KRATAK SADRŽAJ

Urođeni nedostatak zuba predstavlja jednu od najčešćih dentofacijalnih anomalija kod čoveka. Etiologija hipodoncije i dalje nije potpuno definisana i smatra se da su za njenu etiologiju odgovorni brojni genetski i sredinski faktori koji deluju u različitim fazama razvoja zuba. Preko 300 gena povezuje se sa morfogenezom i ćelijskom diferencijacijom u toku razvoja zuba, a prema podacima WNT10A gen je jedan od gena koji ima veoma bitnu ulogu u kontroli odontogeneze. Cilj ovog rada bio je da se prikaže mutacioni status WNT10A gena u porodici sa dijagnostikovanom hipodoncijom/oligodoncijom. U prikazanoj porodici (otac, majka, sin i ćerka) kod dva člana dijagnostikovan je urođeni nedostatak zuba (sin – dva zuba, ćerka – 11 zuba), dok kod roditelja ovaj nedostatak nije zabeležen. Kod svih članova porodice, u okviru egzona 3 WNT10A gena detektovana je mutacija c.682T>A (p.Phe228Ile). Kod majke i oca ova „missense" mutacija je bila u heterozigotnom obliku, dok je kod sina i ćerke utvrđeno prisustvo iste mutacije u homozigotnom obliku. Zabeležene razlike u analiziranoj porodici, od odsustva simptoma do blage hipodoncije i izrazite oligodoncije, mogu biti posledica prisustva c.682T>A (p.Phe228Ile) mutacije, ali takođe i uticaja faktora sredine u toku odontogeneze.

Ključne reči: hipodonciia/oligodoncija; homozigotni; heteroigotni; mutacija; WNT10A gen

UVOD

Urođeni nedostatak zuba predstavlja jednu od najčešćih dentofacijalnih anomalija kod čoveka [1]. Može biti izolovana (nesindromska) ili se može pojaviti u okviru nekog kliničkog sindroma [2]. U zavisnosti od broja zuba koji urođeno nedostaju, različiti termini se koriste da se opiše ova anomalija. Termin hipodoncija se koristi kada urođeno nedostaje jedan do šest zuba (isključujući treće molare), dok termin oligodoncija ukazuje da u denticiji nedostaje više od šest zuba (isključujući treće molare). Termin anodoncija se vezuje za ekstremne slučajeve kompletnog odsustva svih zuba u vilicama [3].

Etiologija hipodoncije i dalje nije potpuno definisana. Smatra se da je za etiologiju hipodoncije odgovorno više faktora : brojni genetski i sredinski faktori koji deluju u različitim fazama razvoja zuba [4, 5].

Kao što je već poznato, preko 300 gena se povezuje sa morfogenezom i ćelijskom diferencijacijom u toku razvoja zuba [6]. Urođeni nedostatak zuba može biti posledica različitih nukleotidnih izmena u genima uključenim u proces odontogeneze. Produkti ovih gena su signalni molekuli i transkripcioni faktori koji kontrolišu ekspresije gena u različitim fazama morfogeneze zuba. Prema podacima, WNT10A je jedan od najvažnijih gena eksprimiranih u epitelnim ćelijama, i u okviru Wnt/ß-catenin signalnog puta njegov signalni protein može aktivirati mezenhimne ćelije u ranoj fazi odontogeneze. Takođe, različite mutacije WNT10A gena mogu dovesti do urođenog nedostatka zuba kod nosioca mutacije [6–9].

Cilj ovog rada bio je da se prikaže mutacioni status WNT10A gena u porodici sa dijagnostikovanom hipodoncijom/oligodoncijom.

PRIKAZ BOLESNIKA

Prikazana je četvoročlana porodica iz Srbije (majka, otac, sin, ćerka) u kojoj je kod dva člana (sina i ćerke) dijagnostikovan urođeni nedostatak zuba. Hipo/oligodoncija je potvrđena ortopantomografskim snimcima i kliničkim pregledom. Utvrđeno je da nijedan od članova porodice nije imao druge manifestacije ektodermalne displazije. Roditelji su u anamnezi negirali postojanje urođenog nedostatka zuba jer ortopantomografski snimci nisu bili dostupni. Kod ćerke je dijagnostikovana oligodoncija – urođeni nedostatak 11 zuba (gornji bočni sekutići, gornji drugi molari, donji centralni i bočni sekutići, donji očnjaci i donji drugi levi molar), dok je kod sina dijagnostikovana hipodoncija donjih centralnih sekutića. Ortopantomografski snimci ćerke i sina sa odontogramima su prikazani na Slici 1. Članovi porodice su za učešće u studiji potpisali informisani pristanak i studija je odobrena od strane Etičkog komiteta Stomatološkog fakulteta u Beogradu.

MUTACIONA ANALIZA

Za dobijanje genomske DNK korišćen je bris bukalne sluzokože. Primenom metode direktnog sekvenciranja urađena je mutaciona analiza egzona 2 i 3 WNT10A gena. Kod svih članova porodice identifikovana je ista mutacija (c.682T>A, p.Phe228Ile). Tačnije, kod majke i oca (I-1 and I-2) identifikovana je heterozigotna „missense" mutacija u okviru egzona 3 WNT10A gena, dok je kod sina i ćerke utvrđeno prisustvo iste mutacije u homozigotnom obliku (II-1 and II-2). Dobijena mutacija dovodi do amino-kiselinske izmene u WNT proteinu, p.Phe228Ile, i smatra se da ima patološki efekat (Tabela 1, Slika 2).

DISKUSIJA

Na osnovu anamnestičkih podataka, kod prikazane porodice nisu bile prisutne manifestacije ektodermalne displazije, ali je kod svih članova porodice, u heterozigotnoj ili homozigotnoj formi, detektovana „missense" mutacija, c.682T>A (p.Phe228Ile) u okviru egzona 3 WNT10A gena. Način nasleđivanja date mutacije u prikazanoj porodici mogao bi da bude autozomno recesivan ili autozomno dominantan sa različitom penetrantnošću gena, s obzirom na to da nema podataka o urođenom nedostatku zuba kod roditelja koji imaju mutaci-

ju. Kao i u našoj analiziranoj porodici, u studiji Kantaputre i Sripathomsawata ista mutacija je prikazana u porodici sa nesindromskom hipodoncijom i bez drugih promena u ektodermalnim tkivima [9]. Naime, u njihovoj studiji detektovana je mutacija c.682T>A kod oca (kome nedostaju gornji prvi premolari) i kod dva sina (jednog sina sa nedostatkom gornjih bočnih sekutića i donjih drugih premolara, a kod drugog sina sa mikrodoncijom donjeg levog drugog premolara). S druge strane, u istoj porodici majka nije imala pomenutu mutaciju i nije imala urođeni nedostatak zuba. Ipak, kod majke je detektovana druga mutacija, p.Asp217Asn, koja je takođe bila prisutna i kod dva sina.

Na osnovu kliničkih podataka i mutacione analize, može se pretpostaviti da je način nasleđivanja c.682T>A mutacije WNT10A gena najverovatnije autozomno dominantan. Takođe, u studiji Bohringa i sar. patogena mutacija c.682T>A detektovana je kod pacijenata sa ektodermalnom displazijom, a isto tako je utvrđeno njeno prisustvo i kod zdravih pojedinaca (0,5%) [10]. Analizirajući fenotipske manifestacije kod pacijenata sa heterozigotnom mutacijom p.Phe228Ile, Bohring i sar. [10] navode da heterozigoti ili ne pokazuju nikakve promene, ili pokazuju štetne fenotipske efekte vezane za zube u manjoj ili većoj meri (mali, konični, oštri gornji bočni sekutići, ili nedostatak istih; ageneza donjeg desnog centralnog sekutića; ageneza dva do šest stalnih zuba isključujući treće molare).

Slično, i u našoj prikazanoj porodici kliničke manifestacije kod nosilaca c.682T>A WNT10A mutacije su različite (heterozigotni roditelji i homozigotni sin i ćerka). Zabeležene razlike u analiziranoj porodici, od odsustva simptoma do blage hipodoncije i izrazite oligodoncije, mogu biti posledica prisustva c.682T>A (p.Phe228Ile) mutacije, ali takođe i uticaja faktora sredine u toku odontogeneze.

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Stem cells in tissue engineering – dynamic cultivation requirement

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SUMMARY

Stem cells have shown great potential for *in vitro* tissue engineering, regenerative medicine, cell therapy and pharmaceutical applications. All these applications, especially in clinical trials, will require guided production of high-quality cells. Traditional culture techniques and applications have been performed for the majority of primary and established cell lines and standardized for various analyses. Still, these culture conditions are unable to mimic dynamic and specialized three-dimensional microenvironment of the stem cells' niche from *in vivo* conditions. In an attempt to provide biomimetic microenvironments for stem cells *in vitro* growth, three-dimensional culture techniques have been developed. In our study advantages of newly developed porous scaffolds as the most promising *in vitro* imitation of niche that provides physical support, enables cell growth, regeneration and neovascularization, while they are replaced in time with newly created tissue was explained. Furthermore, dynamic cultivation techniques have been described, as new way of cell culturing that will be the main subject of our future research. In that manner, by developing an optimal dynamic culturing method, high-quality new cells and tissues would be possible to obtain, for any future clinical application.
Keywords: stem cells; culture technique; scaffolds; ALBO-OS; bioreactor

INTRODUCTION

Stem cells (SCs) have the ability to self-renew and differentiate into mature types of cells that develop all organs and tissues in human body. There are two major categories of SCs defined by their origin and potency - embryonic stem cells, and adult, mesenchymal stem cells [1]. Embryonic stem cells (ESCs) are pluripotent, capable of unlimited self-renewal and differentiation into any type of cell in the body. Mesenchymal stem cells (MSCs) are isolated from adult sources such as bone marrow, adipose, and dental tissue. MSCs are multipotent cells that can differentiate into a limited number of cell types. Also, self-renewal and differentiation potential is dependent on the tissue they are isolated from, and age of a donor. The advantages are accessibility and less ethical concerns for their usage [2, 3]. SCs have shown great potential for *in vitro* tissue engineering, regenerative medicine, cell therapy and pharmaceutical applications. All these applications, especially in clinical trials, will require a guided production of high-quality cells [4].

TRADITIONAL CULTURE TECHNIQUES

SCs are propagated as a monolayer in two-dimensional (2-D) plastic culture plates. 2-D culture techniques and applications have been practiced for the majority of primary and established cell lines and standardized for various analyses, from isolation and characterization of cells, to the studies of diseases development and drug testing [5]. To grow cells on plastic culture dishes, ESCs have to be seeded on precoated surface to aid in attachment. 2-D expansion of ESCs has been improved by applying defined and xenogenic-free culture media and attachment substrates. However, uniform expansion of ESCs is still difficult to achieve as 2-D culture methods for propagation of ESCs are challenging, expensive and require high level of expertise [5, 6]. Differentiation of ESCs has been achieved by using specific induction media into ectodermal, mesodermal, and endodermal lineages. An important advantage of monolayer culture is controlled differentiation of human ESCs that allows uniform treatment for differentiation of cells. However, differentiation in monolayer culture often results in mixed populations of differentiated cells [4, 5, 7].

Unlike embryonal, MSCs have natural ability to adhere to plastic and glass surfaces. Xenogenic substrates are not necessitated for attachment, although they are usually cultured in the media containing animal serum. Use of animal-derived media can potentially transmit pathogens and limit reproducibility between cultures. Recently, xenogenic-free media has become available for cultivation of MSCs [8]. Expansion of MSCs in monolayer has its limitations. Monolayer culture needs routine passaging to maintain self-renewal and potency of cells, which is

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highly inefficient for large-scale expansion of cells. Maintenance of uniform distribution, growth, and harvesting processes is needed and consequently, heterogeneity is minimized and cell yield is high. Phenotypic changes occur in MSCs while culturing in monolayers, and cells' fate and differentiation potential are altered after numerous passages [9]. Despite the limitations, 2-D culture has been used for differentiation of MSCs into many specialized cells, including chondrocytes, osteocytes, adipocytes, cardiomyocytes, smooth muscle cells, and hepatocytes, by using cell-specific differentiation media [10, 11]. Assessment of differentiation stages is commonly done by specific transcriptional gene expression and extracellular matrix (ECM) depositions. The main downside of monolayer differentiation is a lack of providing functionally competent cells. They often differentiate into precursor-like cells, suggesting the possibility to re-differentiate during extended culturing [4]. Prior to clinical application, modification of differentiation protocols should be made. Overall, 2-D culture conditions are unable to mimic dynamic and specialized three-dimensional (3-D) microenvironment of the SC niche from *in vivo* conditions.

THREE-DIMENSIONAL CULTURE TECHNIQUES

In an attempt to provide biomimetic microenvironments for stem cells *in vitro* growth, 3-D culture techniques have been developed. These methods have a common goal to mimic the ECM composition and stiffness of SC niche *in vitro*. Challenge lies in protocol optimization depending on cell type and the aim of analysis [4]. Therefore, the uniform expansion of SCs without loss of genetic stability or differentiation potential has to remain regardless of applied technique.

Static three-dimensional culture

1. Spheroids

Formation of spheroids that consist of cell aggregates and allow cell interactions in the absence of additional substrates is one of the simplest 3-D culturing method. A wide range of adherent cell types have the ability to form spheroids by spontaneous cell aggregation when they are seeded in low-adhesion culture plates in suspension culture, in a form of a hanging drop, or in rotating culture [7, 12]. MSCs are successfully maintained and expanded by spheroid method where they exhibited increased clonal growth and multipotency, and activation of pluripotency genes. However, as in monolayer cultivation, long-term culture of MSCs in spheroids spontaneously led to differentiation [12]. In comparison with monolayer culture, MSCs grown in 3-D spheroids have shown increased chondrogenic and osteogenic differentiation *in vitro*. In particular, chondrogenic differentiation of MSCs has been shown as more effective in high-density cell culture methods utilizing pellet culture or spheroids, in comparison to 2-D culture. Still, this method is not applicable for cell expansion due to the inability to control aggregate size, leading to agglomeration, apoptosis, and inhibition of cell proliferation [13]. Depending on tissue that cells are isolated from, spheroids could consist of heterogeneous population of cells, with different proliferative capacity. Even more important drawback is limited diffusion of oxygen and nutrients in the center of the spheroid, which gradually leads to a hypoxic environment, and at the end, formation of necrotic center. Due to these drawbacks, spheroids have been more successfully employed in study of 3-D cell structures, cell differentiation and cancer biology rather than homogenous cell proliferation and production of high-quality uniform cell cultures [13, 14].

2. Scaffolds

Various natural and synthetic biocompatible and biodegradable materials have been used to mimic the biochemical and biophysical properties of SC niches that stimulates cell proliferation and/or differentiation. Natural biomaterials (agarose, fibronectin, hyaluronic acid, chitosan) *in vitro* often transduce biological signals to cells [15]. As a result, biomaterials can aid in cell maintenance and differentiation. However, problems such as variability and the potential of xenogenic media components to cause disease limit their use [15, 16]. Synthetic polymers (polyethylene glycol, poly-l-lysine, poly-lactic acid, polyglycolic acid, and poly-dl-lactic acid-co-glycolic acid) are the group of artificially made scaffolds with different mechanical properties (pore size, elasticity, adhesion, tensile strength) [17–22]. Biodegradable, porous scaffolds represent the most promising *in vitro* imitation of SC niche that provides physical support, enables cell growth, regeneration and neovascularization, while they were replaced in time with new bone [23, 24, 25]. Innovative scaffold construction that consists of ceramic part mimicking bone structure, and thin polymer layer above that contribute its' better mechanical properties and biocompatibility, showed to be very promising biomaterial for bone tissue engineering (Figure 1) [21, 25, 26]. Scaffolds are commonly used as carriers that mimic ECM, promote expansion, migration, and differentiation of SC. For SC culturing, scaffolds can be prefabricated, afterward, cells are seeded onto the scaffold, and allowed to migrate and proliferate [20, 27, 28]. Scaffolds can incorporate growth factors and cytokines, and provide mechanical stimulation for SC differentiation. On the other side, there are scaffolds with self-assemble encapsulating cells that are incorporated in biomaterial at the time of its fabrication. Due to these advantages, prefabricated scaffolds are commonly used for seeding SCs prior to their differentiation and usage in tissue engineering [28]. We recently reported successful application of composite scaffold, combination of calcium hydroxyapatite and poly (lactic-co-glycolic) acid, named ALBO-OS, as a bone substitute [21, 25]. Scaffold with very high porosity and nanotopology showed to be very suitable for cell adhesion and proliferation (Figure 2), providing larger surface area, allowed better adhesion and provided more area for differentiation of MSCs [24]. Furthermore, it has been shown the formation of new mineralized matrix, osteoconductive and certain

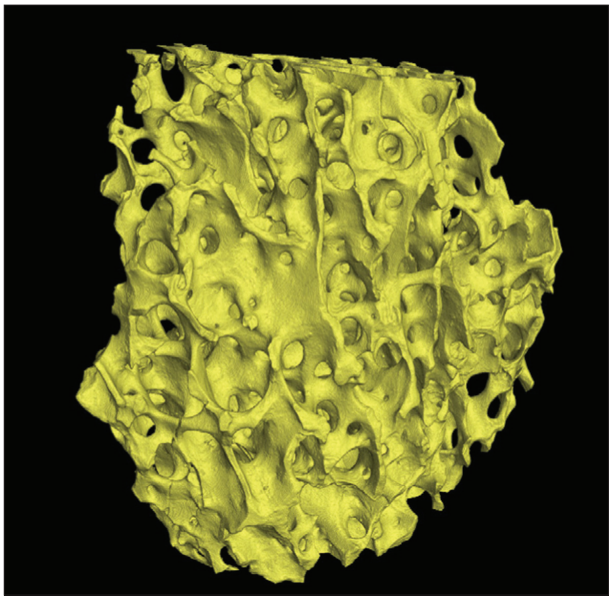

Figure 1. Micro CT volume reconstruction of ALBO-OS carrier; porous biomaterial has ideal micro- and nano-porosity, trabecular thickness, cylindrical shape of the pores, pore diameter and connectivity of bone scaffold model
Slika 1. Mikro CT zapreminska rekonstrukcija ALBO-OS nosača, poroznog biomaterijala idealne mikro i nanoporoznosti, debljine trabekula, pora cilindričnog oblika, dijametra i međusobne povezanosti modela koštanog zamenika

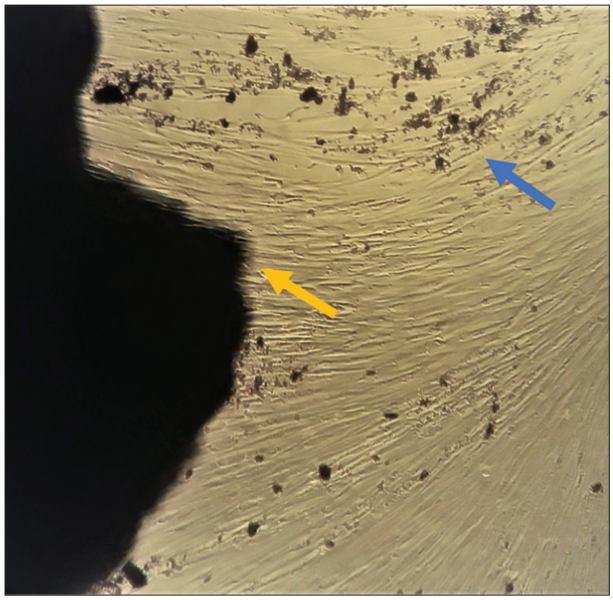

Figure 2. Stem cells from apical papilla (*SCAP*), from 5th passage, after 5 days of cell culture seeded on ALBO-OS carrier. Yellow arrow points tight contact of scaffold and confluent *SCAP*; blue arrow points scaffold particles released in growth medium during culturing covering *SCAP*.
Inverted microscope, 100×
Slika 2. Matične ćelije poreklom iz apikalne papile (*SCAP*), iz pete pasaže, posle pet dana gajenja ćelija na ALBO-OS nosaču. Žuta strelica pokazuje na bliski kontakt nosača i gusto naseljenih *SCAP*; plava strelica pokazuje sitne delove nosača oslobođene u hranljivom medijumu koje prekrivaju *SCAP*.
Invertni mikroskop, 100×

osteoinductive effect (Figure 3). Compressive strength of ALBO-OS showed to be a good mechanical support during the whole period of its transformation into new bone [21, 24, 25].

It has been shown that scaffold mechanical properties stimulate differentiation of MSCs into various lineages. Softer substrates induced MSC differentiation into neural and beta islet cells, chondrocytes and adipocytes. On the other hand, increase in substrate stiffness supported MSC differentiation into myoblasts and osteoblasts. Compressive forces mimicking joint action via mechanotransductive scaffold increased chondrogenic gene expression in MSCs [27, 28]. Since the composition and mechanical properties of biomaterials guide MSCs differentiation into specific cell lineages, it is important to be optimised and easily producible. Cell-cell and cell-ECM interactions in static 3-D culture during maintenance and expansion of

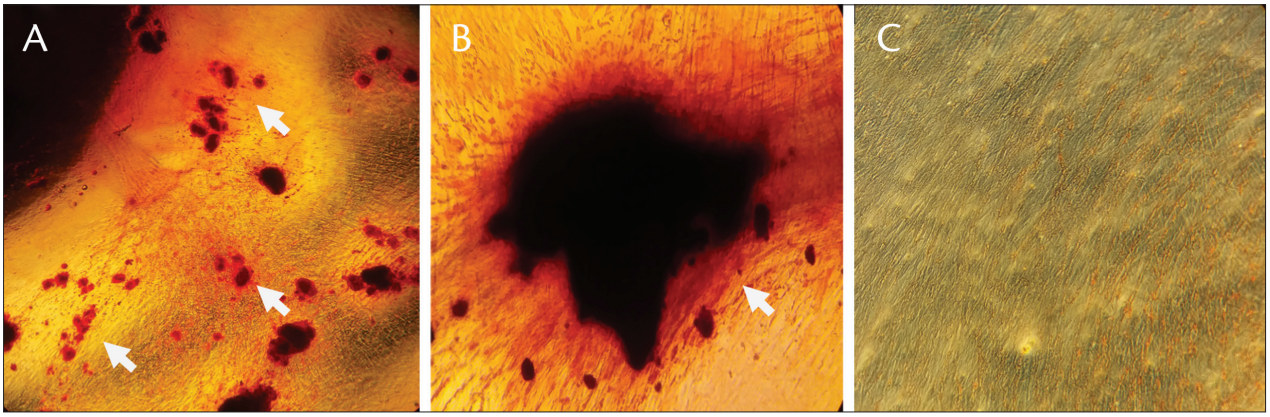

Figure 3. Alizarin Red S staining after 21 days of *SCAP* seeded on ALBO-OS carrier in growth medium (A and B), and *SCAP* in growth medium as a control (C). White arrows point on numerous calcium deposits in the red colored complex of newly deposited mineralized extracellular matrix, suggesting significant osteoinductive potential of ALBO-OS. In control no mineralization was observed except some spontaneous mineralized nodules.
Inverted microscope, 40× (A), 100× (B, C).
Slika 3. Bojenje *SCAP* Alizarin crvenim nakon 21 dana gajenja ćelija na ALBO-OS nosaču u hranljivom medijumu (A i B), i samih *SCAP* gajenih u hranljivom medijumu kao kontrolna grupa (C). Bele strelice pokazuju na brojne kalcijumske nakupine obojene crvenom bojom u kompleksu novostvorenog mineralizovanog vanćelijskog matriksa, ukazujući na značajan osteoinduktivni potencijal ALBO-OS nosača. U kontroli mineralizacija nije uočena, osim pojedinačnih spontano mineralizovanih čvorića.
Invertni mikroskop, 40× (A), 100× (B, C).

MSCs can provide insight on basic cell biology processes and mechanisms. The main challenge of 3-D static cultivation remains extensive cell expansion in individual scaffold constructs. By increasing in size of scaffold, cell growth in the center could be compromised in a similar way as in spheroids. Flow of oxygen, nutrients, and waste in whole scaffold space might be limited [4]. Methods that could provide 3-D cell growth and expansion without nutritional drawbacks are the subject of current investigations.

Dynamic three-dimensional culture

Bioreactors are used to enable, monitor, and control biological processes. In tissue engineering, they enable culturing high densities of cells, allow cell growth and proliferation, and minimise variability observed in traditional culturing. The new way of cell culturing requires defined and well-regulated conditions regarding temperature, oxygen and nutrients concentration, *pH*, as well as metabolites removal. Various types of 3-D culture techniques provide effective cell proliferation and differentiation [29]. There are differences in techniques used to develop bioreactor systems that affect their application.

Several bioreactor designs have been developed during recent years that can be used for expansion and guided differentiation from a single cell to tissue culture. Spinner flasks are one of the simplest and most convenient bioreactor systems. They operate by using local hydrodynamic forces that create shear stress on cells. In the center of the system is cell/scaffold assemble, while magnetic bar provides a constant flow of medium around scaffold. The system is placed in an incubator with controlled conditions. The degree of shear stress is defined by stirring speed. The downsides represent possibility of dense layer formation of cells on surface that could compromise nutrition of cells in the center, and unequal shear stress, with the highest level at the bottom of the flask [30]. Rotating bioreactors were designed for improved control of shear stress by horizontal rotation and simulation of microgravity. In this way limitations regarding the equal flow of nutrients and waste products are reduced [31]. The awareness of limitations of diffusion systems based on rotation led to development of perfusion bioreactors. Flow perfusion systems allow constant exchange of nutrients and waste, providing better control of culture parameters. They enable transport of nutrients and oxygen through the entire scaffold, while media must be changed at regular intervals [32]. Lastly, mechanical force bioreactors have been developed to mimic tissue physiology, by using direct mechanical strain such as compressive and tensile forces. Mechanical stimulation could be in the form of bending, stretching, contraction and compression. It has been shown that direct mechanical stimulation induced proliferation, osteogenic differentiation, and formation of ECM. A drawback could be diffusional limitations in larger constructs [33].

Future prospective research should include culturing of MSCs on porous scaffolds as promising tool for cells expansion and differentiation, in dynamic constructions, as flow perfusion systems, that would provide homogenous culturing conditions. In that manner, high-quality new cells and tissues would be possible to obtain regardless of scaffold size, which represents qualitative foundation for any clinical research.

CONCLUSION

Biomedical applications of stem cells require production of high number of uniform cells that nowadays well exceed millions of cells produced by traditional culturing techniques. Recent studies have shown that transplantation of higher cell concentration had better outcome. Billions to trillions of cells will be required in clinical trials involving cell therapy. Advancements in the developing xenogenic-free media, culturing techniques and devices are of great importance. Large scale of cell culturing demands collaboration between biomedical researchers and engineers, to develop an optimal dynamic culturing method that will provide therapeutic use of stem cells.

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Matične ćelije u tkivnom inženjerstvu – potreba za dinamičnom kultivacijom

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KRATAK SADRŽAJ

Matične ćelije su pokazale veliki potencijal za primenu u tkivnom inženjerstvu u *in vitro* uslovima, regenerativnoj medicini, lečenju matičnim ćelijama i farmaceutskoj primeni. Sve ove grane, posebno primena u kliničkim istraživanjima, zahtevaće kontrolisano stvaranje visokokvalitetnih ćelija. Tradicionalnim tehnikama izolovana je većina primarnih ćelijskih kultura i ćelijskih linija, i vremenom su one postale standardne tehnike za različite ćelijske analize. Ipak, ovi uslovi gajenja nisu u mogućnosti da imitiraju dinamične, trodimenzionalne uslove mikrosredine niše matičnih ćelija iz *in vivo* uslova. U pokušaju da se obezbede navedeni uslovi i u *in vitro* uzgajanju ćelija, razvile su se trodimenzionalne tehnike gajenja ćelija. U ovom preglednom radu opisujemo prednosti novorazvijenih poroznih nosača ćelija, kao ključnih činilaca u imitaciji ćelijske niše koji obezbeđuju mehaničku potporu, omogućuju rast ćelija, regeneraciju i razvoj novih krvnih sudova, dok vremenom bivaju razgrađeni i zamenjeni novostvorenim tkivom. Dalje, tehnike dinamičnog gajenja ćelija su opisane kao vid novog načina gajenja ćelijskih kultura koji predstavlja i pravac naših budućih istraživanja. U tom smislu, razvijajući optimalan, dinamički model gajenja ćelija, biće moguće obezbediti nove ćelije i tkiva visokog kvaliteta za sva dalja klinička istraživanja.

Ključne reči: matične ćelije; tehnike gajenja; nosači; ALBO-OS; bioreaktor

UVOD

Matične ćelije (MĆ) imaju sposobnost samoobnavljanja i usmeravanja u pravcu zrelih tipova ćelija koje grade sva tkiva i organe ljudskog organizma. Postoje dve osnovne grupe MĆ definisane po poreklu i potentnosti – embrionalne matične ćelije i zrele, mezenhimalne matične ćelije [1]. Embrionalne matične ćelije (EMĆ) jesu pluripotentne, sa sposobnošću neograničenog samoobnavljanja i usmeravanja u pravcu svih tipova ćelija prisutnih u organizmu. Mezenhimalne matične ćelije (MMĆ) izolovane su iz zrelih tipova tkiva, kao što su koštana srž, masno tkivo i tkiva usne duplje. MMĆ su multipotentne ćelije koje mogu biti usmerene u ograničen broj ćelijskih tipova. Takođe, sposobnost samoobnavljanja i usmeravanja zavise od vrste tkiva iz kojih su izolovane i starosti davaoca tkiva. Prednosti MMĆ su laka dostupnost i to što njihova primena povlači manje etičkih pitanja [2, 3]. MĆ su pokazale veliki potencijal za primenu u tkivnom inženjerstvu u *in vitro* uslovima, regenerativnoj medicini, lečenju matičnim ćelijama i farmaceutskoj primeni. Sve ove grane, posebno primena u kliničkim istraživanjima, zahtevaće kontrolisano stvaranje visokokvalitetnih ćelija [4].

TRADICIONALNE TEHNIKE GAJENJA ĆELIJA

MĆ se uzgajaju u vidu jednog sloja ćelija, u dve dimenzije (2-D), na plastičnim podlogama ploča i boca namenjenih uzgajanju ćelija. Gajenje ćelija u 2-D uslovima primenjeno je na većini primarnih ćelijskih kultura i ćelijskih linija, i standardizovano za različite analize, od izolacije i karakterizacije ćelija do istraživanja razvoja oboljenja i testiranja lekova [5]. Da bi se uzgajale na plastičnim podlogama, EMĆ se moraju zasejati na prethodno pripremljene površine kako bi se olakšalo vezivanje ćelija za podlogu. Gajenje EMĆ u 2-D uslovima poboljšano je primenom određenih hranljivih medijuma bez prisustva proteina životinjskog porekla i dodataka za poboljšanje vezivanja ćelija za podlogu. Međutim, ujednačeno umnožavanje EMĆ se i dalje teško postiže u 2-D uslovima usled zahtevnih, skupih metoda, koje zahtevaju veliki stepen stručnosti [5, 6]. Primenom posebnih indukcionih medijuma EMĆ su usmerene ka ektodermalnoj, mezodermalnoj i endodermalnoj lozi. Važna prednost gajenja EMĆ u jednom sloju jeste kontrolisano usmeravanje ćelija koje omogućuje podjednaku izloženost ćelija medijumu. Ipak, ovakvo usmeravanje često za rezultat ima mešovitu populaciju zrelih ćelija [4, 5, 7].

Za razliku od embrionalnih, MMĆ poseduju prirodnu sposobnost da se lepe za plastičnu i staklenu podlogu. Proteini životinjskog porekla nisu potrebni za podsticanje lepljenja ćelija za podlogu, ali se ćelije uglavnom uzgajaju u medijumu koji u sebi sadrži serum životinjskog porekla. Primena takvih medijuma može biti potencijalni izvor prenosivih patogena i ograničiti ponovljivost ogleda. U skorije vreme razvijeni su i medijumi za uzgajanje MMĆ bez prisustva proteina životinjskog porekla, što predstavlja neophodan uslov za buduću kliničku primenu MMĆ [8]. Pri gajenju MMĆ u 2-D uslovima javljaju se određena ograničenja. Ćelije gajene u jednom sloju zahtevaju redovno presađivanje kako bi se zadržala potentnost i sposobnost samoobnavljanja, što je veoma neisplativo u slučaju uzgajanja velikog broja ćelija. Neophodno je održavati podjednake uslove raspoređenosti, rasta i prikupljanja ćelija, posledično umanjiti mogućnost heteregenosti u kulturi i obezbediti visok prinos ćelija. Tokom uzgajanja javljaju se fenotipske promene i ćelije gube potencijal rasta i usmeravanja nakon određenog broja pasaža [9]. Uprkos ograničenjima, izlaganjem ćelija posebnim medijumima u 2-D uslovima, MMĆ su usmerene ka različitim tipovima specijalizovanih ćelija, uključujući hondrocite, osteocite, adipocite, kardiomiocite, glatke mišićne ćelije i hepatocite [10, 11]. Procena stadijuma diferentovanosti ćelija se najčešće utvrđuje veličinom ispoljavanja gena specifičnih za određeni tip zrelih ćelija i kvalitativnim bojenjem depozita vanćelijskog matriksa (VĆM). Osnovni nedostatak usmeravanja ćelija u 2-D uslovima jeste izostanak stvaranja funkcionalno aktivnih ćelija.

One često diferentuju u prekursorne ćelije, te postoji mogućnost da tokom vremena dediferentuju [4]. Pre kliničke primene neophodno je izvršiti izmene u protokolima za usmeravanje ćelija. Uopšteno, 2-D uslovi gajenja ćelija nisu u mogućnosti da imitiraju dinamičnu i specijalizovanu trodimenzionalnu (3-D) mikrosredinu niše u kojoj rastu MĆ u *in vivo* uslovima.

TRODIMENZIONALNE TEHNIKE GAJENJA ĆELIJA

U pokušaju stvaranja mikrosredine u kojoj se imitiraju biološki uslovi rasta MĆ u *in vitro* uslovima, razvijene su 3-D tehnike gajenja ćelija. Zajednički cilj ovih metoda jeste imitacija sastava VĆM i rigidnosti niše u *in vitro* uslovima. Izazov predstavlja optimizacija protokola zavisno od tipa ćelija i cilja analize [4]. Stoga, ravnomerno umnožavanje MĆ bez gubitka genetičke stabilnosti ili potencijala za usmeravanje ka specijalizovanim ćelijama se mora očuvati nezavisno od primenjene tehnike.

Statična trodimenzionalna kultura

1. Sfere

Sfere su jedan od osnovnih 3-D metoda gajenja ćelija i predstavljaju nakupine ćelija u kojima je omogućena međućelijska interakcija u odsustvu drugih faktora. Veliki broj različitih tipova ćelija ima sposobnost spontane agregacije kada se zaseju u suspenziji u pločama za gajenje ćelija sa niskim stepenom adhezije, u obliku viseće kapi ili u rotacionim bocama [7, 12]. MMĆ se uspešno održavaju i gaje u sferama, ispoljavajući povećanje klonalnog rasta i multipotentnosti i aktivaciju gena odgovornih za pluripotentnost. Međutim, kao i pri gajenju u 2-D uslovima, dugotrajno održanje MMĆ u sferama spontano dovodi do njihovog sazrevanja [12]. Osteogena i hondrogena diferencijacija je uspešnija kod MMĆ gajenih u 3-D sferama, u poređenju sa 2-D uslovima diferencijacije. Hondrogena diferencijacija se pokazala posebno efikasnom kada su se ćelije gajile u vidu velike nakupine ćelija ili u vidu sfera, u poređenju sa gajenjem ćelija u jednom sloju. Ipak, ovaj metod nije primenljiv za umnožavanje ćelija usled nemogućnosti kontrole veličine stvorene nakupine ćelija, što dovodi do prerastanja, odumiranja i zaustavljanja ćelijskog umnožavanja [13]. Zavisno od tkiva iz kojeg su ćelije izolovane, sfere se mogu sastojati od heterogene populacije ćelija, sa različitim potencijalom umnožavanja. Još važniji nedostatak jeste ograničen protok kiseonika i hranljivih materija u središtu sfere, što postepeno vodi u sredinu sa sniženim nivoom kiseonika i, na kraju, stvaranja nekrotičnog centra. Zbog navedenih nedostataka, sfere se više primenjuju pri izučavanju samih 3-D ćelijskih kultura, usmeravanja ka zrelim ćelijama i biologije tumora, pre nego za umnožavanje ćelija homogene populacije i stvaranja visokokvalitetnih i uniformnih ćelijskih kultura [13, 14].

2. Nosači

Različiti prirodni i veštački biokompatibilni i biorazgradivi materijali se primenjuju za imitaciju biohemijskih i biofizičkih svojstava MĆ niše koja podstiče umnožavanje i/ili usmeravanje ćelije. Prirodni biomatrijali (agaroza, fibronektin, hijaluronska kiselina, citosan) u *in vitro* uslovima često prenose biološke signale ćelijama [15]. Kao rezultat, biomaterijali mogu podstaći održanje ćelija i usmeravanje. Međutim, problem kao što je raznolikost i mogućnost prenosa određenih bolesti preko materijala životinjskog porekla ograničavaju njihovu primenu [15, 16]. Sintetski polimeri (polietilen glikol, polilizin, polilaktična kiselina, poliglikolna kiselina, polilaktid-koglikolna kiselina) predstavljaju grupu veštački napravljenih nosača različitih mehaničkih svojstava (veličina pora, elastičnost, adhezija, zatezna čvrstoća) [17–22]. Biorazgradivi, porozni nosači ćelija predstavljaju ključne činioce u imitaciji ćelijske niše koji obezbeđuju mehaničku potporu, omogućuju rast ćelija, regeneraciju i razvoj novih krvnih sudova, dok vremenom bivaju razgrađeni i zamenjeni novostvorenim tkivom [23, 24, 25]. Inovativni nosač napravljen od keramičkog dela koji imitira koštanu strukturu i tankog sloja polimera preko, koji doprinosi boljim mehaničkim svojstvima i biokompatibilnosti, pokazao se kao veoma dobar biomaterijal namenjen primeni u koštanoj regeneraciji (Slika 1) [21, 25, 26]. Nosači se često primenjuju za imitaciju VĆM, podstiču umnožavanje, migraciju i sazrevanje MĆ. Mogu biti napravljeni od biomaterijala, nakon čega se ćelije zasejavaju, migriraju i zatim se umnožavaju u samom nosaču [20, 27, 28]. Takvi nosači u sebi mogu sadržati faktore rasta i citokine i obezbediti mehaničku stimulaciju za usmeravanje MĆ. S druge strane, u nosače se mogu ugraditi inkapsulirane ćelije u vreme njihovog pravljenja. Napravljeni nosači se često primenjuju za zasejavanje MĆ pre njihovog usmeravanja i primene u tkivnom inženjerstvu [28]. Nedavno smo objavili uspešnu primenu kompozitnog nosača, koji predstavlja kombinaciju kalcijum-hidroksiapatita i polilaktid-koglikolne kiseline, pod imenom ALBO-OS, kao koštanog zamenika [21, 25]. Nosač velike mikro i nanoporoznosti pokazao se kao veoma pogodan za ćelijsku adheziju i umnožavanje (Slika 2), obezbeđujući veliku površinu, bolju adheziju i veći prostor za usmeravanje MMĆ [24]. Nadalje, pokazano je stvaranje novog mineralizovanog matriksa, osteokonduktivno i određeno osteoinduktivno dejstvo na ćelije (Slika 3). U *in vivo* uslovima se pokazalo da ALBO-OS ima zadovoljavajuću zateznu čvrstoću i pruža dobru mehaničku potporu tokom čitavog perioda stvaranja nove kosti [21, 24, 25].

Pokazano je da mehanička svojstva nosača podstiču usmeravanje MMĆ ka različitim ćelijskim tipovima. Mekši supstrati podstiču MMĆ da diferentuju ka neuralnim i beta ćelijama, hondrocitima i adipocitima. S druge strane, povećanje rigidnosti podstiče usmeravanje ka mioblastima i osteoblastima. Studija u kojoj je nosač prenosio mehanički stres na MMĆ silama pritiska koje imitiraju pokret zgloba pokazala je uticaj nosača na povećanje ispoljavanja gena odgovornih za hondrogenu diferencijaciju [27, 28]. Pošto sastav i mehanička svojstva biomaterijala vode MMĆ da diferentuju u posebne ćelijske tipove, važno je da budu dobro definisani i da se mogu jednostavno proizvesti. Interakcija između ćelija i ćelija i VĆM u statičnoj 3-D kulturi tokom održanja i umnožavanja MMĆ može omogućiti uvid u osnovnu biologiju ćelijskih procesa i mehanizama. Glavni izazov statične 3-D kulture ostaje umnožavanje velikog broja ćelija u individualnom nosaču. Kako se nosač uvećava, rast ćelija u njegovom centru može biti ugrožen na sličan način, kao što je to slučaj u sferama. Protok kiseonika, hranljivih materija i produkata metabolizma u čitavom prostoru nosača može biti ograničen [4]. Metode koje mogu omogućiti rast ćelija u 3-D uslovima bez nutritivnih nedostataka predstavljaju predmet trenutnih istraživanja.

Dinamična trodimenzionalna kultura

Bioreaktori omogućuju odvijanje, nadzor i kontrolu bioloških procesa. U tkivnom inženjerstvu omogućavaju uzgajanje velike gustine ćelija, rast i umnožavanje ćelija i umanjuju raznolikost prisutnu u statičnim uslovima uzgajanja ćelija. Novi način uzgajanja ćelija traži definisane i dobro kontrolisane uslove temperature, koncentracije kiseonika i hranljivih materija, pH sredine, kao i uklanjanje produkata metabolizma. Različite vrste 3-D tehnika uzgajanja ćelija obezbeđuju dobro umnožavanje i usmeravanje MĆ ka zrelim ćelijama [29]. Različite tehnike koje su primenjene u razvoju sistema bioreaktora određuju njihovu namenu.

U proteklih nekoliko godina razvijeno je više tipova bioreaktora namenjenih umnožavanju i vođenoj diferencijaciji od pojedinačne ćelije do uzgajanja tkiva. Rotirajuće boce za gajenje ćelija su najjednostavniji i najpraktičniji sistem bioreaktora. Lokalne hidrodinamske sile predstavljaju osnovu sistema i na ćelije deluju silama smicanja. U središtu sistema se nalazi nosač sa ćelijama, dok magnetna mešalica omogućuje stalni protok medijuma oko nosača. Sistem je smešten u inkubatoru sa kontrolisanim uslovima sredine. Vrednost sila smicanja definisana je brzinom mešanja medijuma. Nedostaci ove metode sadržani su u mogućnosti stvaranja gustog sloja ćelija na površini, koje mogu ugroziti ishranu ćelija u dubljim strukturama nosača, kao i stvaranja nejednakih sila smicanja, uz najveće vrednosti na dnu boce u kojoj se ćelije gaje [30]. Iz potrebe za boljom kontrolom sila smicanja osmišljeni su rotirajući bioreaktori sa horizontalnom rotacijom i simulacijom mikrogravitacije. Na ovaj način ograničenja vezana za jednak protok hranljivih materija i produkata metabolizma su smanjena [31]. Kada su se uvideli nedostaci vezani za sisteme zasnovane na rotaciji, došlo je do razvoja bioreaktora sa perfuzijom. Sistemi sa perfuzijom omogućuju stalnu razmenu hranljivih materija i produkata metabolizma, što omogućuje bolju kontrolu parametara za gajenje ćelija. Prenos hranljivih materija i kiseonika se odvija kroz čitav nosač, dok se medijum u sistemu menja u redovnim intervalima [32]. Na kraju, bioreaktori sa dejstvom mehaničkih sila su osmišljeni kako bi se imitirala fiziologija tkiva, primenjujući direktne mehaničke sile zatezanja i pritiska. Mehanička stimulacija može biti u vidu savijanja, istezanja, skupljanja i pritiska. Pokazano je da direktna mehanička stimulacija podstiče umnožavanje ćelija, njihovu osteogenu diferencijaciju i stvaranje mineralizovanog VĆM. Potencijalni nedostatak može predstavljati ograničenje u protoku medijuma kod većih nosača [33].

Buduća prospektivna istraživanja bi trebalo da uključe gajenje MMĆ na poroznim nosačima kao najpogodnijim strukturama za umnožavanje i usmeravanje ćelija u dinamičnim sistemima, kao što je bioreaktor sa perfuzijom, kako bi se omogućili homogeni uslovi gajenja. U tom smislu, biće omogućen razvoj visokokvalitetnih novih ćelija i tkiva nezavisno od veličine nosača, što predstavlja kvalitetnu osnovu za sva buduća klinička istraživanja.

ZAKLJUČAK

Primena matičnih ćelija u biomedicini zahteva gajenje velikog broja uniformnih ćelija, koji premašuje milione ćelija koje je danas moguće gajiti tradicionalnim tehnikama. Nedavne studije su pokazale da transplantacija velikog broja ćelija daje bolji ishod lečenja. Kliničke studije koje će uključiti ćelijsku terapiju će zahtevati milijarde i trilione novih ćelija. Napredak u primeni medijuma bez proteina životinjskog porekla, tehnikama uzgajanja i opremi su od velike važnosti. Gajenje velikog broja ćelija zahteva saradnju istraživača iz oblasti biomedicine i inženjerstva, kako bi se razvio optimalni dinamični metod gajenja ćelija koji će omogućiti sprovođenje ćelijske terapije.

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